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The *in vitro* and *in vivo* efficacy of novel metallo-β-lactamase inhibitors co-administered with meropenem to target CREs

Nakita Reddy

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The *in vitro* and *in vivo* efficacy of novel metallo-β-lactamase inhibitors co-administered with meropenem to target CREs

Nakita Reddy

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A thesis submitted to the College of Health Science, University of KwaZulu-Natal, Westville, for the degree of Doctor of Philosophy in Pharmaceutical Science.

This is to certify that the contents of this thesis are the original, unaided work of Mrs Nakita Reddy, carried out under the supervision of the Catalysis and Peptide Research Unit, at the University of KwaZulu-Natal, Westville campus, Durban, South Africa.

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

Supervisor:

Signed: -------- [redacted] ------- Name: **Prof. T. Naicker** Date: ----------

Abstract

The evolution and phenotypic expression of metallo β-lactamase genes across the world has led to the escalated transmission rates of carbapenem resistance. The effect has crippled the already impaired healthcare system, with the emergence of COVID-19 exacerbating the crisis further. Our plight for a solution to combat antimicrobial resistance has not been greater. One strategy to tackle this non-susceptibility is the development of metallo-β-lactamase inhibitors that can neutralize the metallo-β-lactamase enzyme, thereby allowing the carbapenem antibiotic to elicit its function on the microorganism. Currently, there is no FDA-approved metallo-β-lactamase inhibitor to meet the clinical challenges of drug resistance. In a desperate need to find a candidate drug, research has been initiated into the discovery and development of biologically active inhibitors. Therefore, this thesis focuses on the advances made by our research group, the Catalysis and Peptide Research Unit, in developing novel β-lactam derived inhibitors; NOTA, NO3PY, BP- 1, 6,10 and 14, that re-sensitize the microbe to the efficacy of meropenem. The *in vitro* and *in vivo* activities of the initial chelators, NOTA and NO3PY, were evaluated as potential metallo-β-lactamase inhibitors (MBLIs) against metallo-β-lactamase (MBL) resistant bacteria. Time-kill studies showed that NOTA and NO3PY restored the efficacy of meropenem against all bacterial strains tested. A murine infection model was then used to study both metal chelators' *in vivo* pharmacokinetics and efficacy. NO3PY displayed poor bioavailability at the selected doses using a validated LC-MS/MS method, therefore discouraging the *in vivo* efficacy evaluation. NOTA showed good bioavailability; hence, the *in vivo* efficacy was determined in a murine thigh infection model. The co-administration of meropenem and NOTA (100 mg/kg.bw each) significantly decreased the colony-forming units of *K. pneumoniae* NDM over an eight-hour treatment period. The findings suggested that chelators, such as NOTA, hold strong potential for use as an MBLI in treating CRE infections; however, further preclinical development was needed to improve the pharmacokinetic

properties of these agents to increase their bioavailability and tissue distribution. With this information, our group derivatized NOTA by coupling it to a β-lactam to create the BP series of novel MBLIs. The results generated by the BP compounds have proven to interact synergistically with meropenem, by restoring the MIC of meropenem to therapeutically acceptable concentrations ($\leq$ 2 mg/L) that concur with the breakpoints outlined by CLSI. In addition, the bactericidal activity of the re-sensitized meropenem was evident in the time-kill study over 24 hours. Cytotoxicity assays were further conducted to study the inhibitors, with an outcome in favor of safe administration *in vivo*. The metallo-β-lactamase inhibitors reported herein have demonstrated good potency against NDM-1 and VIM-2 metallo-β-lactamases with a Ki of 25-97μM. Since the BP compounds are metal chelators that function as metallo-β-lactamase inhibitors, it was important to determine the binding specificity of the BP compounds to a physiologically relevant zinc-harboring enzyme, glyoxylase II. At concentrations of up to 500 μM of BP, the activity of glyoxylase II remained unhindered. This confirmed the hypothesis of BP specificity to be exclusive to NDM-1 and VIM-2 metallo-β-lactamases. These findings prompted further interest in the binding exhibited by BP and led to additional studies to address the binding interactions of BP with the metallo-β-lactamases through quenching and computational experiments. Fluorescent quenching experiments investigating the Ka of BP indicated that a higher binding affinity was noted for NDM-1 compared to VIM-2 MBLs, thus implying a stronger interaction with NDM-1. Molecular docking and dynamic simulation experiments shed light on the BPs' mode of action, showing the interaction of the chelators' carboxylic moiety with the Zn^{2+} ions in the MBLs structure. In favor of this BP series as functional inhibitors, *in vivo* efficacy was explored in a murine infection model (BP1 and BP10). In *Klebsiella pneumoniae* NDM infected mice, BP co-administered with meropenem was efficacious in reducing the bacterial load by > 3 $\log_{10}$ units' post-infection, compared to meropenem monotherapy. These findings validate our strategy for derivatizing NOTA into the

series of the BPs, as the bioavailability of NOTA, when coupled to a cephalosporin, improved the overall *in vivo* efficacy, and allowed the drug to be quantified in plasma under the same conditions previously used. This study clearly indicated the influence of the BP compounds in reducing the bacterial burden and the success of employing combination therapy as a treatment alternative. Moreover, the outcome of this preclinical development represents a solid foundation, whereby we can build on our existing knowledge. In aligning with our research goals of alleviating the threat of antimicrobial resistance, coupling β-lactams to a cyclic zinc chelator offers a safe and efficacious solution to meet the calamity that plagues our healthcare system.

Declaration 1-Plagiarism

I, Nakita Reddy declare that

1. The research reported in this thesis, except where otherwise indicated, 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 reference section.

Signed: 

Mrs Nakita Reddy

Date: 01/12/2022

Declaration 2-List of Publications

Details of the contribution to the publications that form part of the research presented in this dissertation (include publications in preparation, submitted, *in press* and published) are as follows:

1. PCT Filing-PCT/IB2022/056748

Peters BK, Arvidsson PI, Kruger HG, Govender T, Naicker T

This patent was filed to protect the intellectual property of the novel BP compounds, described within this thesis.

2. A 2018-2019 patent review of metallo β-lactamase inhibitors

Nakita Reddy, Mbongeni Shungube, Per I Arvidsson, Sooraj Baijnath, Hendrik G Kruger, Thavendran Govender and Tricia Naicker. Expert Opinion on Therapeutic Patents *Published.* 2020. DOI: 10.1080/13543776.2020.1767070
(Impact factor = 6.67)

Nakita Reddy performed the literature search, analyzed the patents, and composed the manuscript. Mbongeni Shungube analyzed the patents, drew the chemical structures, and contributed to writing the synthesis component of the manuscript. Nakita Reddy was also responsible for the revisions and edits. The remaining authors are supervisors on the project and contributed to the conceptualisation of the idea, guidance of the students and funding of the project.

3. The in vitro and in vivo potential of 1,4,7-triazacyclononane-1,4,7 triacetic acid (NOTA) in enhancing the bactericidal activity of meropenem against carbapenem-resistant *Enterobacterales*

Kehinde F. Omolabi, **Nakita Reddy**, Sipho Mdanda, Sphamandla Ntshangase, Sanil D. Singh, Hendrik G. Kruger, Thavendran Govender, Tricia Naicker and Sooraj Baijnath. FEMS Microbiology Letters. *Published*. 2022. https://doi.org/10.1093/femsle/fnac122 (Impact factor = 2.82)

Kehinde Omolabi performed the experiments and composed the manuscript. Nakita Reddy trained Kehinde Omolabi to perform the microbiological work as well as performed the microbiological analysis in the animal studies, wrote parts of the paper and supervised the checkerboard assay. Nakita Reddy was also responsible for the revisions and edits. Sipho Mdanda and Sphamandla Ntshangase assisted with the animal studies and LCMS/MS analysis. The remaining authors are supervisors on the project and contributed to the conceptualisation of the idea, guidance of the students and funding of the project.

4. The *in vitro* and *in vivo* development of a β-lactam-metallo-β-lactamase inhibitor: targeting carbapenem-resistant *Enterobacterales*

Byron K Peters[#], **Nakita Reddy**[#], Mbongeni Shungube, Letisha Girdhari, Sooraj Baijnath, Sipho Mdanda, Lloyd Chetty, Thandokuhle Ntombela, Thilona Arumugam, Linda A. Bester, Sanil D. Singh, Anil Chuturgoon, Per I. Arvidsson, Glenn E. M. Maguire, Hendrik G. Kruger, Tricia Naicker and Thavendran Govender. ACS Infectious Diseases. *In press.* 2022. (Impact factor = 5.08)

[#]Byron Peters and Nakita Reddy contributed equally to this manuscript. Byron Peters contributed to the design of the synthetic route towards the β-lactam-metallo-β-lactamase inhibitor. Nakita Reddy performed the antimicrobial susceptibility tests, enzyme assays and animal studies. In addition, Nakita Reddy analysed the data and composed the manuscript and parts of the supplementary information. Nakita Reddy was also responsible for the revisions and edits. Mbongeni Shungube, Letisha Girdhari and Lloyd Chetty assisted in upscaling the synthesis of the metallo- β-lactamase inhibitor. Thandokuhle Ntombela performed computational studies, Thilona Arumugam and Anil Chuturgoon performed the MTT and LDH assays. Sipho Mdanda, Linda A. Bester and Sanil Singh assisted with the animal trials. The remaining authors

are supervisors on the project and contributed to the conceptualisation of the idea, guidance of the students and funding of the project.

5. Neutralizing carbapenem resistance by co-administering meropenem with novel β-lactam-metallo-β-lactamase inhibitors

Nakita Reddy, Letisha Girdhari, Mbongeni Shungube, Sooraj Baijnath, Sipho Mdanda, Arnoldus Gouws, Thandokuhle Ntombela, Thilona Arumugam, Linda A. Bester, Sanil D. Singh, Anil Chuturgoon, Per I. Arvidsson, Hendrik G. Kruger, Tricia Naicker and Thavendran Govender. *To be submitted.*

Mbongeni Shungube and Letisha Girdhari synthesized the metallo-β-lactamase inhibitor. Letisha Girdhari was responsible for the scale up synthesis. Nakita Reddy performed the antimicrobial susceptibility tests, enzyme assays and animal studies. Nakita Reddy analysed all the data and prepared the manuscript and parts of the supplementary information. Nakita Reddy will be responsible for the revisions and edits. Arnoldus Gouws assisted in the animal experiments, cfu/thigh enumeration tests and LCMS/MS troubleshooting. Thandokuhle Ntombela performed computational studies, Thilona Arumugam and Anil Chuturgoon performed the MTT and LDH assays. Sipho Mdanda and Linda A. Bester assisted with the animal trials. The remaining authors are supervisors on the project and contributed to the conceptualisation of the idea, guidance of the students and funding of the project.

