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MOLECULAR AND CHARACTERIZATION STUDIES OF NEW DELHI METALLO-B-LACTAMASE

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Deidre Natalie Govender

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A thesis submitted to the School of Health Science, University of KwaZulu-Natal, Westville, for the degree of Master of Medical Science (Pharmaceutical Chemistry).

This is a thesis in which the chapters are written as a set of discrete research paper, with an overall introduction and final discussion. Typically, these chapters will have been published in internationally recognized, peer-reviewed journals.

As the candidate's supervisors, we have approved this thesis for submission.

Supervisor:

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CONTENTS

DEDICATION

This thesis is dedicated to my parents. I'm grateful for every sacrifice, prayer, provision, support and love.

ABSTRACT

The increasing rate of antibiotic resistance within human pathogens has caused global concern. Bacterial strains have employed several strategies to resist β-lactam antibiotics, the most prevalent being the expression of metallo-β-lactases (MBLs). The New Delhi (NDM-1) being the most notorious MBL, is able to hydrolyse most β-lactam antibiotics resulting in multiple-drug resistance. NDM-1 renders the β-lactam antibiotic inactive through cleavage of the amide bond. Zinc ions are a key component for this hydrolysis reaction. There are currently no inhibitors available to combat the detrimental effect of these enzymes. Therefore, identification of potential inhibitors is of primary importance within the scientific community. The aims of this study are to investigate the interactions of a recombinant metallo-β-lactamase protein with metal chelator inhibitors using molecular and quantitative techniques. NDM-1 was cloned into the vector pET302 and over expressed in *E. coli BL21 (DE3)* cells. Furthermore, this study reports kinetic inhibition and thermodynamics of recombinant NDM-1 against four metal chelators, namely, BP1, BP9, BP10 and BP11. The NDM-1 gene was successfully cloned and expressed in *E.coli*. The expression yield of the protein was 3.6 mg/L. The kinetic parameters of the recombinant NDM-1 β-lactamase were measured as 29.07 μM and 21.77 s^{-1} of K_m and k_{cat} respectively toward nitrocefin. Inhibition studies showed that the K_i values of BP1, BP9, BP10 and BP11 were 0.24, 0.16, 0.19 and 0.18 μM, respectively. BP9 showed the highest inhibition against NDM-1. Fluorescence studies confirmed that BP9 is a better inhibitor compared to the others, showing a high K_a value (11846.77 μM). The metal chelator BP11, through ITC experiments, demonstrated tight binding for Zn^{2+}. It was also shown in this study that all four compounds inhibit MBLs efficiently. Kinetic studies confirmed that BP9 was the best inhibitor among the other four compounds. Therefore, we can conclude that these potential metal chelators may be used as lead molecules for future drug candidates. Future studies will focus on X-ray crystallography, which involves the study of direct interactions of the drug with specific residues of the protein NDM-1. In addition, a study should be carried out to determine whether the metal chelators have the same effect as in NDM-1 with other MBLs such as IMP, VIM and SPM.

DECLARATION 1-PLAGIARISM

I, Deidre Natalie Govender declare that

1. The research reported in this thesis, except where otherwise indicated, and is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written but the general information attributed to them has been referenced
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Deidre Natalie Govender

Signed:……

Date: 11/04/2019

LIST OF PUBLICATIONS

1. Chelators on β-lactam antibiotics; the way to fight metallo-β-lactamases

Authors: **Deidre N Govender**, Sibusiso Maseko Thavendran Govender, Tricia Naicker, Johnson Lin, Yasien Sayed Glenn E.M. Maguire, Gert Kruger.*

Unpublished

Deidre N Govender: Principal investigator**,** performed the experiments and wrote the paper.

Sibusiso Maseko, Thavendran Govender, Tricia Naicker, Johnson Lin, Yasien Sayed,

Glenn E.M. Maguire and Gert Kruger: Supervised the project.

2. Optimized procedure for recovering HIV-1 protease (C-SA) from inclusion bodies

Authors: **Sibusiso B Maseko**, Deidre Govender, Thavendran Govender, Tricia Naicker, Johnson Lin, Glenn E.M. Maguire, Gert Kruger

DOI: 10.1007/s10930-018-9805-7

Published, the protein journal

Sibusiso B Maseko: Performed the experiments and wrote the paper

Deidre Govender: Helped in some of the experiments for her training and wrote corresponding parts of the paper.

Thavendran Govender, Tricia Naicker, Johnson Lin, Glenn E.M. Maguire and Gert Kruger supervised the project.

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To my heavenly Father, thank you for never leaving me or forsaking me through this journey. Thank you for the strength, the perseverance, and endurance, for if it hadn't been for You, I don't know where I'd be today.

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To my best friend, Keith, thank you for believing in me, and pushing me when I felt like giving up.

LIST OF ABBREVIATIONS

AMA	Aspergillomarasmine A
βLs	β-lactamases
CD	Circular dichroism
CDS	Circular dichroism spectroscopy
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HEPES	*N*-(2-hydroxyehtyl) piperazine- *N'* –ethanesulfonic acid
IMP	Imipenemase
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
IR	Infrared
ITC	Isothermal calorimetry titration
MBL	Metallo–β-lactamase
MDR	Multidrug resistance
MRSA	Methicillin-resistant *Staphylococcus aureus*
MST	Microscale thermophoresis
PCR	Polymerase chain reaction
NDM	New Delhi Metallo–β-lactamase
SBL	Serine-β-lactamase
SDS	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SPR	Surface plasmon resonance
SPRS	Surface plasmon resonance spectroscopy
SUMO	Small ubiquitin-related modifier
TACN	1,4,7-triazacylcononane
TAE	Tris-acetate-EDTA
VIM	Verona Integron
VRE	Vancomycin-resistant enterococci

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CHAPTER ONE

General Introduction and Overview

1. Background

Beginning from the 1920s with the discovery of penicillin, antibiotics have been revolutionary in the field of medicine [1]. Antibiotics have been saving the lives of millions of people every year [2]. It has reduced the incidence of infection and has even been employed prophylactically to prevent infectious diseases [3]. However, a point of crisis has recently emerged whereby antibiotics are no longer effective even against some of the most common infections. The increase of resistance to numerous antimicrobial agents in pathogenic bacteria is emerging as a world-wide health concern [4]. Antibiotic resistant bacterial infections are regarded as one of the leading global causes of patient mortality and morbidity [5].

During the previous decade, the central focus on eradicating multidrug-resistant (MDR) microorganisms were on Gram-positive bacteria viz. methicillin-resistant *Staphylococcus aureus (MRSA)* and vancomycin-resistant enterococci (VRE) [6] Drug companies have improved antimicrobial agents to eradicate these bacteria, giving rise to the development of various new drugs with novel mechanisms of action such as linezolid and daptomycin [7]. In addition to Gram-positive bacteria, MDR Gram-negative bacteria is becoming a serious health concern [8].

In recent reports, the Infectious Diseases Society of America distinctively unveiled three categories of MDR Gram-negative bacilli, specifically, extended-spectrum cephalosporin-resistant *Escherichia coli* and *Klebsiella* spp., MDR *Pseudomonas aeruginosa*, and carbapenem-resistant *Acinetobacter* spp [9]. Unfortunately, there are

no new classes of antibiotics developed for, in particular, MDR Gram-negative bacilli [10].

Many mechanisms exist that render bacteria resistant to β-lactam antibiotics, for example, drug inactivation [11], altered target site [12] and reduced drug accumulation [13]. The expression of β-lactamase is recognised as a leading mechanism of antibiotic resistance against β-lactam antibiotics [14]. The β-lactamase enzymes are responsible for the cleavage of the β-lactam ring thus rendering the antibiotic inactive [15]. They are found commonly within Gram-negative and Gram-positive bacteria [16]. These enzymes are encoded by genes that are situated on either the bacterial chromosomes or plasmids [17]. They consist of two broad families, serine-β-lactamases (SBLs) and metallo-β-lactamases (MBLs). SBL enzymes require serine residues to carry out hydrolysis whereas MBL enzymes require zinc ions [18].

The emergence of antibiotic resistance has accelerated a new era in the development and advancement of novel antibiotics sharing the same target site, the β-lactam ring [19]. Development of potential inhibitors to combat MBL mediated resistance would be a therapeutic measure that can sustain the life span of β-lactam antibiotics [20]. In this approach, the antibiotic is administered in conjunction with the inhibitor, upon which the antibiotic remains effective, as is the case with SBL treatment currently [21]. Currently, there are no commercially available inhibitors of metallo-β-lactamases. Therefore the primary focus of this study is to evaluate novel drugs targeting metallo-β-lactamase producing bacteria.

1.1. Antibiotics

1.1.1. History

The development of antimicrobial agents has emerged as a major scientific accomplishment of the 20th century [19]. In the pre-antibiotic era, pneumonia, tuberculosis and dysentery were the primary reason for high human mortality rates [22]. During that time, there were limited therapeutic measures. Two noteworthy antimicrobial agents, arsphenamine [23-25] and sulphonamides [26, 27], were discovered to treat pathogenic illnesses such as syphilis and urinary tract infections, however these compounds displayed many side effects [28]. During the early part of the 20th century many advancements in public health occurred including vaccinations and sanitation [29]. At the epoch, Alexander Fleming discovered the first β-lactam antibiotic, penicillin G, that changed medicinal history [30]. The usage of antibiotics not only saved many lives by therapeutic measures but also through prevention of nosocomial infections. Penicillin G has been employed in clinical practices from 1941 due to its increased chemical stability over its parent compound. Since then streptomycin, chloramphenicol and tetracycline were discovered [31, 32]. Thereafter several classes of antimicrobial agents have been identified and continue to emerge in recent times accompanied with robust antimicrobials inclusive of novel β-lactams [33].

1.1.2. β-lactam antibiotics

After successful clinical trials, penicillin G was the first antibiotic applied in general medicine. As time progressed, penicillin derivatives were thereafter identified, such as penicillin V [34] and the future appeared very promising [35]. The rise of penicillin resistance bacteria became problematic and were seen in greater numbers worldwide. This prompted researchers to search for novel β-lactams through various microorganisms [13]. As a result, numerous novel classes of β-lactam antibiotics were

discovered namely, the carbapenems [36], cephems [37] and monobactams [38]. Each of these compounds have the common four-membered ring within their structure, which comprises of the β-lactam (CO – NH) bond [39] (figure 1.1). These antibiotics attack the bacterial cell wall by inhibiting peptidoglycan synthesis through covalent binding to the serine residue found at the active site of trans-peptidases, therefore portraying bactericidal activities [40]. Essentially, β-lactam antibiotics are responsible for silencing trans-peptidases by inhibiting the crosslinking step which is crucial for the production of peptidoglycan.

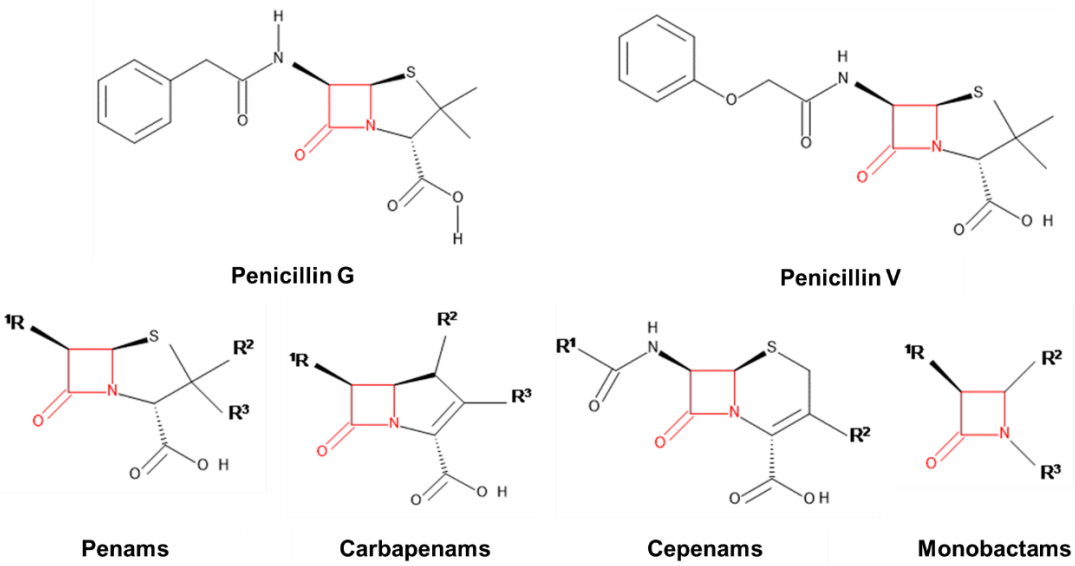

Figure 1.1: Chemical structures of various β-lactam antibiotics with the β-lactam ring in red [3] (open access).

Therefore, development of the bacterial cell wall is prevented which results in bacterial death by changes in osmotic pressure [41]. All of the β-lactam antibiotics have been essential for treating various infections since they have increased solubility, effectiveness, easy administration, low cost and limited side effects [42] .

1.1.3. Antibiotic resistance

The effectiveness of therapeutic agents is generally compromised by the development of resistance [43]. Bacteria can develop resistance through various physiological and biochemical mechanisms such as drug inactivation, alteration of target site and

reduced drug accumulation [4]. Table 1.1 highlights the main three mechanisms of resistance.

Table 1.1: Mechanisms of resistance against antibiotics.

Type	Mechanism of resistance
Drug inactivation	Drug degradation by enzymes such as β-lactamases that hydrolyse β-lactam antibiotics resulting in inactive antibiotics [44].
Alteration of target site	Mutation or modification of target site resulting in the inability of the antibiotic to interact with the target site [45].
Reduced drug accumulation	Efflux pumps or decreased cell permeability results in the decrease of the internal cellular concentration of the antibiotic with high efficiency [46].

The most prevalent mechanism of resistance against β-lactam antibiotics is through the generation of hydrolytic enzymes, β-lactamases [47]. Numerous antibiotics possess hydrolytically susceptible bonds such as amide bonds which are crucial for biological activity. The β-lactamase enzymes hydrolyse the amide bond of the β-lactam ring which results in the inactivation of antibiotics [48].

1.2. β-lactamases

In 1989, there were less than 100 β-lactamases identified worldwide, however, there are currently more than 1800 examples [49]. As mentioned earlier the β-lactamases are divided into two classification schemes established on functional characteristics [33] and sequence similarity [50]. These enzymes are further categorised into four classes: *A*, *B*, *C* and *D* [51]. The classes *A*, *C* and *D* are represented as the serine-β-lactamase whereas class *B* represents metallo-β-lactamases [14].

1.2.1. Serine β-lactamases

Serine β-lactamases (SBL) comprise of key structural features that allow them to recognise and carry out hydrolysis of the β-lactam ring [49]: (1) a polar pocket that has an affinity for carboxyl molecules; (2) an oxyanion that attract and stabilises the carbonyl oxygen of the antibiotic; (3) a catalytic serine residue that attacks the carbonyl carbon which breaks the amide bond; (4) conserved amino acid residues involved in deacylation [52].

All SBL enzymes possess the same α and α/β domain, however they are distinguished by their individual amino acid sequence identity [53]. Regardless of the differences in this sequence, the residues found at the active sites are conserved. There is mechanistic similarity between SBL's and serine proteases [54]. Both enzymes are hydrolases and both make use of acylation/deacylation of a serine residue in their mechanism of action (figure 1.2) [55].

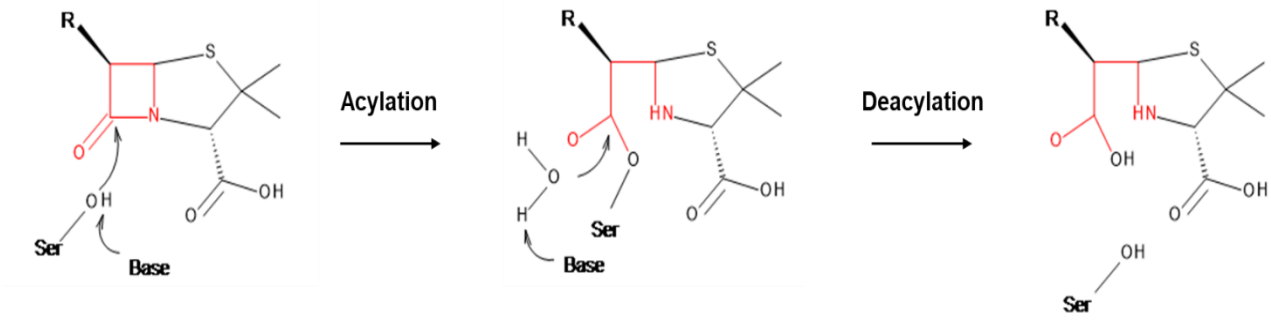

Figure 1.2: General scheme of the catalytic mechanism of SBLs [55] (open access).

As stated earlier, SBLs are categorised into three groups, class *A*, *C*, and *D*. Class A enzymes predominantly hydrolyse penicillin and can be inhibited by clavulanic acid [56] . Class C enzymes preferentially hydrolyse cephalosporin antibiotics but cannot be inhibited by clavulanic acid [57]. Class D enzymes have the ability to hydrolyse

oxacillin. Inhibitors such as clavulanic acid and tazobactam unfortunately have a wide range of efficacies across the general family of serine β-lactamases [58].

1.2.2. Metallo-β-Lactamases

Metallo- β-lactamases (MBLs) are known as the most prevalent β-lactamases. MBLs have the ability to hydrolyse all classes of β-lactam antibiotics, excluding monobactams [59]. Inhibitors that display an effect on serine-β-lactamases do not inhibit MBLs [10]. These inhibitors include; sulbactam, tazobactam or asclavulanate [7]. However metal chelators such as ethylenediaminetetraacetic acid (EDTA) can inactivate MBLs [60]. Since the 1990's, MBLs became a worldwide health concern [61] due to the rapid dissemination of Imipenemase (IMP-) and Verona Integron (VIM-) metallo-β-lactamases. These enzymes are located inside integron structures as gene cassettes and are linked with transposons [62]. The enzymes can be introduced on a bacterial chromosome or a plasmid. The propagation of the genetic material of MBL enzymes to various bacteria is carried out by integrons which is responsible for the circulation of resistance genes and are found in plasmids [63]. This is the formula for the rise of multidrug resistant bacteria.

1.2.2.1. Structure of metallo-β-lactamase

Metallo-β-lactamases portray a vast range of sequences with a low percentage of identity amongst other enzymes [64]. According to X-ray structures many MBLs exhibit similar structures and possess a αβ/βα sandwich feature together with an active site situated at the interface amid domains [65, 66]. This structure holds up to six residues within the active site containing mono- or di- zinc ions for catalytic activity [32].

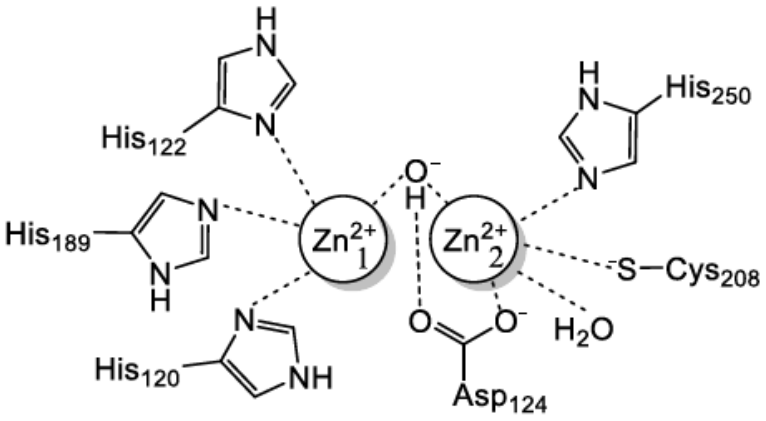

Figure 1.3: Structure of B1 Metallo-β-lactamase and active site [3] (open access)

1.2.2.2. Zinc coordination

The Ambler classification system is employed to place MBLs into three subclasses based on their structure and zinc ion requirement, B1, B2 and B3 [50, 67]. The B1 subclass possesses a zinc ion (Zn1) that attaches to three histidine (His) residues (H116, H118, H196) [68] whilst the second zinc ion (Zn2) binds to three differing residues (D120, C221, H263) [69]. This subclass comprises the most clinically significant members such as VIM [70], IMP [71] and the recently emerged New Delhi MBL (NDM) [61].

The B2 subclass possesses only a Zn2 site, (D120, C221, H263) [72]. The difference between the B1 and B2 subclasses are that the former exemplifies a broader spectrum of activity against β-lactam molecules [73].

The last subclass, B3 possesses a distinguished Zn1 binding site compared to B1 and B2, (H/Q116, H118, H196) and a pronounced Zn2 binding site that excludes a cysteine residue (D120, H121, H263) [74]. The B3 subclass is similar to B1 and B2 with regards to structural homology only and not sequence homology [75]. The overall structure for all three subclasses are analogous [76].

1.2.2.3. Catalytic mechanism

According to Bounaga *et al* [77], a mechanism of action for the monozinc state of subclass B1 has been suggested (figure 1.4). It begins with the binding of a zinc atom with the histidine residues (H116, His118, and His196) together with a water molecule [78]. The zinc ion operates as a Lewis acid and the pK of the water molecule is reduced [79] allowing it to persist as a hydroxide ion at a neutral pH. The carbon belonging to the carboxyl group of the β-lactam ring undergoes a nucleophilic attack by this ion [80]. This results in the production of a tetrahedral intermediary, which is advanced further by the deprotonation of the –OH group performed by an Asp120. This second intermediate is stabilised by interrelation with the same zinc ion. Consequently, the nitrogen of the β-lactam ring is supplied with a proton by the Asp120 residue which facilitates cycle opening and cleavage of the amide bond [81].

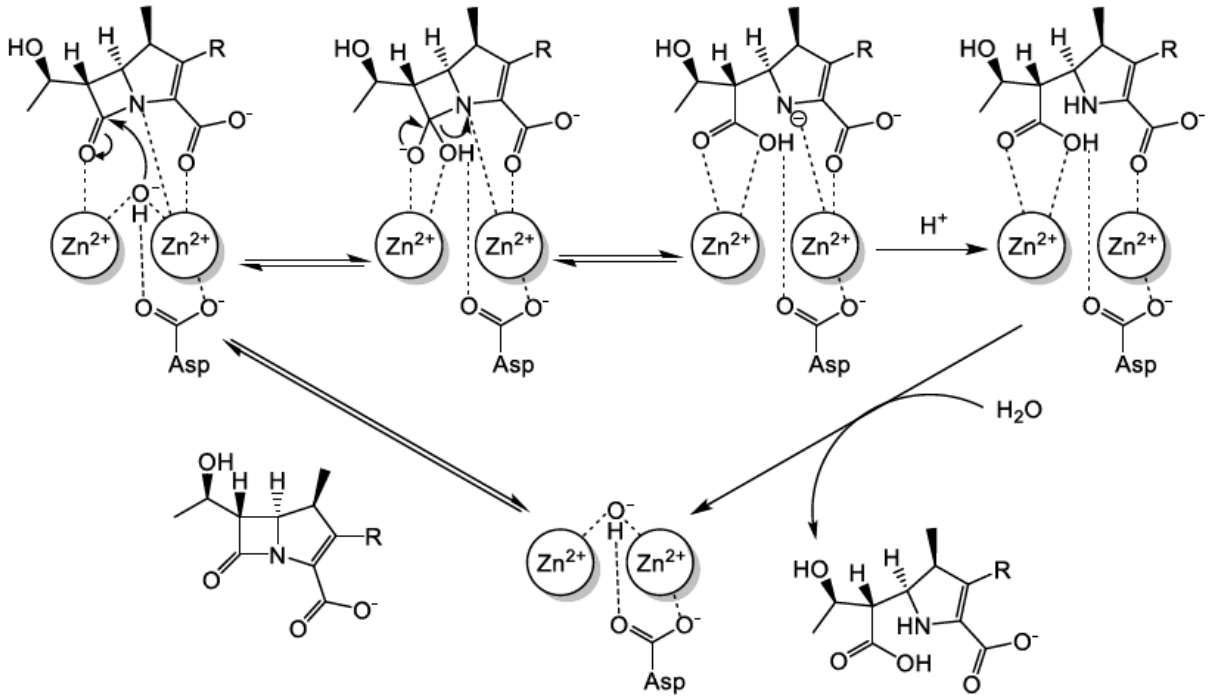

Figure 1.4: Proposed catalytic mechanism of B1 MBL against a carbapenem [82] (open access).

1.3. β-lactamase inhibitors

An excellent strategy to combat the resistant mechanism against antibiotics is the development of inhibitors for the specific enzymes that cause drug resistance. Such agents will prolong the lifespan of current β-lactam antibiotics [23]. Serine β-lactamases inhibitors such as clavulanic acid evidently show that this approach is a success [83]. Clavulanic acid was first serine β-lactamases inhibitor used in combination with amoxicillin in 1984 [84]. This dual therapy paved the way for many more combination therapies such as ampicillin/sulbactam, piperacillin/tazobactam and ceftazidime/avibactam [85]. Unfortunately, despite years of research and development, there are no clinically available inhibitors of MBLs, but there are some that display intriguing potential [86]. From literature, these examples can be placed into groups based on their mechanism: (1) metal ion binding, (2) covalent modification, (3) allosteric inhibitors, and (4) unknown mechanism [87].

Metal binding inhibitors have been regarded as the most promising of these approaches due to the active site and catalytic mechanism being highly conserved within B1 MBLs. Consequently, this group of inhibitors stand a greater chance of broad-spectrum inhibition. Within this approach, there are two essential modes of action; metal ion sequestration and competitive inhibition. EDTA is a well-known metal chelator and has been shown to inhibit MBLs (figure 1.5) [20]. Other examples of potential chelating inhibitors are dipicolinic acid, together with recent examples such as SIT-Z5 and aspergillomarasmine A [88-90].

Despite the discovery of these potent metal inhibitors, there are concerns about their selectivity [73, 90]. Other potential metal inhibitors exist, such as captopril, which forms a ternary complex with MBLs. According to studies, it is found that L-captopril coupled with thiol(ate) can inhibit MBLs through chelating the zinc ions in the active site.

However, this inhibitor coupled with thiol leads to side effects and allergies, making these drugs inadequate for clinical use [91].

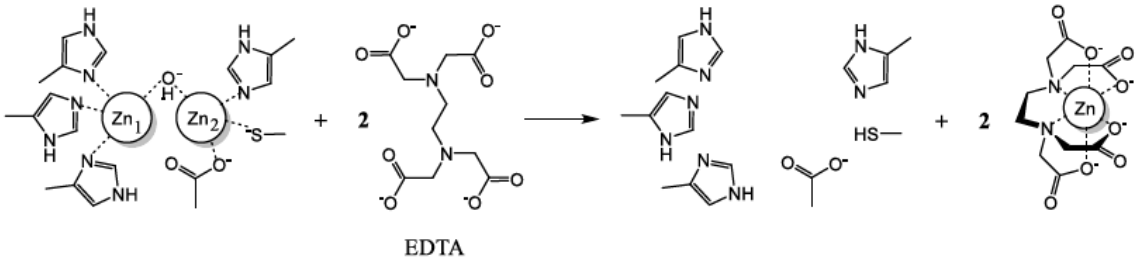

Figure 1.5: Metal ion stripping of MBL by EDTA [3] (open access).

1.4. Analytical methods

Over the years there are many techniques that have been developed to study molecular molecules such as proteins and its application. The following techniques are used to achieve the aims of this study.

1.4.1. Recombinant DNA technology

This technology is employed to treat various severe diseases by generating novel vaccines and pharmaceuticals amongst other broad applications [92, 93]. Recombinant DNA technology entails externally modifying genetic elements to achieve augmented characteristics of a product of interest [94]. It incorporates the insertion of DNA from various sources, containing a particular gene sequence of interest. Restriction digestion is enforced to produce various DNA fragments using specific restriction enzymes for target sequence sites. Thereafter DNA ligase is used to ligate the restricted fragments of the desired gene to the selected plasmid vector. This vector is introduced into an expression host, where it is grown to generate many copies of the inserted DNA fragment in culture. Lastly, clones comprising of the desired gene are chosen and harvested (figure 1.6).