Additional manuscripts not presented as part of this thesis

6. Synthesis and biological evaluation of novel metallo-β-lactamase inhibitors. Mbongeni Shungube, Letisha Girdhari, **Nakita Reddy**, Kehinde F. Omolabi, Ayanda K. Hlophe, Byron B. Peters, Thilona Arumugam, Anil Chuturgoon, Hendrik G. Kruger, Per I. Arvidsson, Thavendran Govender and Tricia Naicker. *To be submitted.*

Mbongeni Shungube synthesized and characterized most of compounds, analysed all the data, prepared the manuscript and the supporting information. Letisha Girdhari and

Ayanda Hlophe and Byron Peters synthesized and characterized some of the compounds. Letisha wrote corresponding parts of the paper and prepared parts of supporting information. Nakita Reddy and Kehinde Omolabi performed the antimicrobial assays on 16 of the synthesized compounds. Thilona Arumugam and Anil Chuturgoon performed cytotoxicity assays. The remaining authors are supervisors on the project and contributed to the conceptualisation of the idea, guidance of the students and funding of the project.

Dedication

This thesis is dedicated to my boys,

Shanav and Chayan

Remember it always seems impossible, until it's done,

Hard work, determination and perseverance will always be the key ingredients in the goal towards your success

Acknowledgements

I would like to thank God for guiding me throughout this journey, for giving me the strength and endurance to persevere, even when at times it felt like there was no way forward.

Other individuals and institutes that have had a positive impact on this research project include:

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- A sincere thank you to Byron Peters for leading the synthesis of the BP compounds.
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- To all my research colleagues, Kamini, Arnoldus, Kamal, Sipho, Sphamandla, Mbongeni, Deidre, Letisha, Lloyd, Kim, Victor and Nivisti thank you for your assistance during the study and for creating a wonderful work environment conducive to research.
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- The research conducted herein is possible through utilization of the University of KwaZulu-Natal's (Westville) equipment and infrastructure

Abbreviations

AMA	Aspergillomarasmine A
AMR	Antimicrobial resistance
AST	Antimicrobial Susceptibility Testing
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
ATP	Adenosine triphospate
βLs	β-lactamases
CDC	Centers for Disease Control and Prevention
Cfu	Colony forming unit
CIC	Critical inhibitory concentration
CLSI	Clinical Laboratory Standards Institute
CMC	Chemistry manufacturing and controls
CNS	Central nervous system
CRE	Carbapenem-resistant *Enterobacterales* / *Enterobacteriaceae*
^{o}C	Degrees Celsius
DMSO	Dimethyl sulfoxide
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
DPA	Dipicolylamine acid
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ES	Enzyme-substrate complex
ESBL	Extended-spectrum β-Lactamases
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
ESI	Electrospray ionization
FDA	Federal drug association
FICI	Fractional inhibitory concentration index
Glo2	Glyoxylase II
HEPES	*N*-(2-hydroxyehtyl) piperazine- *N'* –ethanesulfonic acid

HEPG2	Hepatocellular carcinoma cell line
IC_{50}	Half the maximal inhibitory concentration
IMP	Imipenemase
IND	Investigational new drug application
ISO	International Organization for Standardization
Ki	Inhibition of kinetics
Kg.b.w	Kilograms body weight
Km	Michaelis-Menten constant
Ksv	Stern-Volmer constant
L	Litre
LC	Liquid chromatography
LCMS	Liquid Chromatography Mass Spectrometry
LDH	Lactate dehydrogenase
LoD	Limit of detection
MBC	Minimum bactericidal concentration
MBL	Metallo–β-lactamase
MBLI	Metallo- β-lactamase Inhibitors
MDR	Multidrug resistance
MEM	Meropenem
µg	Microgram
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
µl	Microlitre
µM	Micromolar
mg	Milligram
MIC	Minimum inhibitory concentration
mL	Millilitre
Mm	Millimolar
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MTD	Maximum tolerated dose
m/z	Mass to charge ratio
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NDA	New drug application
NDM	New Delhi Metallo–β-lactamase
nm	Nanometre
nM	Nanomolar
NOTA	1,4,7-triazacyclononane-1,4,7 triacetic acid
NO3PY	1,4,7- tris (2-picolinyl)-1,4,7-triazacyclononane
OD	Optical density
PBP	Penicillin binding proteins
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
%	Percent
PK	Pharmacokinetic
pKa	Acid dissociation constant
p-value	Calculated probability
QTOF	Quadrupole time of flight
RNA	Ribonucleic acid
rpm	Revolutions per minute
SBL	Serine-β-lactamase
SD	Standard deviation
TACN	1,4,7-triazacylcononane
TOF-MS	Time of flight mass spectrometry
TPEN	N,N,N,N-Tetrakis(2-pyridylmethyl)-ethylenediamine

Trp	Tryptophan
USA	United States of America
VIM	Verona Integron
WHO	World Health Organisation
Zn^{2+}	Zinc ions

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Chapter one: Introduction

1.1. Antibiotics

Antibiotics can be defined as chemical substances or bioactive organic molecules that have the ability to alleviate infection elicited by microorganisms [1, 2]**.** It is one of modern medicine's most significant and vital advances and will be described in more detail in the upcoming subsections.

1.1.1. History of antibiotics and the advent of resistance

Scientists have said that life expectancy, in general, would increase by two years if cancer is cured, but adding new antibiotics to treatment regimens would increase life expectancy by ten years [3]. Antibiotics thus possess remarkable potential and are a promising tool for advanced therapeutic targeted delivery. The use of antimicrobial compounds to alleviate infection and diseases dates back centuries. In the pre-biotic era of ancient Egypt and the Far East, people consumed garlic, radish, leeks, and onions for their antibacterial properties. When wounded, some would rub mould onto open cuts and bruises as a treatment strategy [4].

This practice laid the foundation for the "modern antibiotic era" [5], where Paul Ehrlich pioneered the discovery of chemotherapy using an arsenic-based pro-drug called Salvarsan and neosalvarsan (a more soluble and less toxic derivative of salvarsan) to eradicate *Treponema pallidum*, the agent responsible for syphilis [5, 6]. Salvarsan was replaced by Prontosil, a sulphonamide prodrug, first recognized by Gerhard Domagk, who used it to prevent the amputation of his daughter's arm [7]. Thus sulphonamides were the first broad-spectrum antibiotic to produce effective treatment and are still in clinical use [6]. In 1928, Sir Alexander Fleming uncovered penicillin in mould [8, 9], by 1940, penicillin superseded sulphonamide and was available as a prescription for serious infections [10]. During the second World War, penicillin - was instrumental in controlling the

spread of infections [8], however, this was short-lived as resistance to penicillin started to rise [11]. More β-lactam drugs were developed to neutralize the threat of resistance [12]. With the development of new antibiotics, antimicrobial treatment strategies were only effective for a short period before facing the challenges of resistance again [10, 13]. Importantly, except for the anti-tuberculosis drug bedaquiline [14] belonging to the diarylquinoline class [15], there has not been a new class of antimicrobials discovered since 1984 that is safe to administer with high efficacy for the treatment of carbapenem-resistant infections [16, 17], resulting in the uprise of the post-antibiotic era [18].

Now in the post-antibiotic era declared in 2020 [19, 20], antimicrobial resistance (AMR) has escalated to an alarming rate globally [21], while the antibiotic pipeline for the discovery of new drug candidates seems to run dry [20]. This rise in resistance is primarily due to the misuse of antibiotics by practices of over-prescribing and self-medicating, as well as exposure to nosocomial infections [22], with the causative agents responsible, frequently termed "superbugs" [23], The term "superbugs" refers to bacteria that are specifically non-susceptible to the antimicrobials used in their treatment [24]. A large proportion of the current microbial superbugs belong to the *Enterobacterale* family of bacteria [25]. These bacteria include species of *Escherichia*, *Klebsiella*, *Salmonella, Enterobacter,* and *Proteus*, among others [26]. The distinctive characteristics of these bacteria include the presence of an outer membrane, a thin layer of peptidoglycan in the cell wall, and observation of a rod-shaped morphology under the lens of a microscope [26].

The World Health Organization (WHO) already in 2017 categorized these superbugs as an urgent threat that needs immediate attention [27]. In 2019, these priority organisms were ranked fifth in the top ten as global health threats [28, 29]. This urgency is reinforced by the limited number of available therapeutic agents to treat these microbial infections and the simultaneous spike in

resistance patterns to current antimicrobials on the market, including the last line of defence, carbapenems [30, 31]. Therefore, an improvement in the efficacy of current antimicrobials and the development of new drug candidates is desperately needed to combat antibiotic resistance and eradicate the responsible micro-organisms.

β-lactamases neutralize β-lactam antibiotics, rendering them ineffective against infections. Figure 1 represents the introduction of new β-lactam candidates over the years, the emergence of resistant β-lactamase genes expressed in bacteria, and the advancement of co-administering a β-lactamase inhibitor with a β-lactam drug to achieve a therapeutic effect [32]. Classification of β-lactamases categorises them as either serine β-lactamases (further discussed in section 1.3) or metallo-β-lactamases (detailed in section and 1.4) (Table 1) [33].

The exploitation of combination therapy offers many benefits over monotherapy such as, optimised treatment, increased potency from synergistic effects, and suppression of drug resistance [34]. Over the years many combinations of a β-lactamase inhibitor and β-lactam drug, have been successfully developed to target serine β-lactamases. However, there is a desperate need for a metallo-β-lactamase inhibitor to be developed that can be co- administered with a carbapenem, to deliver an effective treatment strategy [35].