Many expression systems exist for heterologous protein production, namely; bacteria expression system, yeast expression system, baculovirus expression and mammalian expression system. *Escherichia coli* is one of the most common systems due to its high propagation rate, well-illustrated genetics and accessibility of high cloning vectors [95]. It is essential that expression strains comprise of genetic elements necessary for expression such as replicon, promoter, affinity tags, tag removal and a selection marker. *E. coli* BL21 is highly prevalent, since it is inexpensive, able to propagate in minimal media, implausible to strive in host tissues and contains the necessary genetic elements mentioned above for expression. One of the most frequently employed expression system for recombinant protein expression is the T7 promoter-based pET expression system [96]. An essential feature of the pET system is that expression is induced by introducing a source of T7 RNA polymerase in the host cell. The T7 RNA polymerase is selective and active that most of the cell's resources are converted to target gene expression. The desired product can contain more than half of the total cell protein a few hours following induction thus making this system very useful [95].

Even after careful selection of a plasmid and host, it cannot be predicted if the desired protein will be produced in abundance and in an active soluble form. If there is no or low expression, using a defined media with glucose as a source of carbon may overcome this challenge. If there is inclusion body formation due to incorrect folding, the media can be supplemented with chemical chaperones and cofactors [94].

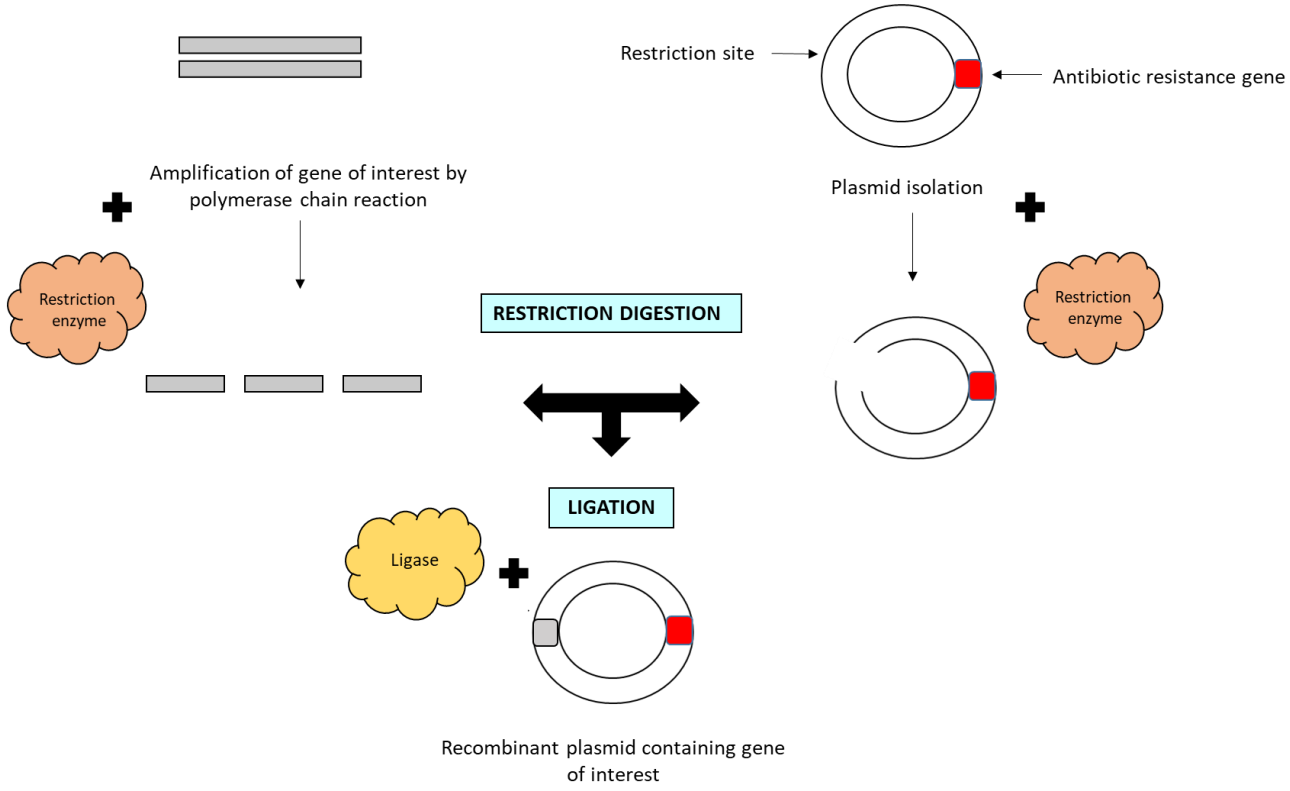

Figure 1.6: Schematic representation of recombinant DNA technology (adapted from [97]).

1.4.2. Overexpression of recombinant strain

Protein production involves manufacturing of a specific protein [98, 99]. It is accomplished through the expression of a manipulated recombinant gene within an expression system (organism), resulting in a high production of a particular protein of interest [100]. Protein production is carried out by transcription of target DNA to messenger RNA (mRNA), thereafter translation of mRNA into polypeptide chains [101]. This conclusively folds into a functional protein and utilized as a target for drug design [102]. Metallo-β-lactamases are a target for drug discovery. Hence, production of large amounts of this protein is required for research purposes.

1.4.3. Enzyme purification

Protein purification is essential for the function, structure and various interactions of the proteins [98]. Separation of proteins from various sources which was genetically engineered and produced by a host cell is accomplished through chromatographic

techniques [103]. The particular technique is selected depending on the characteristic of the protein [104]. The principle of the form of chromatography we are employing is where a mixture of molecules containing the protein of interest are adhered to a solid stationary phase and then segregated upon movement with the assistance of a liquid mobile phase [105]. One of the separation techniques is dependent on size of the molecules, which is called size exclusion. Molecules are separated according to their size or molecular weight and is usually applied to large molecules such as proteins [106]. This technique entails trapping smaller molecules in the pores of the stationary phase. The pores within the stationary phase possess different sizes which determines the size of the macromolecule. Another technique, hydrophobic interaction chromatography (HIC) (also know as 'salting out') involves separation according to the charge or hydrophobicity of the molecules. Proteins containing a hydrophilic and hydrophobic region are subjected to a HIC column under high salt buffer conditions. The salt reduces the solvation of sample solutes and exposes the hydrophobic regions along the surface of the protein [94]. This aids the adsorption of the hydrophobic regions to the hydrophobic regions on the solid support, which results in precipitation of the protein out of the solution. Lastly, affinity chromatography is used for the purification of biological molecules within a mixture by exposing molecular properties. Biological macromolecules such as enzymes and proteins interact with various molecules with high specificity through different types of bonds and interactions (hydrogen bonding, ionic interaction, disulphide bridges and hydrophobic interactions etc.) [103]. The high selectivity of affinity chromatography is caused by permitting the desired protein to interact with the stationary phase and be bound within the column in order to be separated from undesired molecules.

Affinity chromatography provides high selectivity, resolution and capacity in protein purification. It has the advantage of using the proteins biological structure for purification [107].

This technique employs affinity tags for the expression of proteins, such as His-tags which are commonly found in MBLs. Histidine is involved in the coordinate bond with metal ions. Therefore, if histidine molecules are inserted at the end of protein, the affinity of the protein for the metal ion is increased and purification can be easily carried out. When a protein has a His tag and is subjected to a carrier on which a metal ion such as nickel is immobilized, the histidine residue chelates the metal ion and binds to the carrier. Other proteins do not bind to the carrier, it can be removed by washing the carrier with a suitable buffer. Thereafter, by removing imidazole, the protein with the His tag is recoverable with high purity [105].

1.4.4. Characterization of enzymes

A range of biochemical techniques are applied to study the nature of living cells and to characterize certain molecular constituents that perform cellular functions [108]. Cellular functions involve accelerated chemical transfigurations that do not typically take place within a physiological environment [109]. The word enzyme is assigned to a macromolecular gene product that elevates the rate of a particular chemical reaction [110]. Characterization of various enzymes remain a necessity for the development of biological sciences and for profiling of proteins (physical, chemical and biological properties) [111].

Essential kinetic principles are elucidated for comprehending chemical reaction rates and the effects of the enzymes on the rates [110]. Enzymatic activity is interpreted as a quantification of the amount of enzyme present in a reaction. This can lead to determination of specific activity [112]. Specific activity for the enzymes is expressed

as units per mg enzyme protein. The enzyme unit is described as the amount of product released from a particular substrate per unit time. Specific activity is essential for the determination of the purity of a given mixture [113]. It is determined by dividing the number of units/mL by the protein concentration in mg/mL to get µmol/min/mg.

The determination of enzyme kinetic parameters, such as K_m and k_{cat} values are crucial in characterization of enzymes. The K_m value is equal to the substrate concentration at which the enzyme converts the substrate into a product at half its maximal rate and is therefore related to the affinity of the substrate for the enzyme [109]. The catalytic rate k_{cat} is the rate at which the product is formed when the enzyme is saturated with substrate and is therefore a reflection of the enzyme's maximum rate. The k_{cat}/K_m rate is called the specificity constant measure of how efficiently an enzyme converts a substrate into product [111]. The Lineweaver-Burk plot is employed as a graphical representation to determine the various kinetic parameters and for the analysis of the Michaelis-Menten equation:

$$V = Vmax[S]/ (Km + [S]) \quad (1)$$

For this study, it is important to characterize the recombinant MBL using a chromogenic substrate such as nitrocefin [114].

1.4.5. Circular dichroism spectroscopy (CDS)

As stated, characterization of proteins is crucial in the field of proteomics and structural genomics [115]. Circular dichroism (CD) is a useful tool to establish the secondary structure and to evaluate folding and the binding properties of proteins [116]. The amides of the polypeptide backbone of proteins contain chromophores. When the chromophores are aligned in arrays, "exciton" interactions causes their optical transitions to shift or split into multi-transitions [117]. As a result, different structural elements possess certain CD spectral characteristics [116]. A CD spectrum is thus

dependent on protein conformation, therefore CD can be employed to determine the structure of an unknown protein and estimate conformational changes that are caused by temperature, binding or mutations [117].

1.4.6. Surface plasmon resonance spectroscopy (SPRS)

SPRS is a technique employed for the analysis of biomolecule interactions including the determination of kinetic parameters [118]. This technique is widely utilized as a label-free biophysical process [119]. SPR is an excellent tool for studying protein-protein and protein-DNA interactions, peptide inhibitor studies, absorption rate of chemical molecules and to examine the binding of proteins to lipid membranes (figure 1.7) [120].

This optical technique is employed for the interaction detections of two varied molecules whereby one is mobile and the other is immobilized on a thin gold sensor film. At this surface layer, a change in the binding phases, result in a change in the refractive index. Thereby, alterations in the SPR signal communicate these events [121].

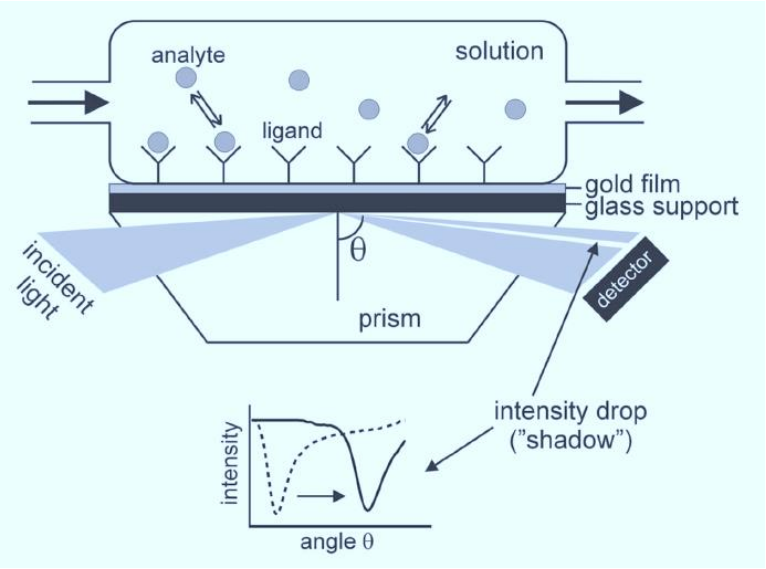

Figure 1.7: Diagrammatic representation of the interactions on the gold layer of a surface plasmon resonance spectrometer. SPR rises when light is internally reflected

from a metal-coated interface between two media of different refractive index. Upon interaction between an immobilized ligand and an analyte in solution occurs, the shadow is sifted on the detector [121] (Open access).

1.4.7. Microscale thermophoresis (MST)

Techniques to analyse proteins and their interrelations with various molecules are essential in order to evaluate cellular functions and for the production of pharmaceuticals [122]. Current advances have shown thermophoresis may supply valuable analyses of such interactions. MST is a technique that involves the stimulation of the movement of molecules by temperature gradients (figure 1.8) [123]. MST is a tool employed to characterize proteins however such investigations are troublesome due to weak thermophoresis response of proteins. In addition, only weak conformational changes upon binding are achieved by low molecular weight binders. An advantage of this technique is that it requires minimal sample and is contact free, which reduces contamination [124]. MST is measured within capillaries which contain fluorescence (aromatic amino acids of target molecule) that is excited and quantified through the same optical device.