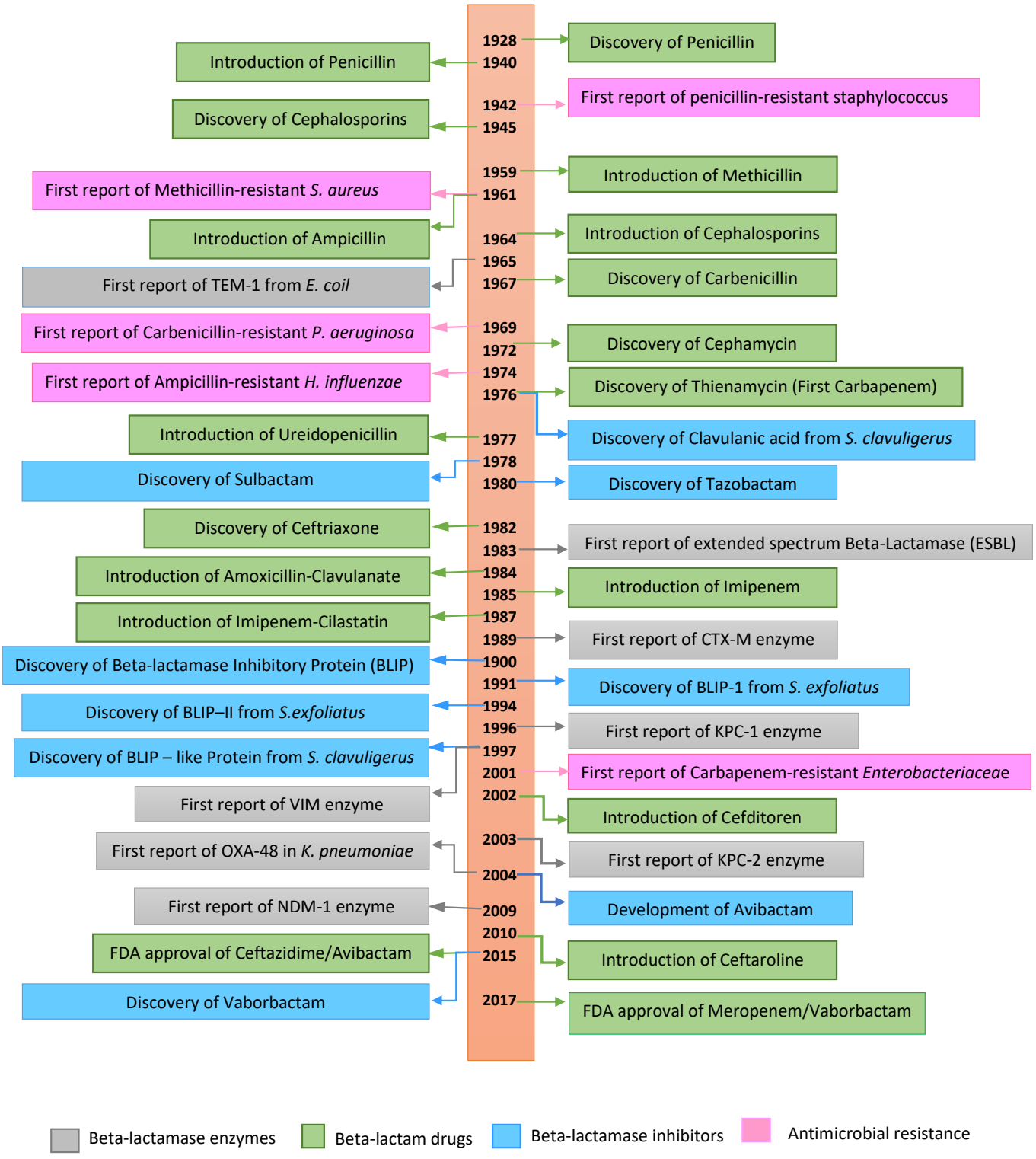


Figure 1: Timeline representing β-lactam drug discovery, inhibitors as well as the advent of resistance (adapted from Eiamphungporn *et al.*, 2018 [36], open access).

The next subsection focuses on the details of the various antimicrobial classes that belong to the β-lactam family of drugs. This subsection aims to provide an understanding of the spectrum of activity exhibited by these antimicrobial classes, to further understand the impact bacterial resistance exerts on the effectiveness and shelf-life of these drugs.

1.1.2. β-lactams

β-lactams are the oldest and most frequently consumed family of antibiotics [37] due to their therapeutic efficacy and rare toxicity exhibited [38]. The family of β-lactam antibiotics comprises of four classes of antimicrobial compounds, which are differentiated based on their chemical structure. These four classes include penicillin, monobactam, cephalosporin and carbapenem [39]. All members possess a β-lactam ring, that is a distinguishing characteristic from other antimicrobial classes [3].

The β-lactam ring in cephalosporins is fused to a six-membered dihydrothiazine ring that contains a sulphur atom [40]. Because there is a possibility of two variable substituents R^1 and R^2 occurring at C3 and C9 respectively (Figure 2), cephalosporins have become a diverse class of β-lactams that are widely prescribed. Based on the antimicrobial activity, cephalosporin derivatives can be categorised into five generations [41].

First generation cephalosporins have a limited spectrum of activity and mainly target gram-positive cocci, except for enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA) [42]. They cannot enter the central nervous system (CNS) and are therefore, not regarded as primary drugs of choice to treat CNS infections [43].

Second generation cephalosporins, have the benefits of the previous generation, with the addition of activity against gram-negative rods, except for *Pseudomonas aeruginosa* [44]. The third

generation of these drugs have increased activity, even against *Pseudomonas aeruginosa* can penetrate the CNS at concentrations high enough to treat bacterial meningitis [45]. The fourth generation only includes clinically available cefepime and cefpirome that exhibit activity against resistant *Citrobacter* and *Enterobacter spp.* [46]. Lastly, the fifth generation was mainly synthesized to target resistant bacterial strains such as MRSA [47].

Penicillin was the pioneering β-lactam class and possesses a common characteristic five membered thiazolidine ring [48, 49], whilst carbapenems were the last class discovered. The β-lactam ring of carbapenems is fused to a five membered ring that constitutes only carbon atoms with a double bond occurring at C2 and C3 (Figure 2) [50]. Carbapenems are often referred to as drugs of "last resort" because of their good tolerance, broad spectrum of activity, and fewer resistance mechanisms as opposed to penicillins and cephalosporins [51].

The first monobactam synthesized was aztreonam, possessing superior antibacterial activity, compared to the bacterially produced monobactams discovered in 1979 [52]. All monobactams possess a characteristic monocyclic β-lactam ring and have activity towards aerobic gram-negative rods [53], except for those rods producing serine β-lactamases [44]. The potent activity directed towards these rod-shaped bacteria is facilitated by the aminothiazole oxime moiety present on the acyl side chain [52, 54].

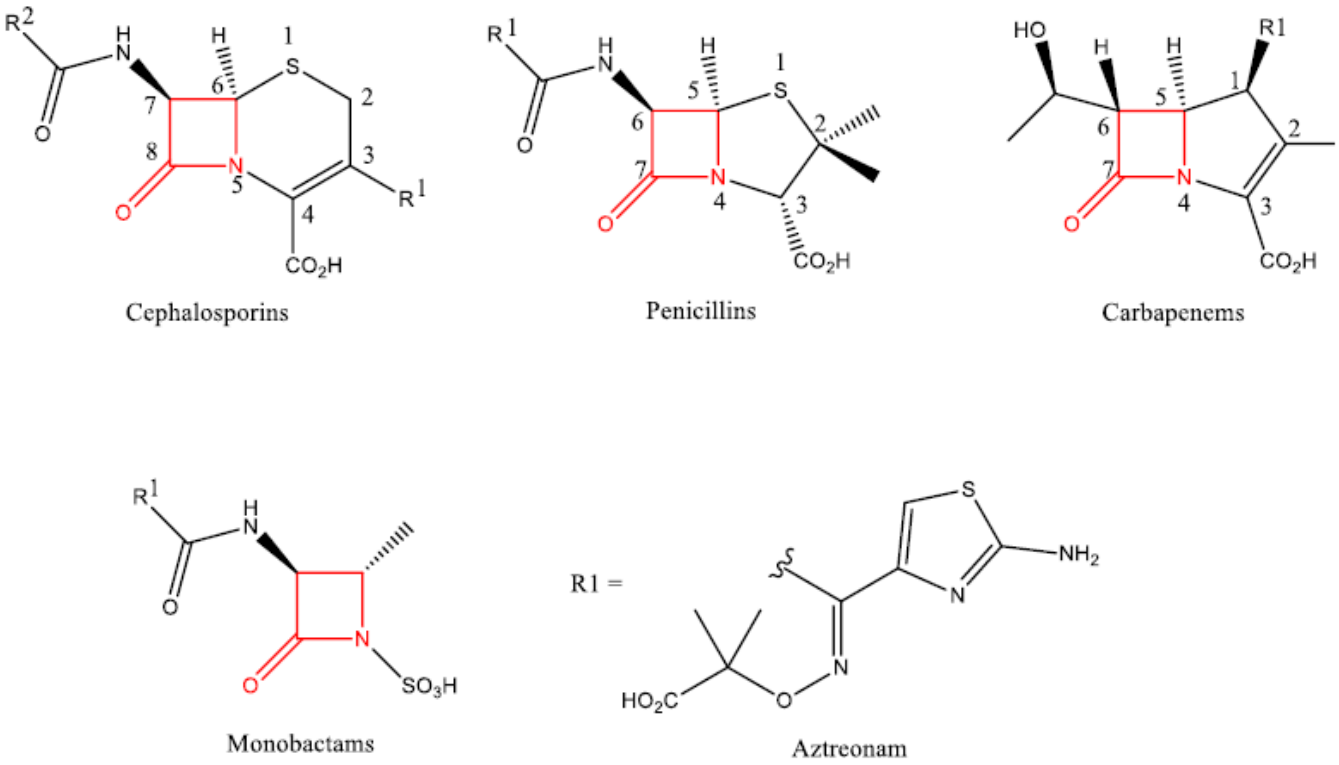
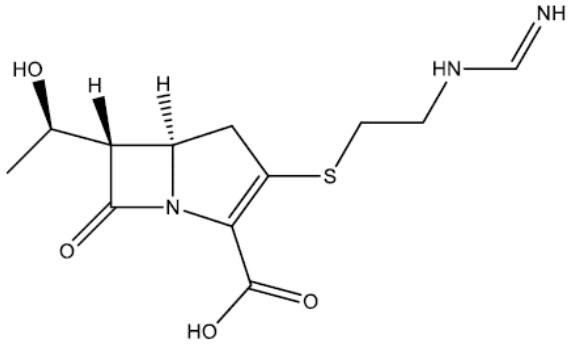


Figure 2: Scaffold for the β-lactam family of antibiotics, the β-lactam ring is depicted in red and atoms have been numbered (adapted from [55], open access).

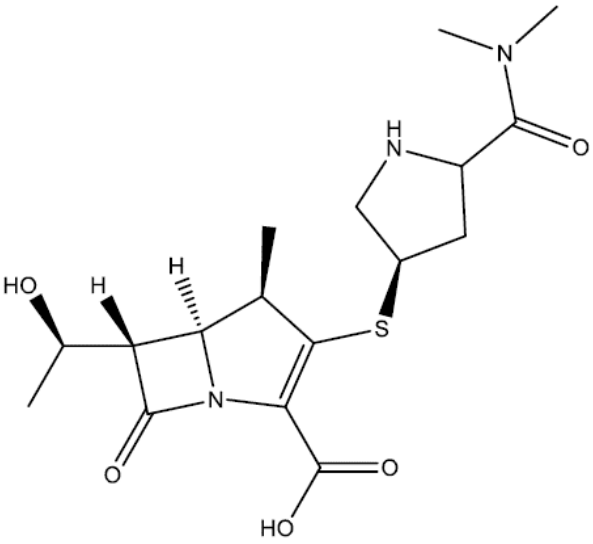
All clinically available carbapenems are administered intravenously, except for tebipenem that is undergoing phase three clinical trials as an oral drug [56, 57] (Figure 3). However, the evolution of multi-drug resistant mechanisms has endangered even carbapenems as many bacteria are resistant to all available antimicrobials in the clinic [58].

The first monobactam synthesized was aztreonam, possessing superior antibacterial activity compared to the bacterially produced monobactams discovered in 1979 [52]. All monobactams possess a characteristic monocyclic β-lactam ring and have activity towards aerobic gram-negative rods [53], except for those rods producing serine β-lactamases [44]. The aminothiazole oxime

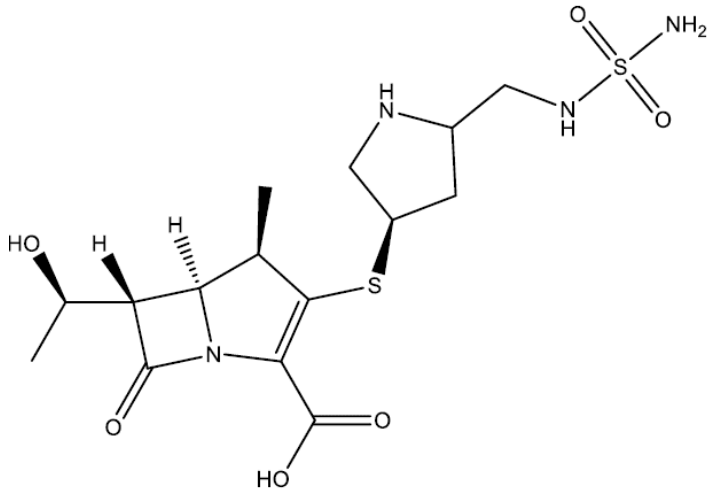
moiety on the acyl side chain facilitates the potent activity directed towards these rod-shaped bacteria [52, 54].



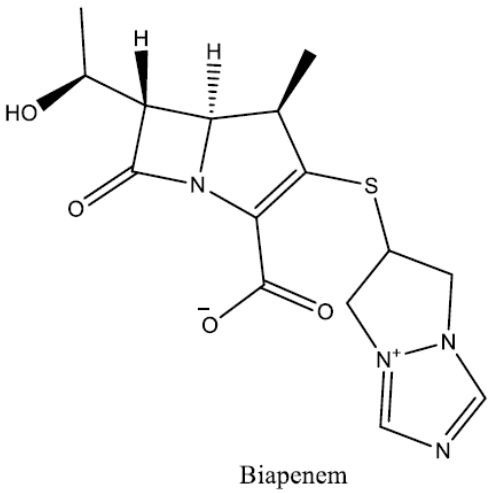
Imipenem



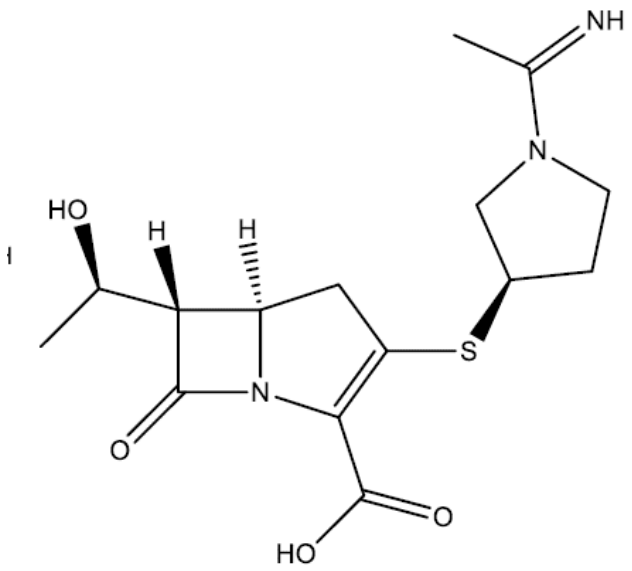
Meropenem



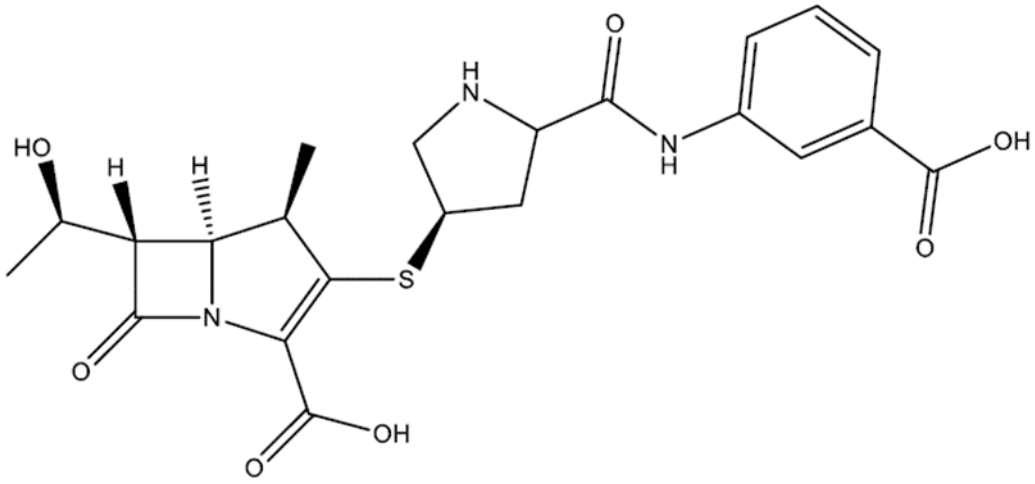
Doripenem



Biapenem



Panipenem



Ertapenem

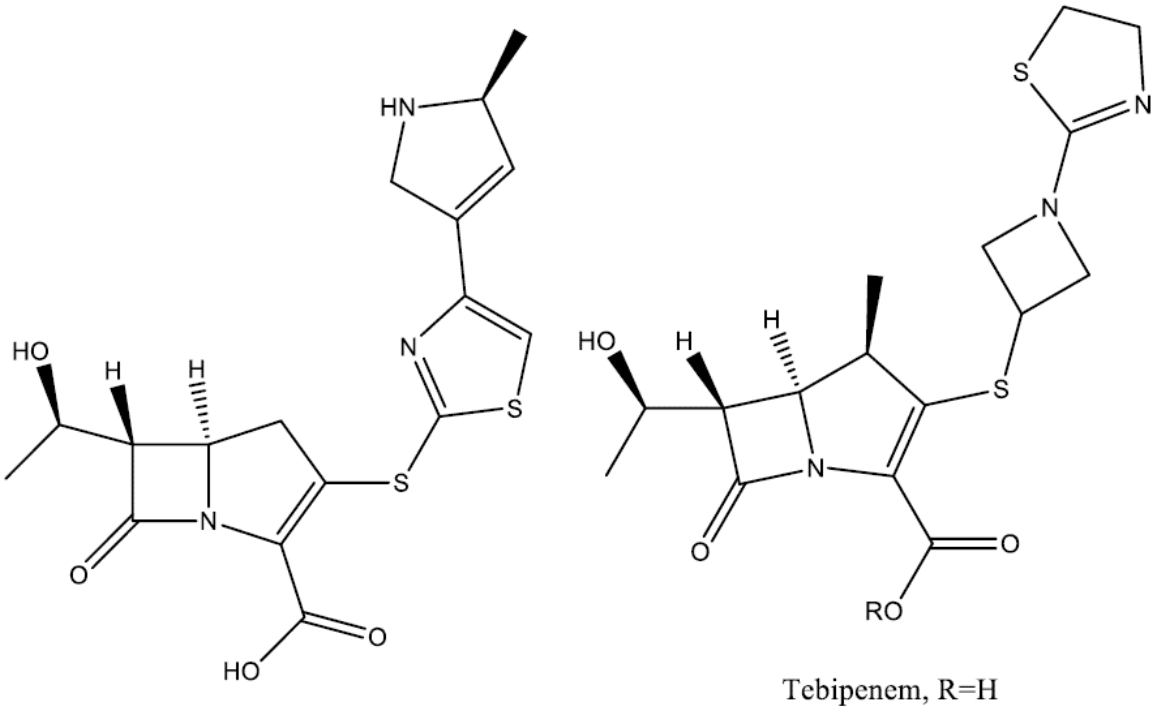


Figure 3: Carbapenems clinically available and tebipenem (in clinical trials) [50] (permission granted, license number, 5043601004745).

Antibiotics are classed according to their mode of action in relation to the infection caused. The different antibiotic classes are unique by their chemical composition and mechanism of action that allows them to target a specific site in the microorganism, that will annihilate the microbe. For instance, aminoglycosides target protein synthesis, fluoroquinolones target DNA replication, and the β-lactams target cell wall synthesis. The next subsection will provide more information on how β-lactams target bacterial cell wall synthesis.

1.1.3. Mechanism of action of β-lactam antibiotics

β-lactams' function by binding covalently to penicillin-binding proteins (PBPs) that are present in the peptidoglycan layer of the bacterial cell wall, thereby preventing cell wall synthesis from occurring [3, 59]. PBPs production varies among bacterial species and their affinity to bind to

various β-lactams [3]. Peptidoglycan is the primary component of the bacterial cell wall. It is responsible for driving resistance to osmotic pressure as well as retaining the strength and integrity of the cell wall [60]. It is comprised of alternating *N*-acetyl muramic acid (NAM) and *N*-acetyl glucosamine (NAG) sugars in which NAM forms a pillared structure with a longitudinal pentapeptide chain (Figure 4).