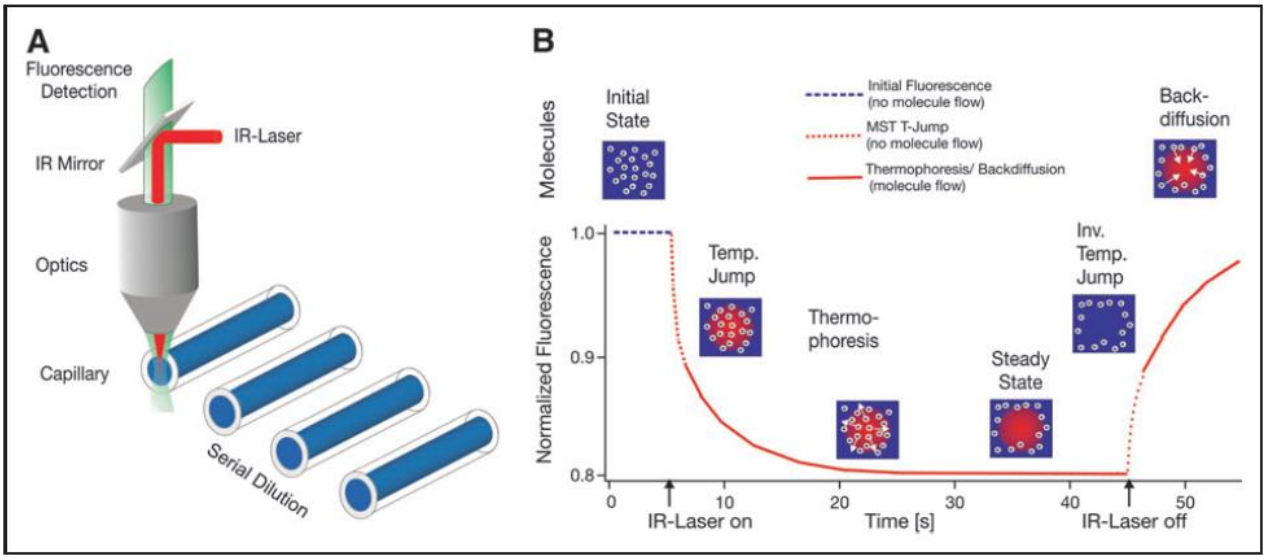

Figure 1.8: Systematic representation of microscale thermophoresis. An infrared (IR) laser is employed to heat the samples within the capillaries and is examined by fluorescence. Change in fluorescence represents the T-jump and thermophoresis directly at varied time scales. At the beginning of the reaction, the molecules are uniformly dispersed and a steady "initial fluorescence" is quantified. When the IR-laser is switched on, a rapid T-jump is visualised, a thermophoretic motion follows. The measurement of the decrease in fluorescence is measured for approximately 30 s. Switching off of the IR-laser results in an inverse T-jump, consequently, a backdiffusion of molecules is observed which is stimulated by mass diffusion [125] (open access).

1.4.8. Isothermal titration calorimetry (ITC)

ITC is regarded as a powerful tool that measures macromolecular interactions [126]. Amongst various measurement techniques for binding, ITC is the only one that can quantify both the magnitude of the binding affinity (K_d), the magnitude of enthalpy (ΔH) and entropy (ΔS) changes (figure 1.9) [127]. Other thermodynamic parameters can be determined such as Gibb's free energy (ΔG) from the following relationships [127]:

$\Delta G = -RT\ln K_a$

$\Delta G = \Delta H - T\Delta S$

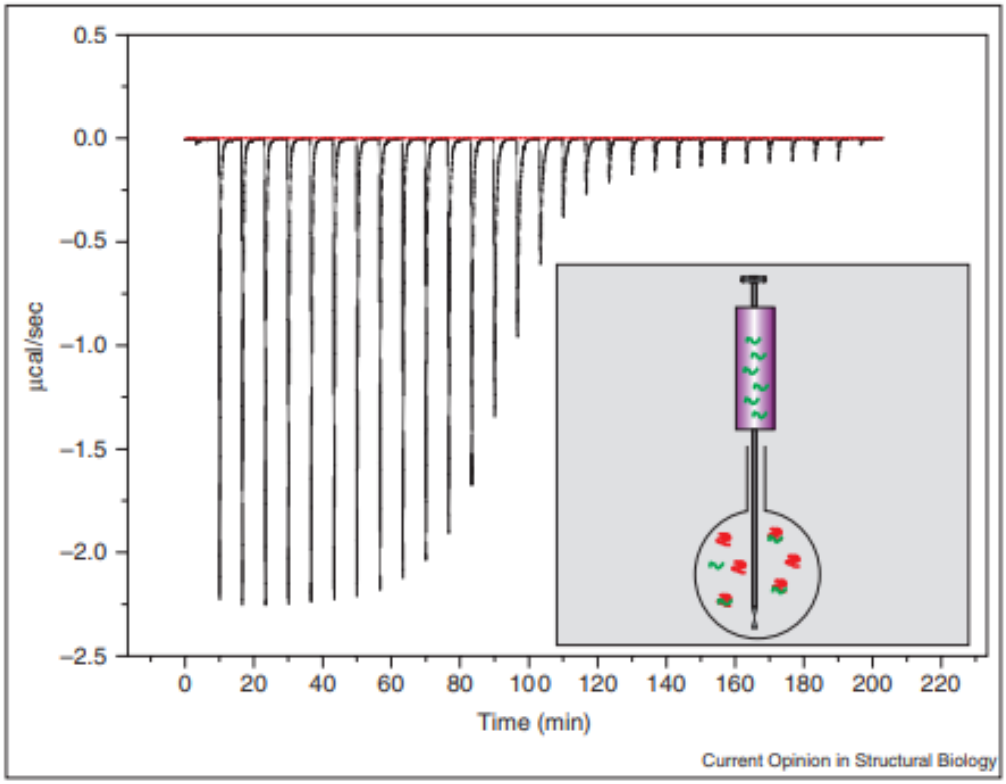

Figure 1.9: Systematic representation of isothermal titration calorimetry. [127] (open access).

In relation to figure 1.9, the inset represents the configuration of an ITC reaction cell. The volume of the cell is 1.4 mL and contains the protein solution (red). The injection syringe stirs the solution ensuring proper mixing and contains the ligand (green). During specific intervals, small volumes of the ligand solution are added into the sample cell, which results in titration heat effects. Upon saturation of the protein, residual heat effects are generated from dilution of the protein, which is subtracted before analysis. The thermogram shows the sequence of peaks, whereby each peak corresponds to a single injection. The y-axis is the time dependence of electric power needed to maintain the constant temperature difference between the reaction and reference cells.

1.4.9. Fluorescence quenching

Fluorescence quenching is a key tool for pharmaceutical detection applications. Proteins comprise of three aromatic amino acid residues, namely; tryptophan, tyrosine and phenylalanine which may contribute to their intrinsic fluorescence [70]. The tryptophan residue possesses a stronger fluorescence and higher quantum yield compared to the other aromatic amino acids. Tryptophan has an excitation of 280 nm, displaying a peak at 330 - 360 nm. Tryptophan fluorescence quenching of proteins by various quenchers such as drugs during protein-ligand interactions has been studied to confirm the binding site, to investigate the mechanism of protein-ligand binding and to determine possible conformational changes of a protein [128].

1.5. Aims and objectives study

Metallo-β-lactamases are enzymes that render microorganisms resistant to various β-lactam antibiotics. This study involves isolating the gene encoding this enzyme, cloning, expressing, purification of metallo-β-lactamase and subsequently, to study the kinetics of inhibition of MBL by potential chelators.

1.5.1. Aim

To determine the inhibitory effect of metal chelator inhibitors against a recombinant metallo-β-lactamase protein.

1.5.2. Objectives

- To clone and overexpress NDM-1 in *E.coli* BL21 cells.
- To purify NDM-1 using affinity chromatography
- To study the kinetics of inhibition of novel metallo-β-lactamases inhibitors on NDM-1

1.6. Outline of the thesis

The thesis is presented in a paper format in which each chapter is dedicated to addressing one or two research questions. In the first and last chapters, a general introduction and overall conclusion are provided, respectively, for the entire study.

The outline is therefore highlighted:

Chapter 1: Introductory background and where the main direction of the study is highlighted.

Chapter 2: Chelators on β-lactam antibiotics; the way to fight metallo-β-lactamases and drug resistance.

Chapter 3: Overall conclusion on the research outcome.

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CHAPTER TWO

Chelators on β-lactam antibiotics; The way to fight metallo-β-lactamases.

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Key words, NDM-1, Inhibition, Kinetics, Thermodynamics, ITC

Abstract

New Delhi Metallo-β-lactamase (NDM-1) is one of the most prevalent metallo-β-lactamases (MBLs), since it hydrolyses the most commonly employed β-lactam antibiotics. The rise of such β-lactamases (βLs), has given rise to the emergence of multi-drug resistant bacteria. It is amongst one of the most well characterized βLs but unfortunately, there are no clinically available inhibitors for NDM-1. Recombinant metallo-β-lactamase NDM-1 was obtained to evaluate potential metal chelators as inhibitors. The kinetic parameters of the recombinant metallo-β-lactamase NDM-1 were found to be 29.07 μM and 21.77 s^{-1} of K_m and k_{cat} respectively using a chromogenic substrate, nitrocefin. Herein, we present four metal chelators designated as BP1, BP9, BP10 and BP11, which we evaluated for their potential to inhibit NDM-1. The K_i values were 0.24, 0.16, 0.19 and 0.18 μM, respectively. BP9 was the best inhibitor for NDM-1 enzyme. The dissociation equilibrium constants (K_d), association equilibrium constants (K_a) and stoichiometry of the compounds with the respective target, NDM-1 were determined using fluorescence quenching. Fluorescence studies also demonstrated that BP9 has the highest K_a value (11846.77 μM). The chelator alone was then further investigated for its affinity to Zn^{2+} through isothermal titration calorimetry studies (ITC). BP11 was curated due to excess availability. The presumptive mechanism of action for our agents was given further credence by the large binding constants obtained in these experiments. We can therefore conclude that these inhibitors have potential as future drug candidates.

1. Introduction

β-lactams are the most widely used class of antibiotics [1] and the cornerstone for treating infections that are caused by both Gram-negative and Gram-positive bacteria [2]. These antibiotics are known for their high efficacy and low toxicity in humans [3]. Effective carbapenems, such as meropenem, are regarded as “last resort” agents against multidrug-resistant bacteria since they are stable in the presence of bacterial β-lactamases [4]. The constant use of carbapenems has led to the emergence of carbapenems- resistant Gram-negative pathogens [5]. Carbapenemase-producing bacteria are a global threat [6]. These enzymes are the primary mechanism of resistance [7]. They inactivate antibiotics by hydrolysing the C-N amide bond of the bicyclic β-lactam ring of antibiotics [8]. Carbapenamases are divided into two groups based on their molecular properties and amino acid sequences, serine β-lactamases (SBLs) and metallo-β-lactamases (MBLs).

Serine β-lactamases require a serine residue at the active site which acts as a nucleophile in the cleavage of the amide bond of β-lactam antibiotics and can be inhibited by several inhibitors, such as, clavulanic acid, avibactam, tazobactam and sulbactam in clinical trials [9]. On the contrary, metallo-β-lactamases are more detrimental due to their high activity and broad substrate spectrum [10] and therefore pose as a major health risk [11]. MBL-producing bacteria can inactivate several β-lactam antibiotics, such as carbapenems, cephalosporin, penicillin and aztreonam [12]. MBLs are zinc dependant and require these ions for the hydrolysis of β-lactam antibiotics.

An MBL named New Delhi metallo-β-lactamase (NDM-1) was discovered in 2008 in a Swedish patient returning from India [13]. Since then, NDM-1 has spread rapidly throughout the world, except Antarctica [14], making its first appearance in South Africa in September 2011 [15]. The NDM-1 protein is a Zinc dependent MBL belonging to the subclass B possessing a Zn^{2+} at the active site which is required for catalysis

[10]. The enzyme is responsible for the degradation of all classes of β-lactam antibiotics except monobactams [16].

A proven approach to restore the activity of antibiotics and combat antibiotic resistance is through the combination of a metal inhibitor and the β-lactam antibiotic [17]. A range of compounds including peptides, chemically synthesized inhibitors and natural products have been reported [18]. There are no current drugs or drug combinations available clinically to restore the efficacy of β-lactam antibiotics in strains that contain MBLs.

Even though there are no clinically available MBL inhibitors, there are potential inhibitors such as aspergillomarasmine A (AMA) which is produced by a fungus. This compound displays inhibition of NDM-1 and VIM-2 and restores the activity of meropenem [19]. However, the synthesis of this natural product is complicated. Recent studies within our group have investigated a zinc chelating moiety, 1,4,7-triazacylcononane (TACN) for its inhibitory activity against MBL-producing bacteria [20]. TACN is a cyclic organic compound that is derived from cyclononane. According to the study, this compound restores the activity of meropenem and therefore inhibiting the MBL-producing bacteria. Despite the inhibitory activity of this compound, TACN is not bioavailable.

Herein, we investigate the effect of novel metal chelators attached to antibiotics on the metallo-β-lactamase protein, NDM-1.

2. Materials and methods

2.1. Bacterial strain, antibiotics and other reagents

All bacterial strains and restriction enzymes were purchased from Thermo scientific. (USA) Ampicillin and nitrocefin was purchased from Sigma-Aldrich. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from ThermoScientific. Inhibitors were synthesized at Catalysis peptide research unit (CPRU)

2.2. Synthesis of the metal chelators/ inhibitors

The metal chelators/inhibitors were synthesized by the Catalysis and Peptide Research Unit of the University of Kwa-Zulu Natal, Westville, Durban, South Africa for the purpose of this study. Our previous studies indicated that the chelators alone exhibit very good β-lactamase inhibition however, poor pharmacokinetics were observed, hence we envisaged the need to explore conjugation of the chelator to enhance its properties, this was achieved by attaching a chelator to known antibiotic cores. (The synthetic route of these structures and above mentioned results will not be disclosed due to filing the corresponding patent and the requirement to protect the intellectual property.)