During cross-linking, each amino acid residue of the pentapeptide chain is gradually eliminated via hydrolysis by PBPs [61], leaving alanyl-alanine (alanine dimer formed by D-alanine-D-alanine) at the terminal end of the pentapeptide [60]. Final stage PBPs such as carboxypeptidases and transpeptidases recognize this alanyl-alanine and mediate cross-linking of the new peptidoglycan layer to the cell wall [62]. Nordmann *et al*. [58] reported a structural similarity of β-lactam antibiotics to that of D-alanyl-D-alanine dipeptide. This structural similarity facilitates the binding of β-lactams to the active site of PBPs (Figure 4). Inhibition of these PBPs, results in the accumulation of cell wall precursors, which in turns activates the cell's autolytic system which results in bacterial cell lysis [58].

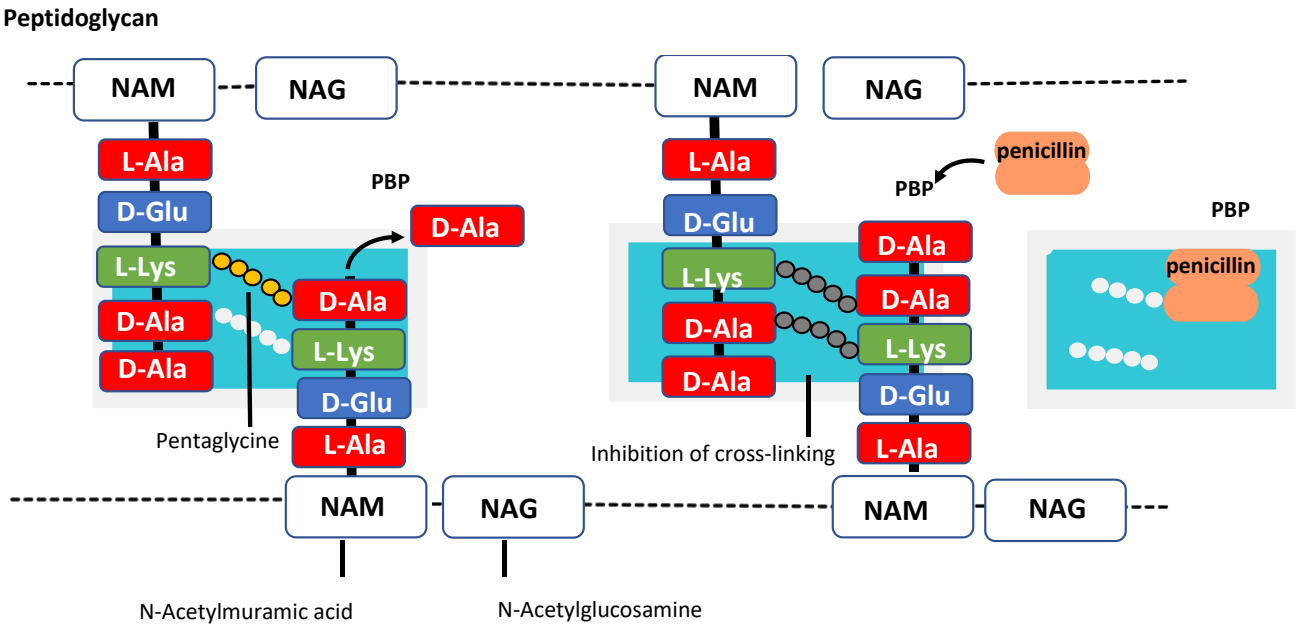


Figure 4: A diagrammatic representation of the inhibition of peptidoglycan synthesis, adapted from Sawa *et al.*, 2020 [60] (open access).

Understanding bacterial resistance is crucial in the attempt of finding suitable drug candidates. Bacteria have evolved and have developed more than a single strategy to overcome the effects of chemical agents. Section 1.2 will highlight the significance of these advances made by bacterial pathogens that have allowed them to proliferate.

1.2. Bacterial resistance

Bacterial resistance can be inherent or acquired from mobile genetic elements, and is used to overcome stressful environmental conditions, such as repeated exposure to antimicrobial agents. Thus, this section focuses on the resistance mechanisms bacteria possess and how they use it as a survival strategy.

1.2.1. Bacterial structure

It is a known fact according to the enormous number of scientific reports, that gram negative bacteria are more resistant to antibiotics than gram positive bacteria [63-65]. This is primarily due to an outer membrane in gram negative bacteria, which acts as a hydrophobic permeability barrier preventing detergents and heavy metals from gaining access to the cell [58, 66, 67].

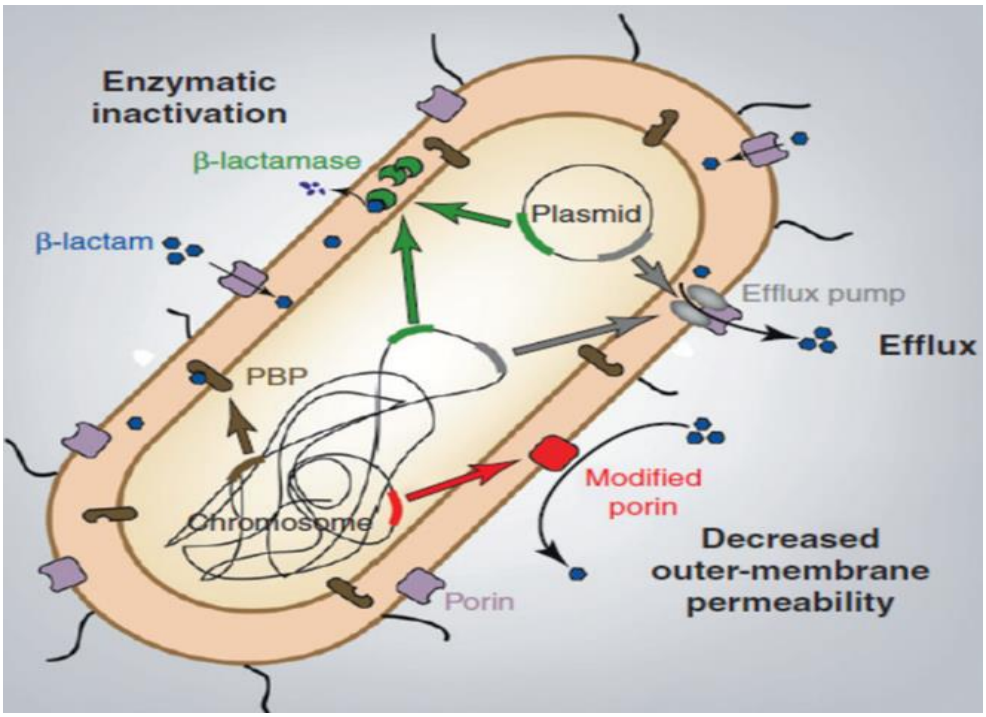


Figure 5: Bacterial structure including the various resistance mechanisms exhibited [58], (permission granted, license number, 5043591470663).

The outer membrane comprises proteins called porins, which are hydrophilic channels that facilitate the transport of nutrients, antibiotics and other essential molecules [68, 69]. However, they are impermeable to hydrophilic solutes [70]. Porins possess membranes that have multiple functions, such as cell adhesion and antibiotic binding [70]. When faced with antibacterial pressure from bactericidal compounds, bacteria develop a survival strategy to combat this pressure [70]. They do this by changing the structural configuration of the porin membrane (Figure 5), thus

resulting in resistance to the bactericidal compound [70]. In the *Enterobacterales* family, Omp (outer membrane protein) F and OmpC are the main porins involved in antibiotic uptake [69].

1.2.2. Efflux pumps

Another mechanism of resistance that provides a thriving area of research is multidrug efflux pumps exhibited by all bacteria and eukaryotes [71-73]. Efflux pumps are responsible for expelling toxic compounds and antimicrobials from the cell (Figure 4), thereby resulting in a decrease of drug accumulation as well as the minimum inhibitory concentration (MIC) [74]. There are five families to which multidrug efflux pumps belong [75, 76], namely; the major facilitator superfamily (MFS) [77], the resistance-nodulation-division (RND) family [78], the ATP binding cassette (ABC) family[79], the multidrug and toxic compound extrusion (MATE) family [80] and the small multidrug resistance (SMR) [81] family (Figure 6) [70, 76].

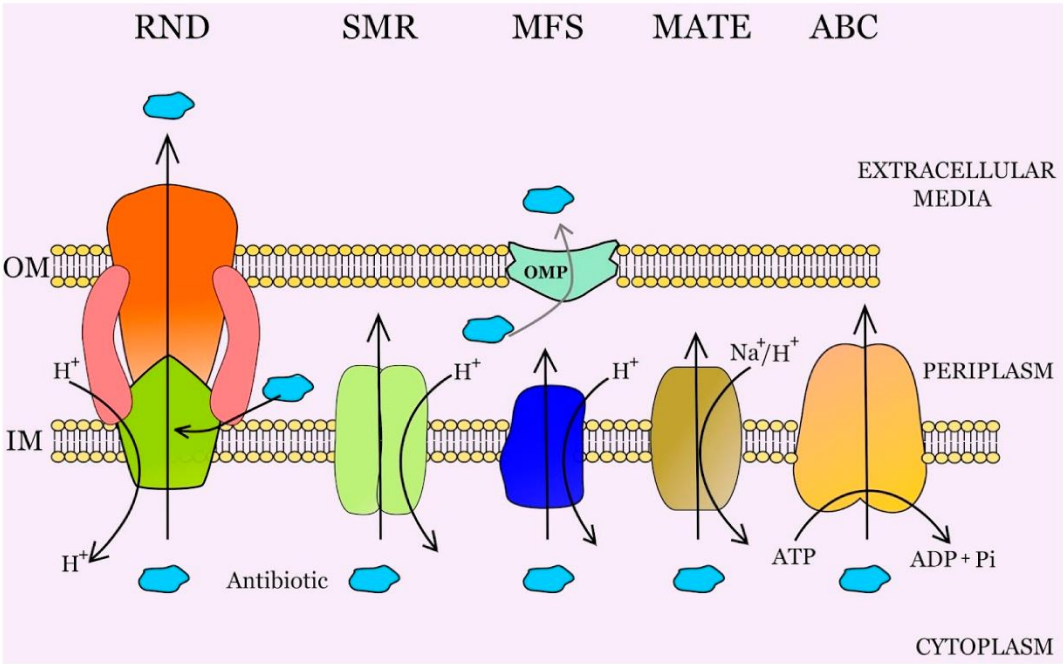


Figure 6: The five major efflux pump families. IM: inner membrane. OM: outer membrane. OMP: outer membrane protein[75] (open access).

Whilst the expression of one efflux pump can trigger resistance to many antibiotics, studies on clinical *Pseudomonas aeruginosa* isolates have documented over-expression of multiple efflux pumps concurrently to antimicrobials [75]. The RND family of efflux pumps has a tripartite system, which facilitates the expulsion of compounds across the outer and inner membrane [82]. The RND family is responsible for expelling β-lactam antibiotics by using a proton motive force [64, 71] and are exclusively found in gram negative bacteria [75].

1.2.3. Enzyme production

Resistance to β-lactam drugs are primarily facilitated by the production of β-lactamases (Figure 5) [83]. These enzymes hydrolyse the amide bond in the β-lactam ring, thus rendering the β-lactam ineffective [84]. The production of AMR in *Enterobacterales* and other closely related bacterial species is largely facilitated by the transfer and expression of genes encoded on plasmids, and transposons, among others (mobile genetic elements) [85, 86]. β-lactamases classification is founded either by their amino acid structure described by Ambler [87] or the Bush and Jacoby [88] molecular-biochemical classification [89]. According to the Ambler classification system, class A, C, and D, are serine β-lactamases since a serine residue is present at the catalytic site. Class B however, is referred to as metallo-β-lactamases and has the metal zinc, at the catalytic site [90, 91]. The Jacoby-Medeiros classification scheme categorizes β-lactamases according to three groups based on substrate degradation of the β-lactam and inhibitory effects [26] (Table 1).

Table 1: βeta-lactamase classification, adapted from Bush and Fisher [92]

Bush-Jacoby Group	Ambler Class	Defining Characteristic(s)	Representative enzymes
1	C	Cephalosporin and cephamycin hydrolysis with higher k_{cat} than penicillins, high affinity for aztreonam, not inhibited by CLA[a] and TZA[b]	AMP-C, CMY-2, FOX-1, P99
1e		Penicillin, cephamycin, ESBLs[c] and monobactam hydrolysis. Not inhibited by CLA and TZA	GC1, CMY-37
2a	A	Penicillin hydrolysis. Inhibited by CLA and TZA	PC1 and other *Staphylococcal* penicillinases
2b		Penicillin and early cephalosporin (e.g., cefazolin) hydrolysis. Inhibited by CLA and TZA	SHV-1, TEM-1, TEM-2 TLE-1 (TEM-90)
2be		Penicillin, ESBL and monobactam hydrolysis. Inhibited by CLA and TZA	CTX-M-15, SHV-5, TEM10, VEB-1
2br		Penicillin and early cephalosporin hydrolysis. Not well Inhibited by CLA	TEM-30, TEM-76, SHV-10, SHV-26
2ber		Penicillin, ESBL and monobactam hydrolysis. Less efficient inhibition by CLA and TZA	TEM-50, TEM-68, TEM-89
2c		Carbenicillin hydrolysis. CLA is an effective inhibitor	PSE-1, CARB-3
2d	D	Cloxacillin or oxacillin hydrolyses. Not frequently inhibited by CLA	OXA-1, OXA-10

2de		Penicillin and ESBL hydrolysis. Not frequently inhibited by CLA	OXA-11, OXA-15
2df		Carbapenem, cloxacillin/oxacillin hydrolysis. Not frequently inhibited by CLA	OXA-23, OXA-48
2e	A	Cephalosporin hydrolysis. CLA and TZB inhibition but not aztreonam	CepA
2f		Penicillin, cephalosporin, cephamycin and carbapenem hydrolysis, poorly inhibited by CLA and TZA	IMI-1, KPC-2, SME-1, GES-2, KPC-3
3a	B	Except monobactams, all β-lactams are hydrolyzed. Not inhibited by CLA and TZA. Inhibited by EDTA and metal ion chelators	NDM-1, VIM-1, IMP-1, L1
3b		Carbapenem hydrolysis. Not inhibited by CLA and TZA. Inhibited by EDTA and metal ion chelators	CphA, Sfh-1

a: Clavulanic acid, *b*: Tazobactam, c: Extended Spectrum β-lactamases, cephalosporins and monobactams that have a side chain extending from the β-lactam ring, containing an aminothiazoleximе moiety

Group one originally involved chromosomal genes and comprises cephalosporinases with similar structural-molecular characteristics and are known as class C. Group two comprises class A and D serine β-lactamases that are structurally similar but distinct from the serine β-lactamases found in group one. Group three corresponds to class B of the classification scheme and the enzymes are termed metallo-β-lactamases [60]. This project will focus on enzyme resistance; therefore β-lactamases will be further discussed in detail.

1.3. Serine β-lactamases

Serine β-lactamases (SBL's) have an α and α/β domain with the active site located in a groove between the 2 domains. Although all SBL's have the same domain and conserved active site, they share as little as 15% amino acid sequence identity [93, 94]. SBL's initiate β-lactam hydrolysis based on the following key motifs that help in recognition of the β-lactam antibiotic[88]: a.) a polar pocket that functions as a magnet to the β-lactam carboxyl moieties; b.) an oxyanion with affinity for stabilising the carbonyl oxygen of the β-lactam ring; c.) a catalytic serine hydroxyl residue that targets the carbonyl carbon atom, leading to breakage of the amide bond; d.) conserved amino acid residues that partake in diacylation, that is absent in PBPs [95]. SBL's hydrolyse β-lactams by utilizing a serine nucleophile at the active site which forms an acyl-enzyme intermediate, a catalytic water molecule activated by a general base, resolves this acyl-enzyme intermediate through the deacylation step (Figure 7) [96]. In class A SBL's the active-site serine is initiated by Lys73 and Glu166, whilst Lys67 and Trp150 residues are involved in class C activation [97, 98]. Class D's general base utilized is Lys73, an unusual *N*-carbamylated lysine residue [99].

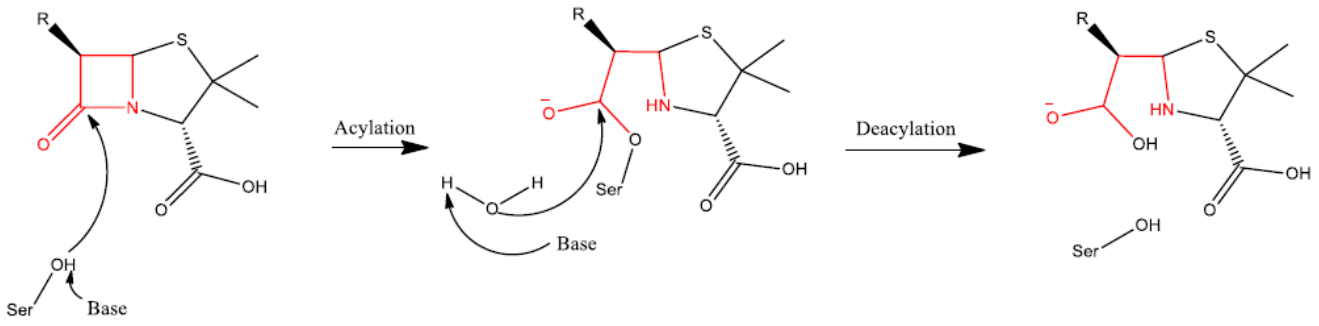
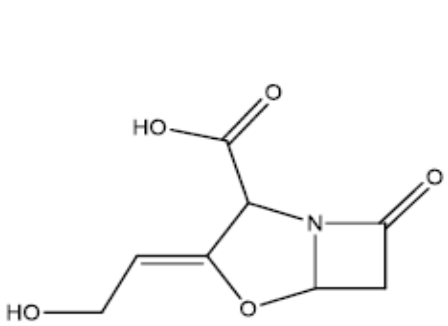


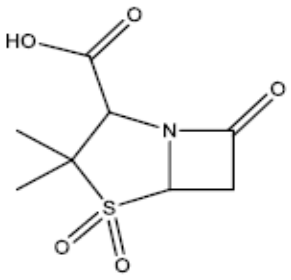
Figure 7: The catalytic mechanism of SBL's with the β-lactam ring depicted in red [96] (open access).

Olivanic acid, a natural product, was the first serine β-lactamase inhibitor reported in 1976, however scientists lost interest in this compound due to poor stability and the inability of the

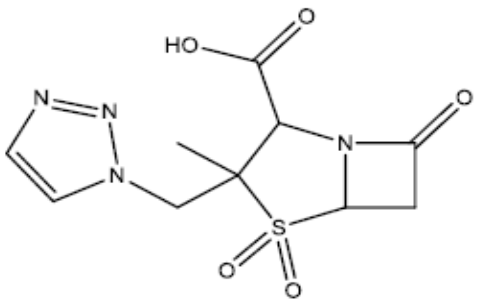
inhibitor to penetrate the bacterial cell wall [50]. Shortly after that research for a suitable β-lactamase inhibitor was re-ignited with the discovery of clavulanic acid from *S. clavuligerus* in 1977 [100], followed by tazobactam and sulbactam in the 1980's [101, 102]. Clavulanic acid is a clavam [103] and is known to target some of the SBL's belonging to class A (Table 1). Whilst sulbactam and tazobactam are penicillanic acid sulphones [103] that are small molecules resembling penicillin's structure [104]. β-lactamase inhibitors provide little to no intrinsic activity on its own, however when co-administered with a β-lactam drug, a potent combination results, for example clavulanic acid and amoxicillin [34]. Many FDA-approved SBL inhibitors clinically available for commercial use (Figure 8).



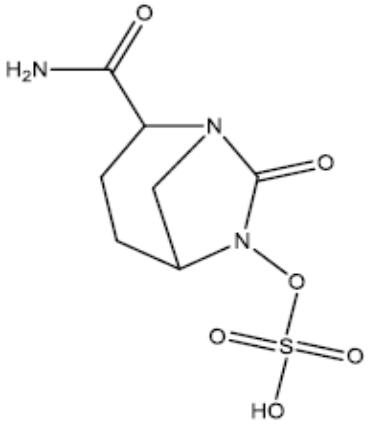
Clavulanic acid



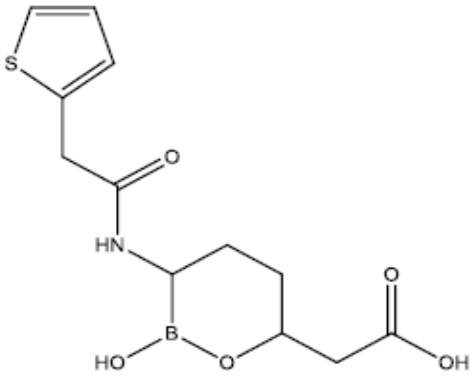
Sulbactam



Tazobactam



Avibactam



Vaborbactam

Figure 8: SBL inhibitors clinically available (re-drawn from Eiamphungporn *et al.*, 2018 [36], open access).