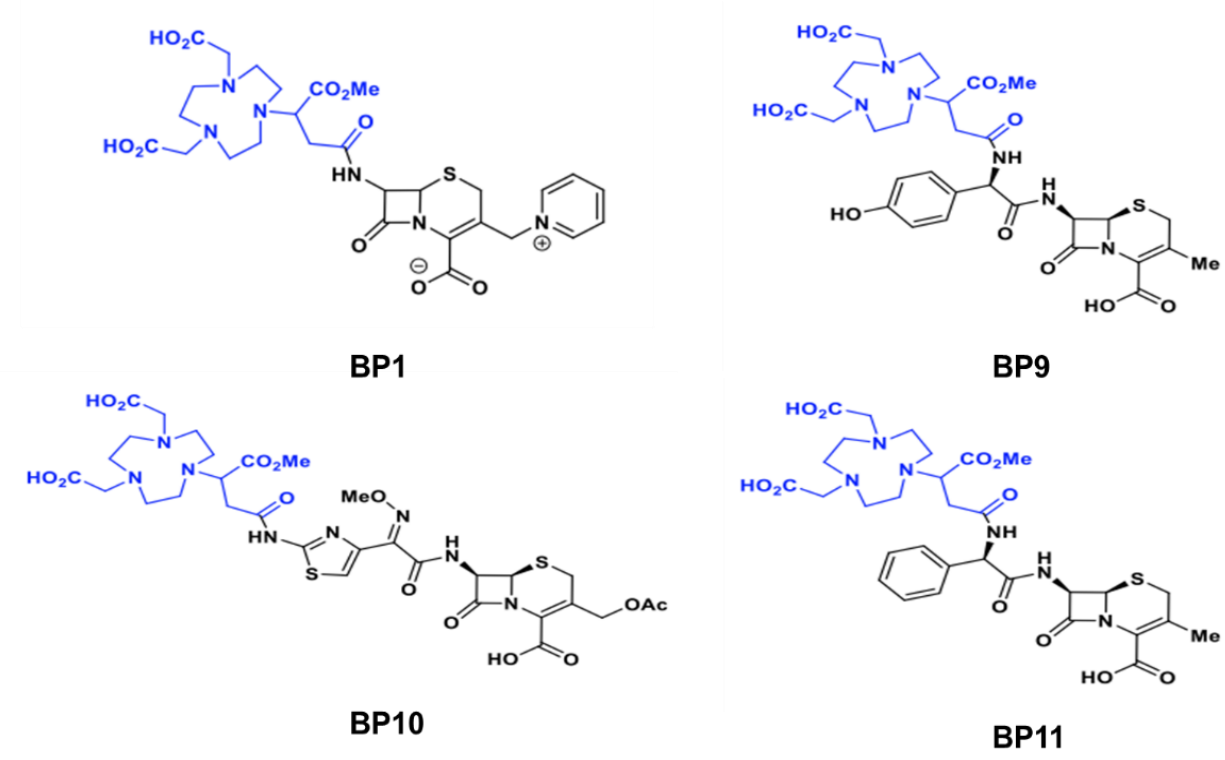

Figure 2.1: Structure of the metal chelators attached to β-lactam antibiotics.

2.3. Cloning of NDM-1

The metallo-β-lactamase gene was synthesized by GenScript USA Inc (GenBank Accession number NC_023908). NDM-1 genes were amplified by performing colony PCR with primers containing EcoRI and XhoI restriction sites in the forward and reverse primers respectively (forward: 5' – GAA TTC TCA GCG CAG CTT GTC GGC CAT – 3' and reverse 5' – CTC GAG TCA ATG GAA TTG CCC AAT ATT ATG – 3'). These genes were cloned in pET302 champion vector. The amplified genes and vector were restricted and purified using a DNA Clean & Concentration™ kit (Zymo Research) as per manufacturer's instruction. Thereafter, the restricted gene and vector were ligated and transformed into BL21 (DE3) pLysS using a heat shock method. Transformed cells were plated in antibiotic selection plates and grown overnight at 37°C. Positive clones were confirmed through colony PCR and restriction digestion.

2.4. Agarose gel electrophoresis

A 1 % (w/v) agarose gel in Tris-acetate-EDTA buffer (TAE) was prepared as a 50 times dilution from stock solution containing 242 g Tris, 57.1 mL acetic acid and 100 mL 0.5 M EDTA at pH 8 [21]. The DNA fragments were visualised by staining the gel using 0.5 µg/ml ethidium bromide, and ran at 100 V, 64 mA for approximately 45 min. The gel was removed from the electrophoresis tank and viewed using Syngene Gel documentation system (Vacutec South Africa).

2.5. Expression and purification of NDM-1

Competent cells *E.coli* BL21 (DE3) were transformed with the recombinant plasmid containing bla_{NDM-1} and grown overnight in an antibiotic selection plate (ampicillin 50 µg/mL). A single colony was used to inoculate 10 mL Luria-Bertani (LB) medium containing ampicillin and grown overnight at 37˚C. The overnight culture (1 %) was used to inoculate a fresh 1 Litre LB medium containing ampicillin. The culture was incubated for 2-3 hours at 37˚C until culture reached an OD_{600nm} of 0.6. A concentration of 0.4 mM IPTG was added into the culture and incubated overnight at a predetermined temperature, 16˚C. The cells were harvested by centrifugation (8000 x *g*, 4˚C and 5 min) and the resulting pellet was stored at -20˚C. The pellet was thawed and resuspended in 200 mL Tris-HCl (pH 7.5). The cells were disrupted by sonication (Omni International Sonic Ruptor 400 Ultrasonic homogenizer) with an operating frequency of 50 kHz. The homogenized sample was subjected to 30s on/30s off pulses for 20 min. To reduce thermal effect, samples were placed on ice. Sonicated samples were subjected to centrifugation (10 000 x *g*, 4˚C and 15 min) to remove bacterial debris. The resulting supernatant (crude protein) was analysed using SDS-PAGE.

The crude protein (20 mL) was loaded onto a cobalt chromatography column (Thermo Scientific) (5 mL), which was pre-equilibrated with equilibration buffer (20 mM sodium phosphate, 300 mM NaCl and 5 mM imidazole). After loading the column was then washed with two column volumes of the same buffer. Bound proteins were eluted with

elution buffer (20 mM sodium phosphate, 300 mM NaCl and 300 mM imidazole). The concentration of purified protein were measured using NanoDrop. In addition, the metallo-β-lactamase activity of the protein fractions were determined using a chromogenic substrate, nitrocefin. Protein purity was verified using SDS PAGE.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The purified protein was subjected to SDS-PAGE analysis according to literature [22]. The protein purity was resolved using 12 % acrylamide gel. 25 µg of protein sample was used to carry out gel electrophoresis. To prepare loading the SDS-PAGE, 30 µL of the protein sample was re-suspended using 5 µL of gel loading buffer which was then boiled for 10 minutes. The gel was submerged in 1 x SDS running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS) at 80 V. Thereafter, the gel was stained using Comassie blue R250 (1 g of Coomassie blue R250 in 1 L of destaining solution) for 2 hours and then de-stained with a de-staining solution (40 % (v/v) methanol and 10 % acetic acid (v/v)) for approximately 2 hours. The gel was then viewed and captured using Syngene Gel documentation system (BioGenie System 2). The molecular weight of NDM-1 was confirmed using a standard protein marker (BioRad).

2.7. Kinetic studies

The kinetic method was adapted from literature [23]. The enzymatic activity of NDM-1 was determined using a chromogenic substrate. Nitrocefin is a cephalosporin that has a conjugated dinitrostyrene group. Upon hydrolysis of the C-N bond of the lactam, the absorbance changes, which causes a shift of colour from “yellow” to “red”. This assay was performed using a 96-well plate. Hydrolysis of the chromogenic substrate was characterised by a decrease in absorbance at 490 nm. Catalytic properties (K_m, k_{cat}, and k_{cat}/K_m) of NDM-1 were determined. To each well, 0.5 µL of nitrocefin (final concentration of 0.1 mM for K_m) and NDM-1 (0 µM – 5 µM for k_{cat}), was added to a final volume of 100 µL with 50 mM *N*-(2-hydroxyehtyl) piperazine- *N'* –ethanesulfonic acid (HEPES). Under Michaelis-Menten reaction conditions, the enzyme parameters were determined (Eq. 1) and Lineweaver-Burk plots were developed from the data (Appendix: Figure S1). All enzyme activity assays were performed at 37˚C on a Jasco V-630 spectrophotometer.

$$V = V_{max}[S]/ (K_m + [S]) \quad (1)$$

2.8. Inhibition studies

The inhibition studies were conducted at 37˚C by measuring the rate of nitrocefin hydrolysis using 7 µM of purified NDM-1 in the presence of the four inhibitors. Four metal chelator inhibitors were used in this study, BP1, BP9, BP10 and BP11. The initial stock of inhibitor concentration (1000 µM) was made up in HEPES, pH 7.0. Approximately eight different inhibitor concentrations were selected (0-500 µM). Each well contained 5 µL of NDM-1 stock, 0.5 µL of 0.1 mM nitrocefin, metal chelator (0 – 500 µM) and made up to a final volume of 100 µL using HEPES buffer.

2.9. Thermodynamic studies

2.9.1. Fluorescence quenching

Thermodynamic studies were carried out according to literature [24]. A Jasco V-630 spectrofluorimeter (Jasco International Co., Ltd, Japan) was used to perform spectrofluorometric studies. Such studies allows for the determination of tertiary structural alterations in MBL NDM-1 by the inhibitors. The experiment was carried out by analysing the interaction between each metal chelator inhibitor and the purified enzyme. Tryptophan residues in the NDM-1 proteins were excited at 295 nm (representing a local probe). The emission intensity of the tryptophan residues was monitored at 482 nm. The concentration of the metal chelator (in 1 µL portions of stock solution added) was increased incrementally to NDM-1 (1 µM) in HEPES solution. The stock solution of metal inhibitor concentration (~ 2.7 mM) was made up in 50 mM HEPES buffer in a final volume of 100 µL. The NDM-1 and inhibitor solution was incubated for 10 min, after which, the change in fluorescence was recorded. At increasing concentrations of the metal chelator, a decrease in fluorescence indicated inhibitor quenching by the tryptophan fluorophores of the NDM-1 enzyme. Stern-Volmer equations 2 and 3 were used to calculate various thermodynamic parameters derived from the resultant plots. Equations are shown as follows:

$$F_0 / F = 1 + K_{SV}[Q] \qquad (2)$$

$$\text{Log } [\Delta F/F] = \text{Log } [k_a] + n\text{Log } [Q] \qquad (3)$$

Where F_0 and F represents the fluorescence intensities in the absence and presence of a quenching agent BP1, BP9, BP10, BP11. K_{SV} is the Stern- Volmer constant and was calculated from the slope of Fo/F vs [Q], whereas, the slope and intercept of plot log ($\Delta F_o/F$) vs log [Q] determined n, which is the number of binding sites of NDM-1 and K_a values as shown in Table 2.3.

2.9.2. Isothermal titration calorimetry (ITC)

ITC experiments were conducted using a MicroCal Auto-ITC200 instrument. Thermodynamic parameters such as the measurement of binding affinities, enthalpy (ΔH) and entropy (ΔS) changes were determined. Prior to performing the experiments, the metal chelators and zinc chloride were dissolved using Tris-HCl buffer (50 mM, pH 8.0) and degassed using a sonication bath. The zinc chloride solution (100 µM) was titrated into BP1, BP9, BP10 and BP11 (10 µM), with 30 aliquots of 2 µL with 180 s between injections. All the experiments were carried out at 25˚C. The generated ITC profiles were integrated using Origin 7.0 software. From the thermodynamic parameters and equation 4, the Gibbs energy changes (ΔG) were calculated:

$$\Delta G = RT\ln K_d \qquad (4)$$

3. Results

3.1. PCR amplification and cloning of NDM-1

Metallo-β-lactamase NDM-1 was cloned into the expression vector pET302. Colony PCR and restriction digestion was conducted to confirm the presence of *bla*$_{NDM-1}$ metallo-β-lactamase resulting in 770 bp as shown in the PCR products, respectively shown in Figure 2.2.

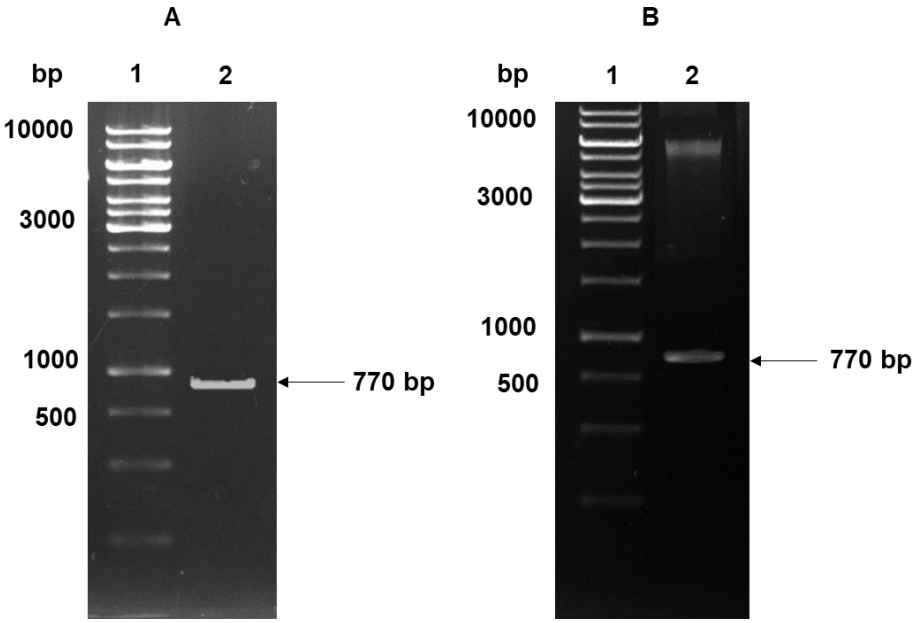

Figure 2.2 Colony PCR (Fig. 2.2A) and restriction digestion (Fig. 2.2B) confirmed the presence of *blaNDM-1* metallo-β-lactamase resulting in 770 bp.

3.2. Expression and purification of NDM-1

The cloned blaNDM-1 gene on expression plasmid vector pET302, was successfully expressed in *E.*coli BL21 (DE3) cells at 16°C using 0.4 mM IPTG for 18 hours. The NDM-1 enzyme was purified by cobalt affinity chromatography. Approximately 3.6 mg of purified enzyme was obtained per litre of culture. The degree of purity, as analysed by SDS-PAGE, was ≥ 95 % (Figure 2.3), and NDM-1 protein migrated with an apparent molecular mass of 27.9 kDa (Figure 2.3). Lane 4 represents the wash step whereas lane 5 represents the purified protein.