Turning the focus to the main protagonist of this research, the metallo-β-lactamases, the next section provides some insight into the history, structure and functioning of these enzymes, when harboured in a prokaryote host. Information on our current stance with this challenging enzyme and the potential candidates representing hope for further clinical assessment is also detailed in this section.

1.4. Metallo-β-lactamases

Metallo-β-lactamases (MBLs) were initially discovered in 1966 by Sabath and Abraham (Bc11 from *Bacillus ceres*), an early 20 years (Figure 1) before carbapenems were first implemented into clinical practice [105]. However, since they were chromosomally encoded in non-pathogenic soil bacteria, they were not considered a serious threat to antimicrobial therapy [106, 107]. Surprisingly, this changed in the 1990's as the spread of verona integron-encoded (VIM) and imipenemase (IMP) MBL's disseminated among *Enterobacterales*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa* strains [108, 109], leading to a global health concern [110].

Except for monobactams, and the recently approved cefiderocol [111] MBL's hydrolyse all β-lactam classes and are thereby the most prevalent β-lactamase group [112]. Although MBL's are incapable of hydrolysing monobactams, many bacteria co-harbour SBL's and MBL's, therefore limiting the use of monobactams as an effective treatment strategy against MBLs [113]. LYS228 however, is a monobactam currently in phase two trials that can de-activate MBLs and SBLs [114].

Metal stripping chelators such as EDTA, inhibit MBL activity [32] by removing metal ions from the active site [115], but due to the high toxicity exhibited, it is unsafe for clinical use [116]. Metal

stripping agents offer poor selectivity and off-target specificity, with implications of damage to the physiological system through de-activation of essential metalloproteins [115, 117] Metal ion binding chelators L-captopril and D-captopril have produced excellent activity against MBLs [118]; however, they are known to form ternary complexes with the metal ions and suffer non specificity [115, 118]. Hence, there are no MBL inhibitors that are currently effective for clinical use, chapter two describes a review paper on recent advances made towards finding such an inhibitor within the patent literature in this field.

1.4.1. MBL Structure and arrangement of zinc ions

Metallo-β-lactamases have a characteristic αβ/βα sandwich fold. They are further categorized into three subclasses, B1, B2 and B3 based on the number and co-ordination of zinc ions at the active site as well as the substrate profile [55]. The B1 subclass utilizes two zinc ions at the active site, has a large substrate profile and is therefore the most clinically catastrophic subclasses [119]. At the first zinc binding site, one zinc molecule is bound to His116, His118, His196 and a bridging water molecule. At the second binding site, one zinc molecule is coordinated to Asp120, Cys221, His263 and a second water molecule [120].

Members of this class are known for the global spread of their resistance genes on mobile genetic elements and include NDM [62], IMP [121], VIM [109], SPM [122] and CcrA [123]. Subclass B2, are chromosomally encoded MBL's with a narrow substrate profile that is exclusive to carbapenems. In the B2 subclass the second zinc binding site is utilized with metal ligands Asp120, His121 and His263 (Figure 9) [55]. The first binding site has an Asn116 substitution instead of His116, this leads to a conformational change of binding site one, such that binding another zinc ion will deactivate the MBL [124].

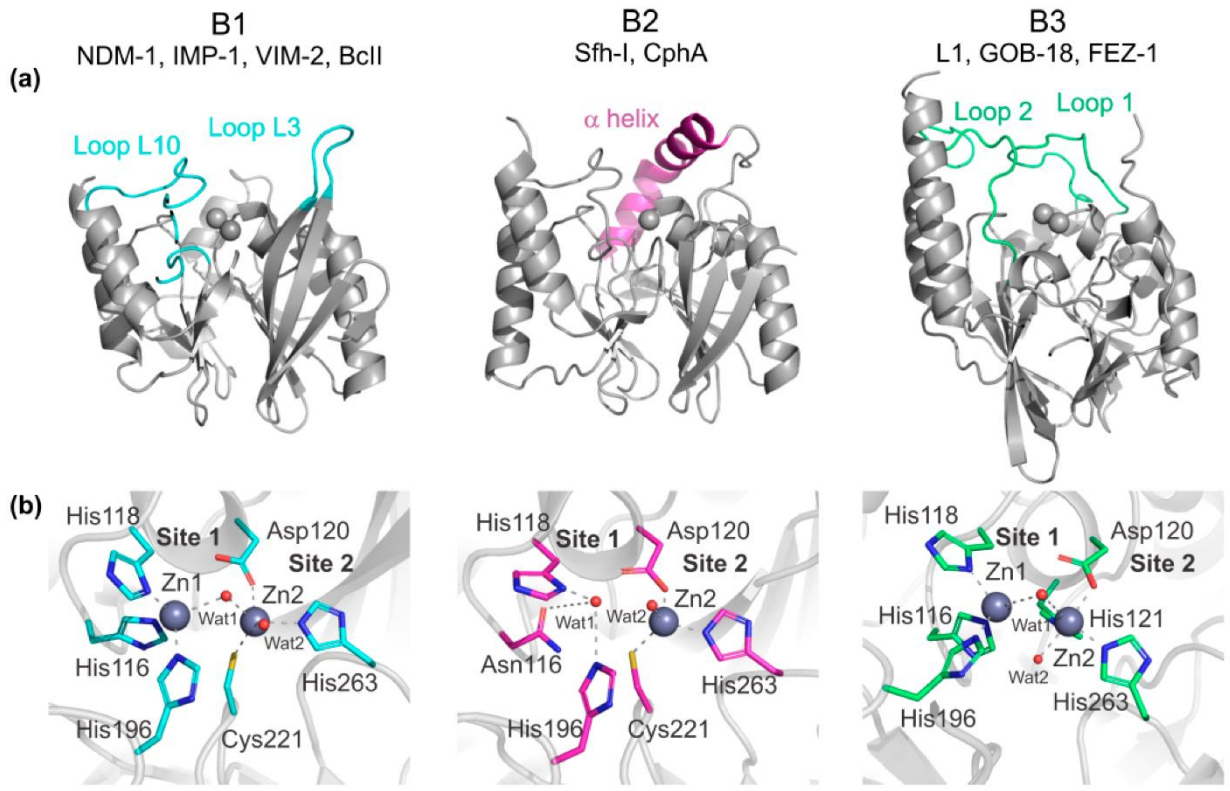


Figure 9: Subclasses B1-B3 and the interactions at the binding sites [55] (open access). (a) Overall protein structures. Helices and loops involved in interacting with substrate are depicted in color. (b) Metal ligand interactions are shown in colour, zinc ions are illustrated as grey spheres, whilst water molecules are presented as red spheres. Dashed lines represent interaction of the zinc ions. For subclass B2, Sfh-1 is used to illustrate one zinc ion at the binding sites.

Members of subclass B2 include CphA from *Aeromonas hydrophila*, IMiS from *Aeromonas sobria* and Sfh-1 from *Serratia fonticola [119]*. The metal ligands of binding site one are conserved for subclass B3 but differs to subclass B1, in that the second binding site utilizes Asp120, His121 and His263 as metal ligand residues [125]. Subclass B3 has a large substrate profile with only 9 residues preserved with subclass B1 and B2 [126]. MBL's in B3 includes; GOB from *Elizabethkingia meningoseptica* [126], FEZ from *Legionella gormanii* [127] and L1 from *Stenotrophomonas maltophilia* [128, 129].

1.4.2. Catalytic mechanism of MBLs

The role of zinc in MBL catalysis is advantageous in that it can function as a Lewis acid and bind strongly to suitable sites by exhibiting flexibility in the co-ordination geometry of these sites [130, 131]. By functioning as a Lewis acid, zinc arranges itself on the amide carbonyl oxygen of the β-lactam ring, resulting in an electron deficient carbonyl carbon [132, 133]. This leads to a nucleophilic attack and stabilization of the negatively charged carbonyl oxygen, which forms a tetrahedral anion [134]. This co-ordination is orchestrated by a concentrated zinc-bound hydroxide in which the pK_a of the attached water has been lowered [135]. Lastly co-ordination of the leaving amine nitrogen to the metal ion (Asp120) or the metal-bound water molecule, exhibits a catalytic reaction whereby the amine nitrogen departing group is protonated and results in the breakdown of the tetrahedral intermediate [135, 136]. This process opens the β-lactam ring (Figure 10).

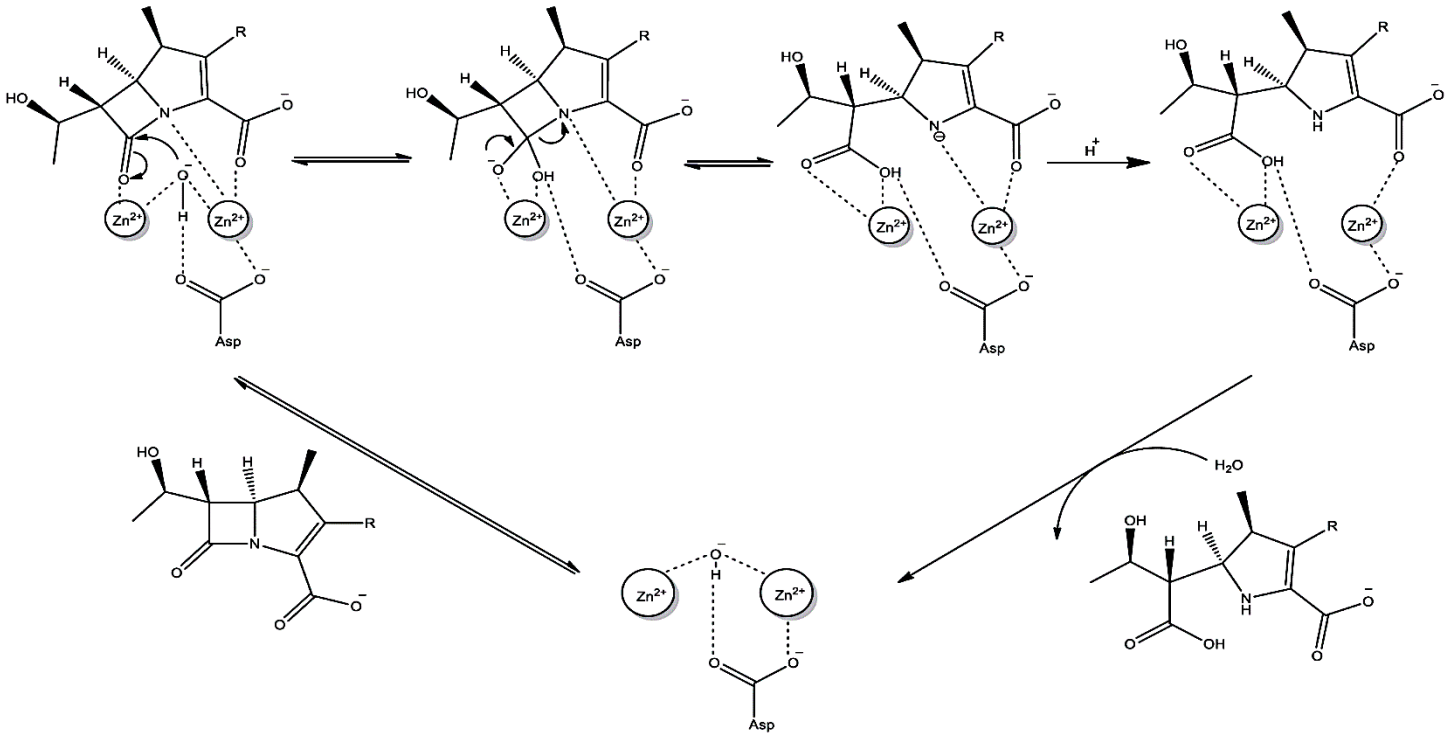


Figure 10: General catalytic mechanism of subclass B1 MBL inactivating a carbapenem antibiotic [134] (open access).