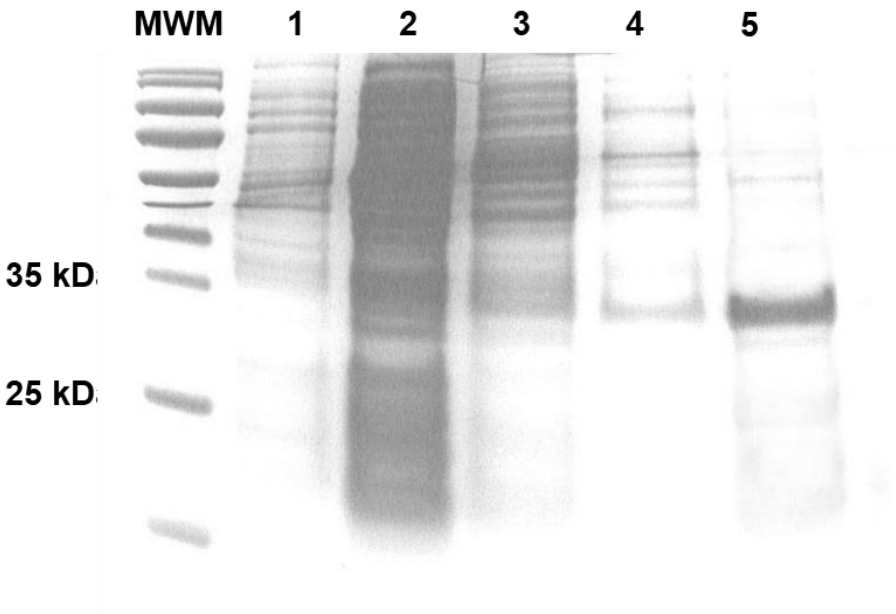

Figure 2.3: Over expression and purification of MBL NDM-1. The bacteria was grown at 16 °C for 18 hours with IPTG A) MWM, molecular weight marker, Lane 1: Un-induced NDM-1, Lane 2: Induced NDM-1, Lane 3: Unbound protein, Lane 4-5: Purified NDM-1.

3.3. Kinetic studies

The kinetic parameters, K_m, k_{cat} and k_{cat}/K_m of NDM-1 were determined using a chromogenic substrate, nitrocefin. Under selected environmental conditions, the enzyme hydrolysed nitrocefin and showed good affinity and catalytic activity with the substrate (Table 2.1).

Table 2.1: Enzyme kinetic parameters of NDM-1 protein using chromogenic substrate nitrocefin at 37˚C (n=3).

Parameter	NDM-1
K_m (μM)	29.07±1.36
k_{cat} (s^{-1})	21.77±1.02
k_{cat}/K_m (μM^{-1} s^{-1})	4 x 10^{-4}±0.18X10^{-4}
V_{max}	22.68±1.12

3.4. Inhibition studies

An irreversible reaction was observed. A summary of the K_i values of metal chelators against NDM-1 were calculated from Eq. 1 are shown in Table 2.1. A graphical example of K_{obs} determination is shown in Figures 2.4, 2.5, 2.6 and 2.7, of which was used to determine K_i (Appendix: Figure S2). BP9, BP10 and BP11 are regarded as effective and had K_i values of 0.16, 0.19, 0.18 μM, respectively, BP9 being the most potent metal chelator. The least potent of the inhibitors was BP1 having a higher K_i value, 0.24 μM.

Table 2.1: Kinetics of inhibition for the NDM-1 protein against various metal chelators) (n=3).

Inhibitors	K_i (μM)
BP1	$0.24\pm0.84X10^{-2}$
BP9	$0.16\pm0.69X10^{-2}$
BP10	$0.19\pm1.4X10^{-2}$
BP11	$0.18\pm1.1X10^{-2}$

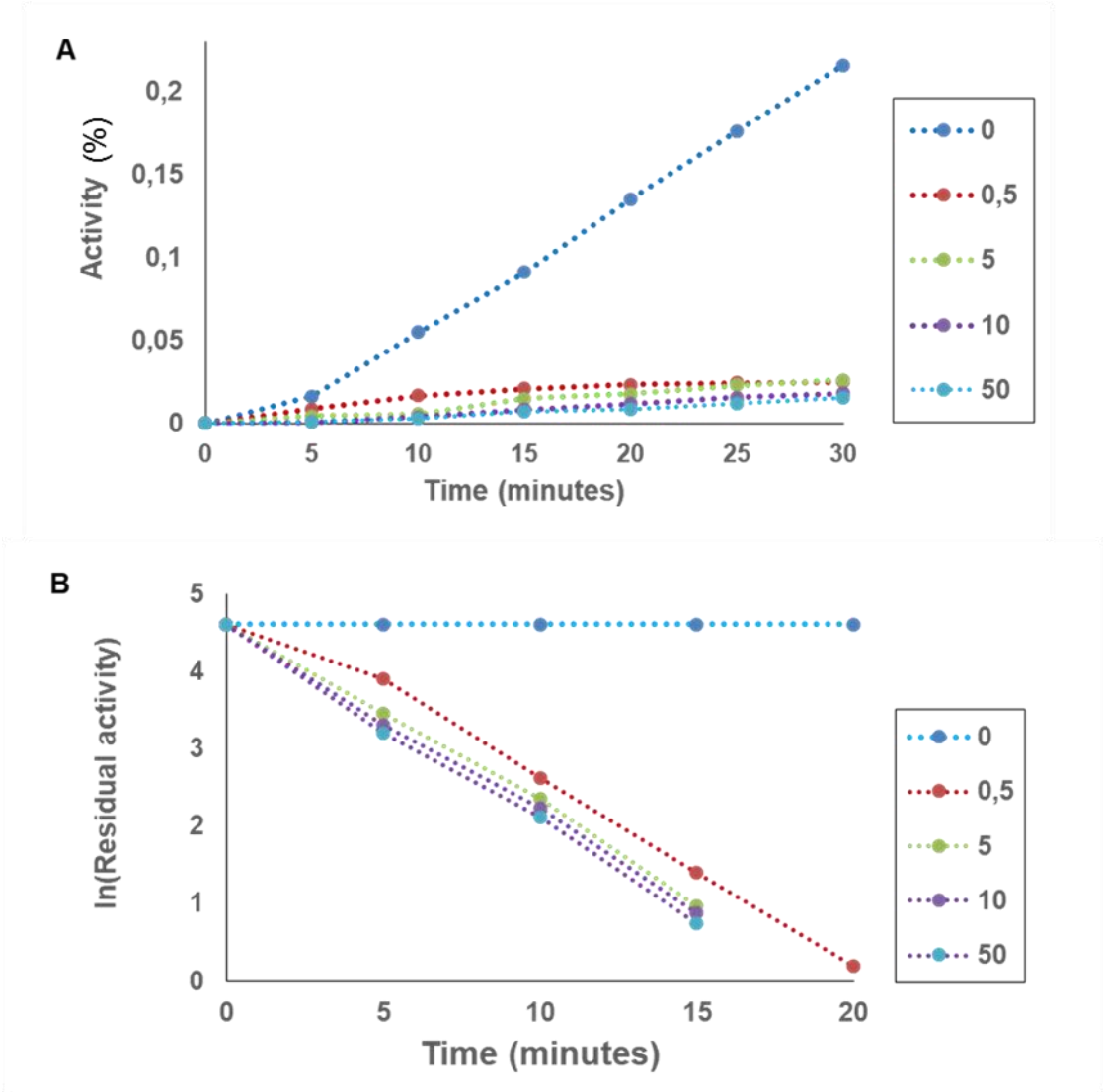

Figure 2.4: Kinetics of inhibition. (A) Plot of activity versus drug concentration for BP1. (B) Plot of In (Residual activity) against time to determine K_{obs} for BP1.

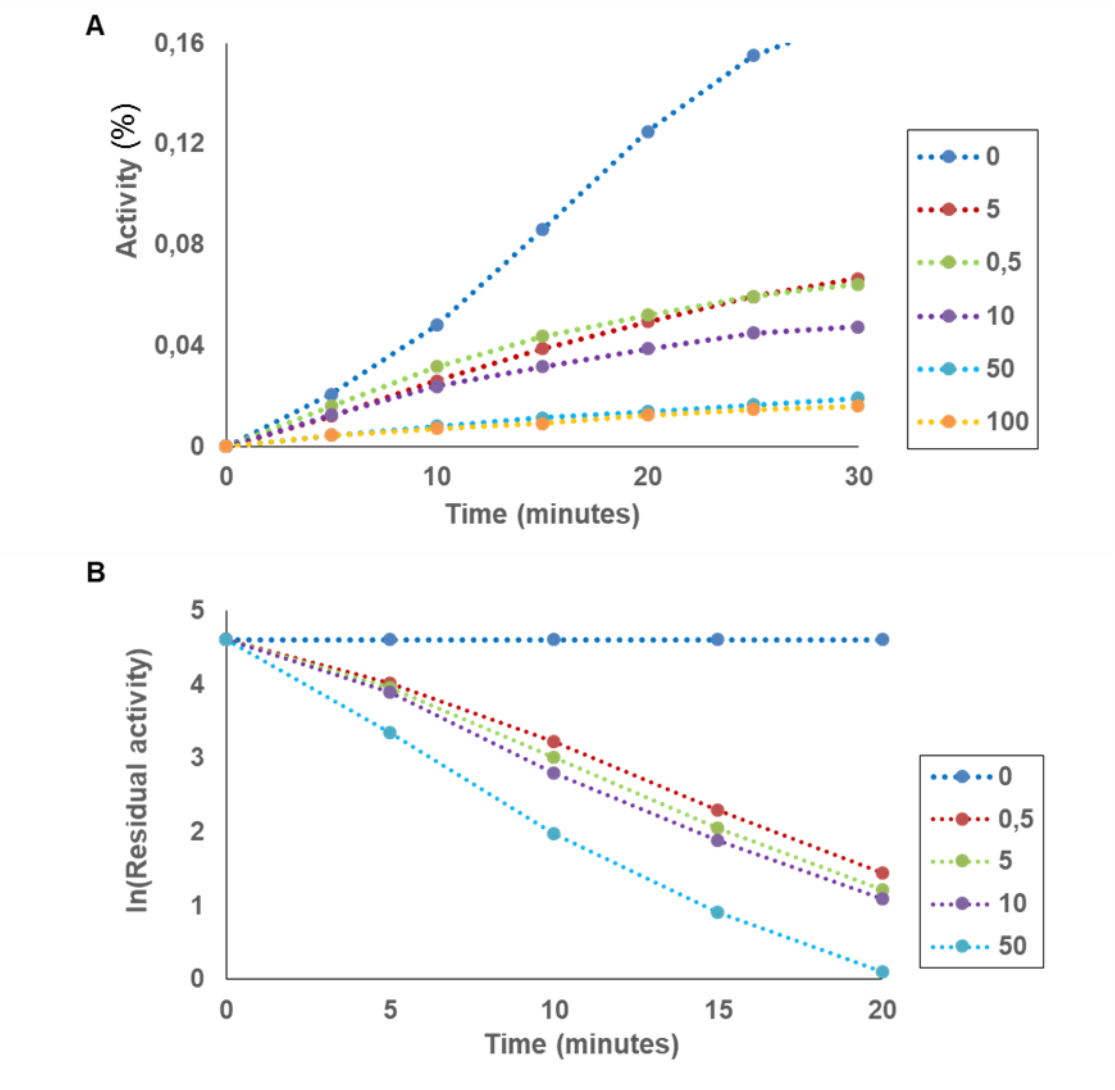

Figure 2.5: Kinetics of inhibition. (A) Plot of activity versus drug concentration for BP-9. (B) Plot of In (Residual activity) against time to determine K_{obs} for BP9.

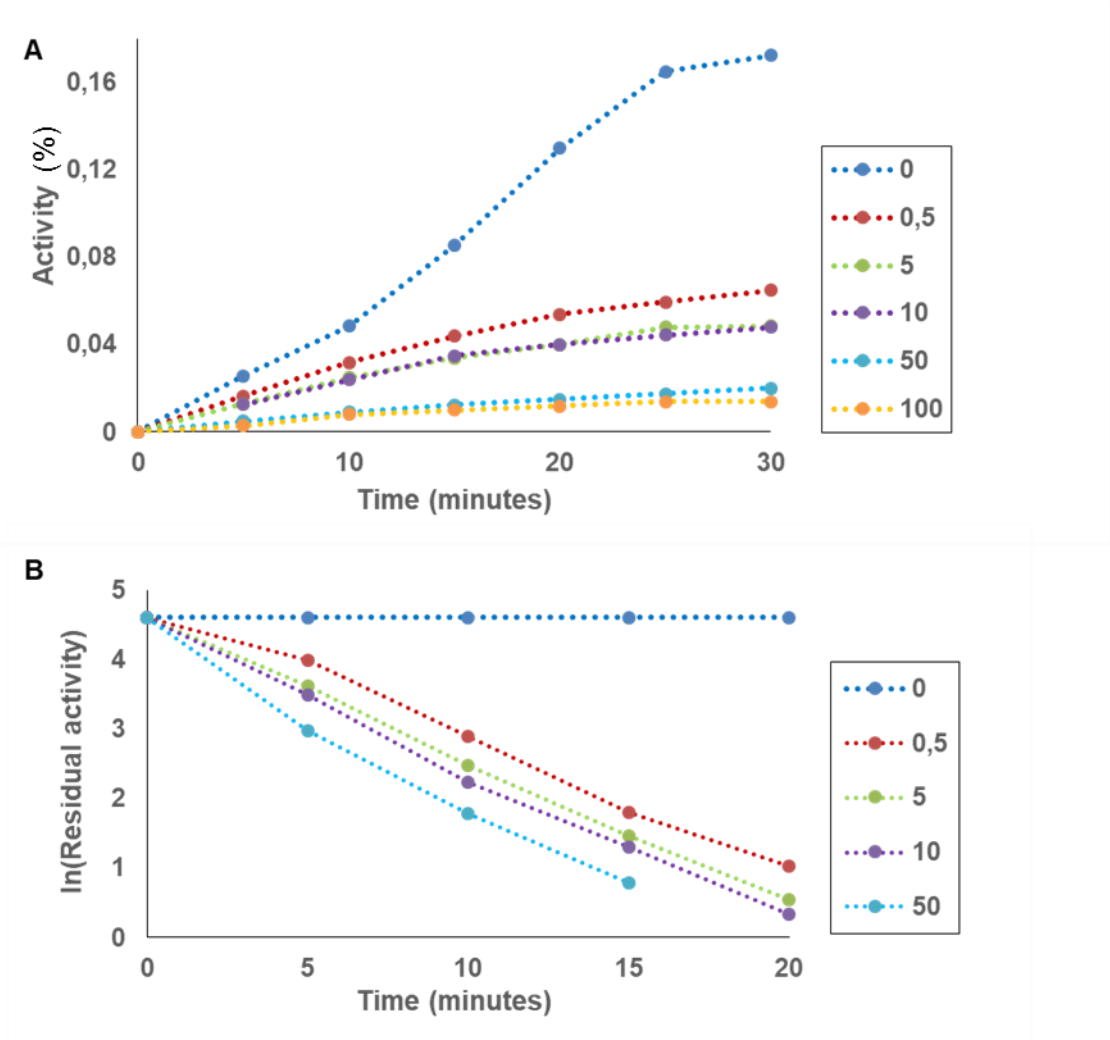

Figure 2.6: Kinetics of inhibition. (A) Plot of activity versus drug concentration for BP10. (B) Plot of In (Residual activity) against time to determine K_{obs} for BP10.

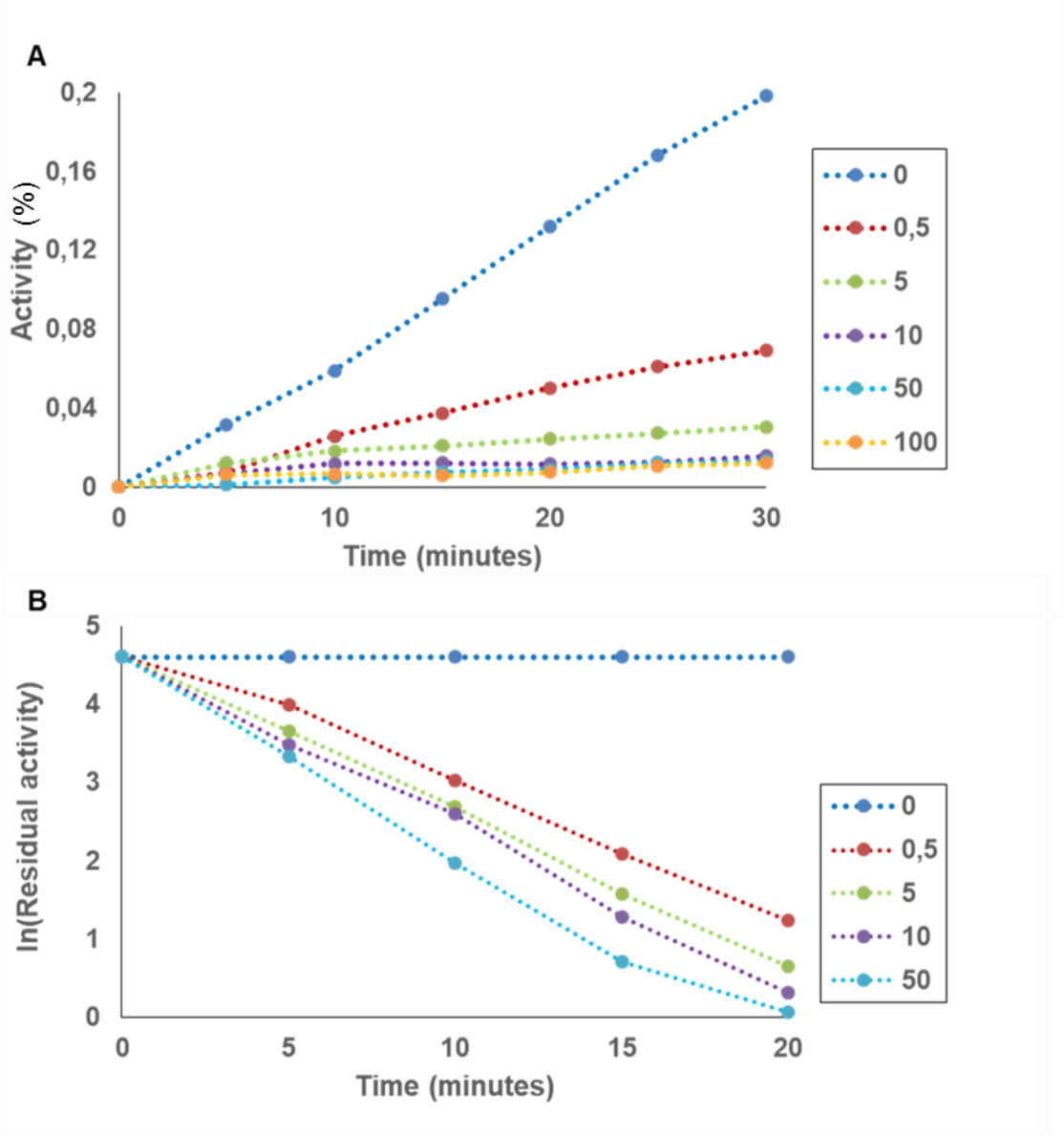

Figure 2.7: Kinetics of inhibition. (A) Plot of activity versus drug concentration for BP11. (B) Plot of In (Residual activity) against time to determine K_{obs} for BP11.

3.5. Thermodynamic studies

3.5.1. Fluorescence quenching

Inhibitor binding thermodynamics of NDM-1 was attained by employing fluorescence quenching. The tryptophan fluorophore molecules within NDM-1 that are relatively near the active site. In particular, Trp-93 is close to the active site (Appendix: Figure S3), therefore we expect that this residue is more likely to be affected by the inhibitors. In this study, the addition of each of our agents to the MBL protein resulted in fluorescence quenching with linear Stern-Volmer plots (Figure 2.8), and allows for the determination of the Stern-Volmer quenching constants (K_{sv}) from the slope (Eq. 3). Table 2.3 shows a summary of the thermodynamic parameters. BP9 was found to have the strongest interaction with NDM-1 (K_a value of 11846.77 µM).

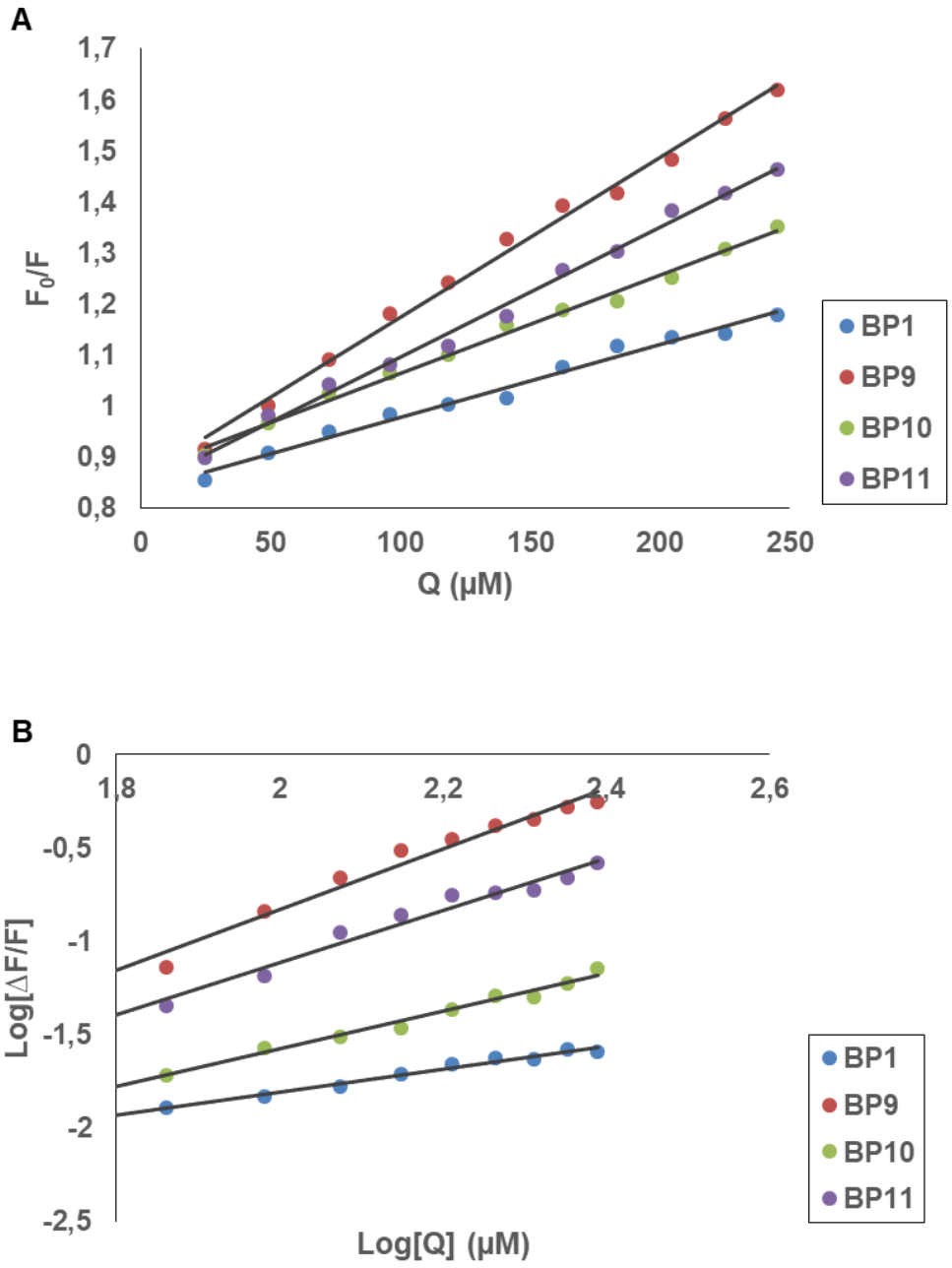

Figure 2.8: BP1, BP9, BP10, BP11 induced fluorescence quenching of NDM-1 at 298 K. (A) Examples of Stern-Volmer plots. (B) Modified Stern-Volmer plots using log function to determine K_d. The concentration of NDM-1 was 1 μM.

Table 2.3: A summary of thermodynamic parameters for the four potential metal chelator inhibitors (n=3).

	BP-1	**BP-9**	**BP-10**	**BP-11**
K_{SV} **(μM^{-1})**	0.0014±0.0000	0.0031±0.0001	0.0019±0.0000	0.0025±0.0001
K_a **(μM)**	1086.43±24,55	11846.77±334,07	3979.24±72,02	8256.57±203,94
K_d **(M)**	$9.20x10^{-4}$±$2.08x10^{-4}$	$8.44x10^{-5}$± $0,238x10^{-5}$	$2.50x10^{-4}$±$0,45x10^{-4}$	$1.20x10^{-4}$±$0,0029x10^{-4}$
n	0.614	1.623	1.011	1.401
R^2	0.9781	0.9688	0.9795	0.9652

3.6. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry **(**ITC) is used to determine the measurement of binding affinities, enthalpy (∆H) and entropy (∆S) changes. All BP compounds have a similar structure. They share the same chelator and vary with the attached β-lactam antibiotic. BP11 was curated for ITC experiments based on its excess availability.

A calorimetric titration profile is generated of Zn^{2+} with BP11 at 20˚C (Figure 2.9). Each peak represents a single injection of Zn^{2+} into un-complexed metal chelator solution. A negative heat deflection was observed when zinc ions was introduced into the metal chelator solution at respective temperature. This indicates that the binding between Zn^{2+} and inhibitor is an exothermic process. Table 2.4 summarizes the thermodynamic parameters for the binding of zinc ions to the metal chelator. In these experiments, BP11 obtained a K_d value of 2.3 x 10^{-5} (Table 2.4).

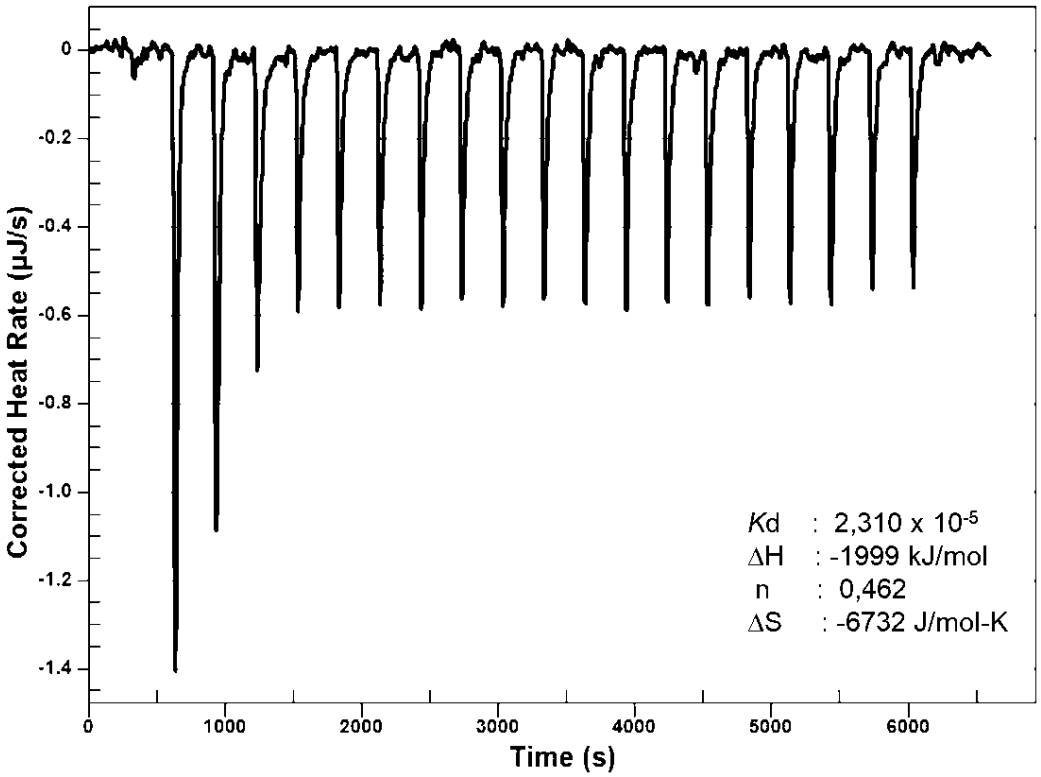

Figure 2.9: Isothermal titration thermogram for binding of Zn^{2+} by BP11.

Table 2.4: Thermodynamic parameters for the binding of BP11 with Zn^{2+} as estimated by ITC.

Thermodynamic parameters	**BP11, Zn^{2+}**
K_d (µM)	2.3×10^{-5}
∆H (kJ/mol)	-1999
n*	0.462
∆S (J/mol-K)	-6732
∆G (kJ mol^{-1})	-26.00

**Number of binding sites*

4. Discussion

Metallo-β-lactamases NDM-1 is an enzyme that causes resistance by hydrolysis of the amide bond of β-lactam antibiotics. With no clinically available inhibitors for MBLs, this raises great concern. This limitation has led us to investigate potential metal chelator inhibitors for NDM-1. The NDM-1 protein was successfully expressed at a low IPTG concentration (0.4 mM) at 16 °C overnight. The expression yield of NDM-1 was lower (3.6 mg/L) than previously reported (95 mg/L) [9]. It has been reported in literature that bigger fusion tags such as glutathione transferase (GST) and small ubiquitin-related modifier (SUMO) can increase protein production yields [10]. The expression of NDM-1 did not involve the use of GST or SUMO fusion tags hence the low production yield.

Minimal inhibitory concentrations (MICs) of meropenem co-administered with BP compounds against carbapenamase-producing organisms was previously conducted. All four BP compounds showed synergistic inhibition activity (≤ 16 μg/mL) and were able to completely restore the activity of meropenem (0.06 – 0.125 μg/mL) (unpublished data).

NDM-1 catalysis and steady-state kinetic parameters were determined against a chromogenic substrate nitrocefin. The enzyme hydrolysed the tested substrate and displayed activity (table 2.1). Our results are comparable to literature [26], however the kinetic parameters in this study displays a higher K_m compared to literature, indicating the enzyme investigated has a lower affinity.

Development of metallo-β-lactamase inhibitors are crucial to restore the efficacy of current β-lactam antibiotics. Here, we performed inhibition studies to determine the potency of four metal chelators, BP1, BP9, BP10 and BP11 that are attached to β-lactam antibiotics. Amongst these metal chelator inhibitors, BP1 exhibited a large K_i value (0.24 μM), compared to the other BP compounds. This indicates a lower affinity

to the NDM-1 enzyme. BP9 gave the best inhibition, which was indicated by a low K_i value of 0.16 μM. This means that a low concentration of BP9 is required to cause inhibition of NDM-1. In comparison to the previously mentioned chelator, TACN, a K_i value of 0.044 μM was obtained [20]. This is far lower than the K_i value we obtained, however, there are structural differences between TACN and BP compounds. TACN is solely a chelator, whereas BP compounds are designed as a chelator that is attached to a β-lactam antibiotic. The attachment of an antibiotic to the chelator allows permeation through bacterial cell walls (unpublished results).

Fluorescence quenching experiments can be useful in studying the interaction of inhibitors and the protein. This effect was used to examine the binding affinity and efficiencies of the metal chelator compounds, with the recombinant protein NDM-1. The NDM-1 contains tryptophan fluorophore molecules, especially those that are near the active site; the inhibitors therefore act as intrinsic quenchers and as a result, decrease the quantum yield. The intensity of quenching is directly proportional to the K_{sv} value [24]. From the results, dynamic quenching was observed. The Stern-Volmer quenching constants exhibited the same trend as the kinetics of inhibition data for all compounds. BP9 was once again the best inhibitor with a K_{sv} value of 0.0031 μM^{-1}. The order was the same as for the inhibition experiments (Table 2.3).

The binding affinity of Zn^{2+} to metal chelator and thermodynamic parameters were determined by ITC. Thermodynamic parameters such as enthalpy ΔH and entropy ΔS were determined for the chelator and a representative calorimetric profile is shown in Figure 2.9. The enthalpy represents the amount of heat released per mole of ligand bound and is determined from the isotherm. Therefore, an ITC experiment provides useful information about the binding interactions which helps understand the interactions and thermodynamic mechanism. To the best of our knowledge, a study of Tehrani and Martin is the only study conducted that reports ITC data where zinc is

titrated into thiol based MBL inhibitors [25]. They show ΔG values of -26.81 kJ.mol^{-1} and -28.57 kJ.mol^{-1}. Interestingly, the ΔG value obtained in this study was in the same range (Figure 2.9). In addition to the ITC results obtained, we have demonstrated that the metal chelator binds to zinc ions and indeed to the enzyme pocket.

5. Conclusion

In this study, we have demonstrated that all BP compounds inhibit NDM-1 within a reasonable range. Based on this study, BP9 is the best inhibitor. It can be presumed that the chelators also demonstrate the ability to restore the activity of β-lactam antibiotics. The ITC results indicated binding of the metal chelator to zinc ions. We may presume the other chelators bind like BP11. This provides evidence of a presumptive mechanism (which is that of metal sequestering or irreversible inhibition) appears to be operating with these molecules. It will be interesting to know the exact mechanism of how these inhibitors interact with the MBLs. Future studies will involve X-ray crystallography to investigate how the inhibitors interact with specific residues in the protein.

6. References

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CHAPTER 3

Overall conclusion of the research outcome

1. Conclusion

Antibiotic resistance is a worldwide burden and is becoming a major public health concern [1]. The production of β-lactamases by Gram-negative and Gram-positive bacteria has compromised the efficacy of β-lactam antibiotics [2], leading to antibiotic resistance and causing several severe infections [3]. The enzyme, β-lactamase is responsible for catalysing the hydrolysis of the amide bond found in the β-lactam ring of β-lactam antibiotics [4]. Metallo-β-lactamases (MBLs) are a major clinical issue since they are able to hydrolyse nearly all β-lactam antibiotics, including the 'last resort' antibiotic, carbapenems [5]. New Delhi Metallo- β-lactamase-1 (NDM-1) is a recent emerging MBL and exhibits increased hydrolytic potency against most clinically β-lactam antibiotics [6].Unfortunately, to date, there are no clinically available MBL inhibitors that can restore the efficacy of β-lactam antibiotics [7]. Therefore, many studies are aimed at drug discovery and development of MBL inhibitor in order to combat this clinical burden [8]

In this chapter a summation of the thesis is presented. The first chapter provides a literature review of metallo-β-lactamases. The introduction chapter focuses on antibiotic resistance and briefly discusses the various mechanisms involved in the emergence of resistance. It also makes mention of the problem statement with emphasis on NDM-1 MBL and the lack of inhibitors in literature. Chapter 1 also briefly discusses β-lactamase inhibitors and their mechanism of action. An overview of analytic techniques is also provided in this chapter.

Chapter 2 reports a procedure for cloning, expression and purification of NDM-1. Expression of the NDM-1 protein was expressed at low concentrations of IPTG and

resulted in a lower expression yield 3.6 mg/L compared to previous studies (95 mg/L) [9]. This result was ascribed to the absence of fusion tags. Fusion tags such as glutathione transferase (GST) and the small ubiquitin-related modifier (SUMO) are known to increase the protein production yield [10]. The enzyme was fully characterized and the kinetic parameters were determined (K_m, k_{cat}, and k_{cat}/K_m).

The next phase of this study was to determine the effect of four novel metal chelator inhibitors (BP1, 9, 10 and 11) against the recombinant protein, NDM-1. The studies showed that BP9 gave the best inhibitory effect as seen by the low K_i (0.16 μM). Fluorescence studies revealed that BP9 had the strongest interaction to NDM-1 whilst BP1 had the weakest binding. ITC demonstrated that the metal chelator binds to the zinc ions and to the enzyme pocket. As a result, we may presume that the other BP chelators will bind like BP11. This provides evidence that a presumptive mechanism (either by metal sequestering or competitive inhibition) appears to be taking place with these molecules.

We can conclude that these metal chelators can be used a lead molecules for future drug candidates. Future studies will focus on X-ray crystallography which entail the study of direct interactions of the drug with specific residues of the NDM-1 protein. Additionally, a study should be conducted to determine whether the metal chelators have the same effect as in NDM-1 with other MBLs such as IMP, VIM and SPM.

2. References

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APPENDIX

1. Supplementary Information for Chapter Two

Chelators on β-lactam antibiotics; the way to fight metallo-β-lactamases.

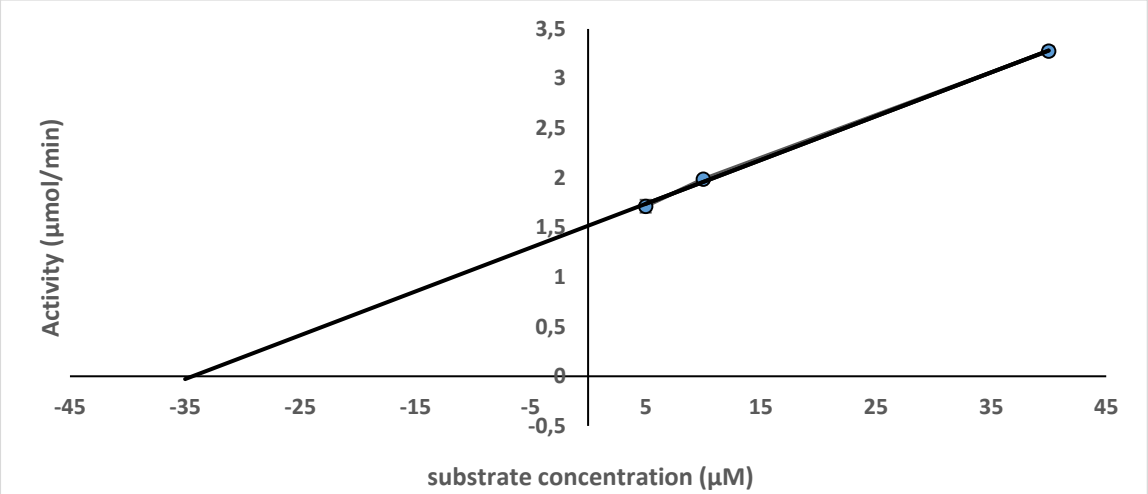

Figure S1: Lineweaver-Burk plot for the determination of Vmax and Km values of NDM-1.

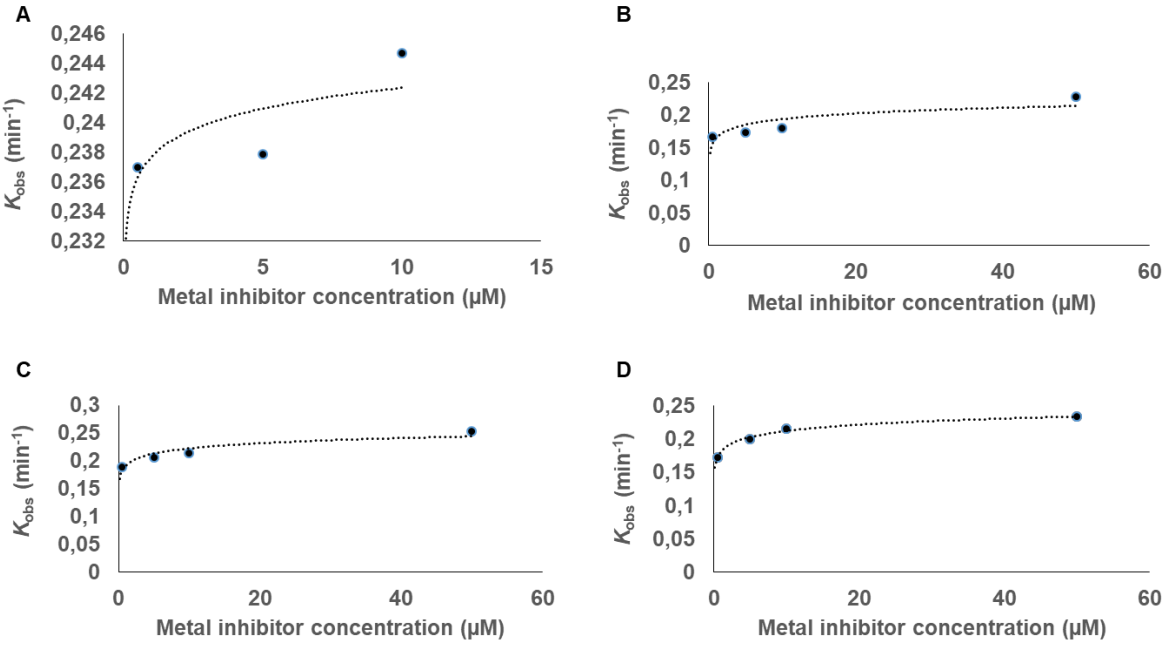

Figure S2: The hyperbolic plot of K_{obs} of BP compounds against inhibitor concentrations. (A) BP1 (B) BP9 (C) BP10 (D) BP11.

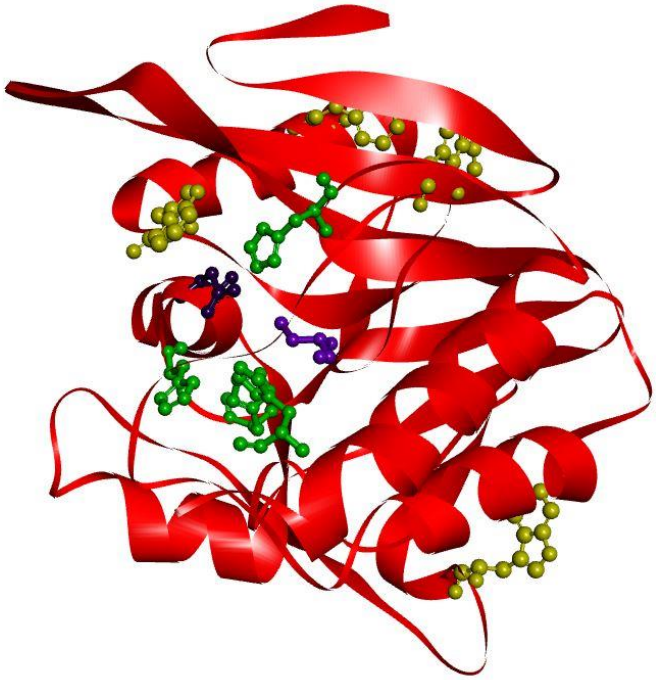

Figure S3: A ribbon presentation of NDM-1. Shown in yellow are the tryptophan molecules, cysteine is represented by purple, shown in green are the histidine residues and dark blue shows asparagine. (created using UCSF Chimera version 1).