Enzymes are pertinent to the survival of all life forms and occupy a diverse array of roles that can either be essential to survival or detrimental to many cellular systems. The next focus area will encompass enzyme expression principles used to upscale the quantity of enzyme required for experimental testing procedures.

1.4.3. Enzyme production through recombinant DNA technology

Many proteins are not available to scientists in an abundant amount for various experimental procedures. Therefore, recombinant DNA technology offers a solution to increase protein production in a suitable host and facilitate the protein purification process. Three components generally govern over expression of a protein, namely [137]:

a.) A gene of interest [137]. This can be selected through enzymatic cleavage of various DNA segments by restriction endonucleases [138]

b.) A vector in which the gene can be cloned into [137]. This is facilitated by DNA ligase joining the gene fragments and fixing them into a vector [138]

c.) An expression host that can produce enough good quality protein [137]. This is facilitated by introducing the vector into a host organism, multiplication of the expression host containing the incorporated gene, followed by selection and harvesting of clones and protein purification [138].

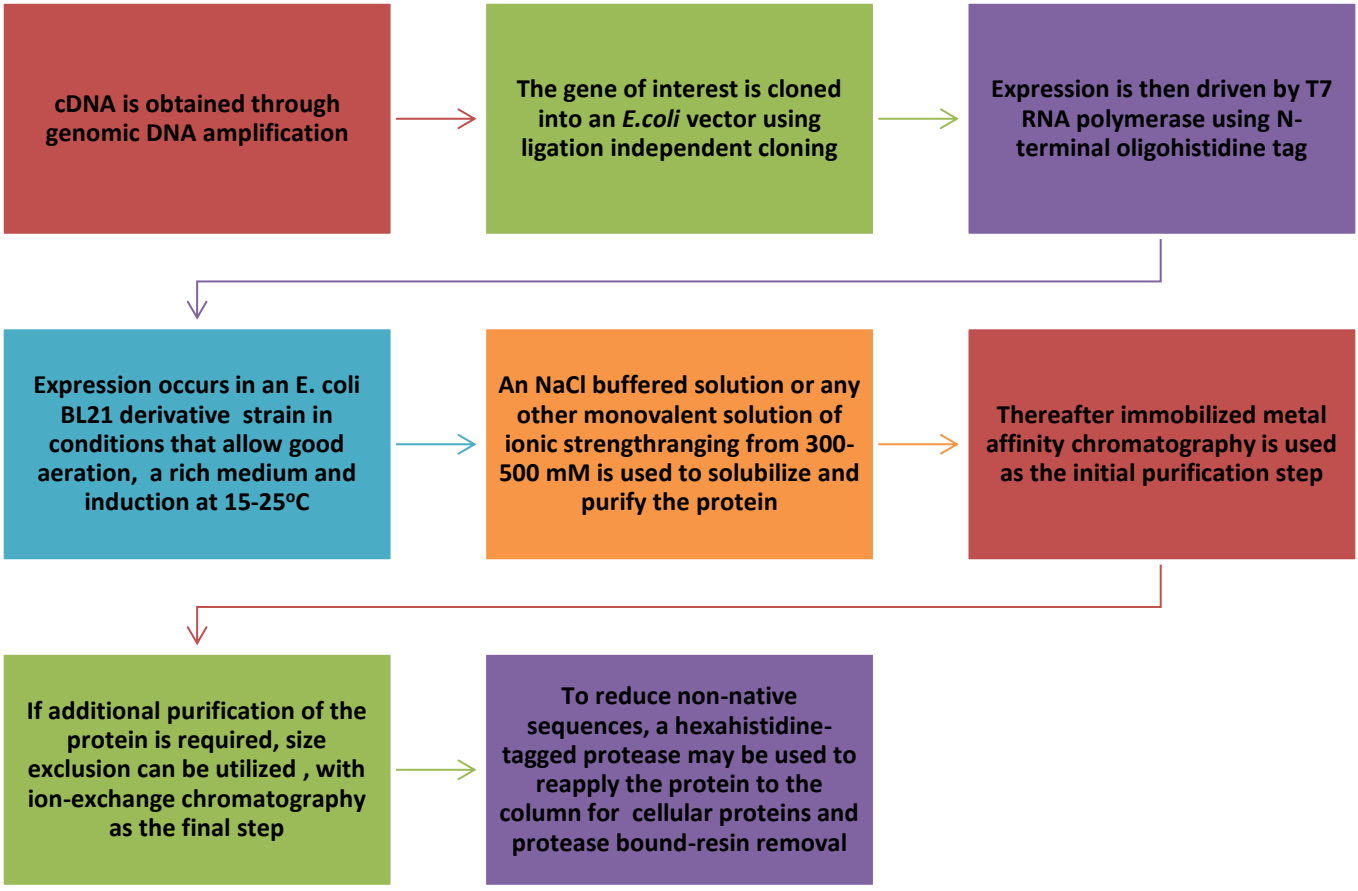


Figure 11: Consensus protocol adapted from literature [139].

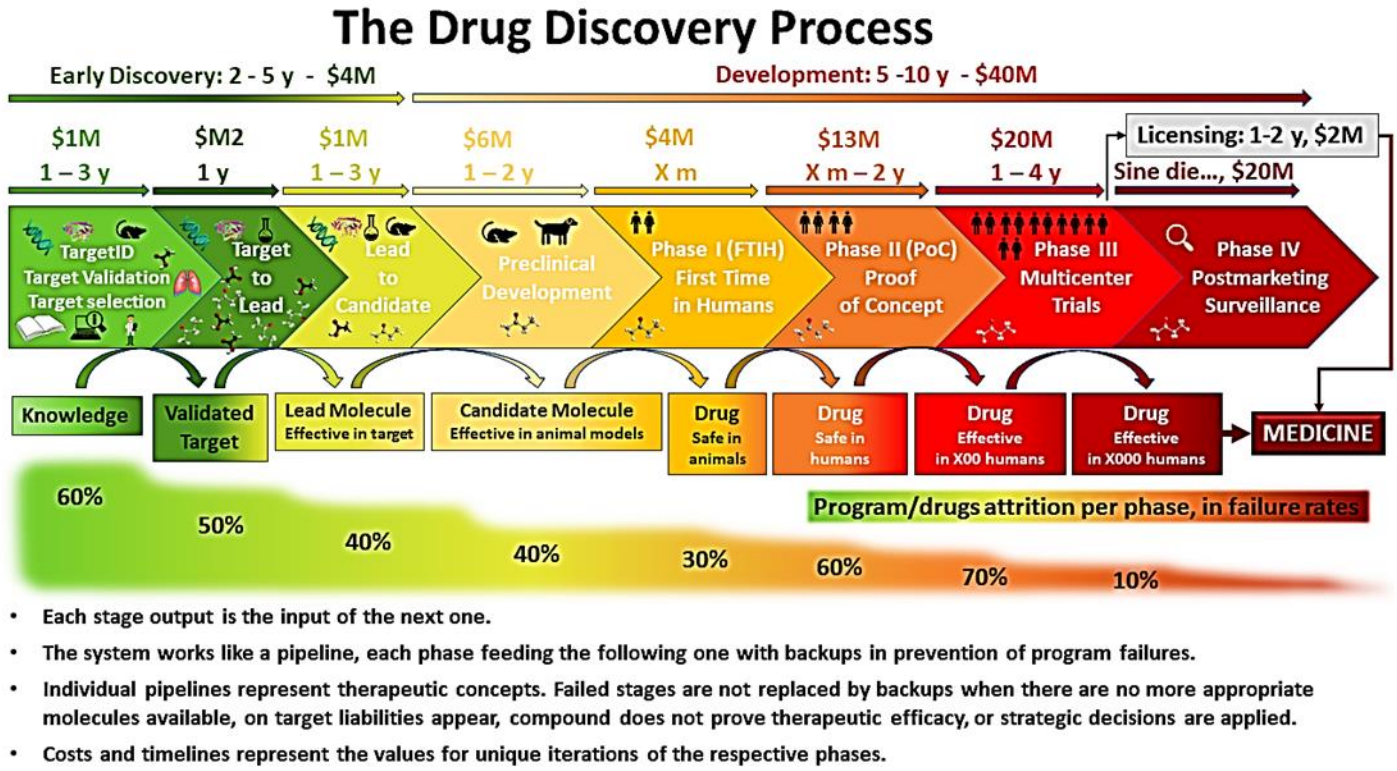
Various hosts can be used as an expression system such as *Escherichia coli, Pseudomonas spp* [140]*, Lactococcus lactis* [140, 141], *Saccharomyces cerevisiae* [142, 143] and Baculovirus for insect cell lines [144]. However, *Escherichia coli* is often the preferred expression host, factored by benefits such as simple yet fast expression, cost effective and versatile in that many different types of proteins can be produced [145, 146]. Generally, enzymes are synthesized according to the description provided in Figure 11, however for ease of convenience for this study we purchased the required enzymes, as they are readily available from suppliers.

1.4.4. The use of metal chelators as MBLI's

Two types of MBLI's exist: small molecules dependent on zinc or those independent [137] of zinc as their mechanism of action [134]. Those dependent on zinc include metal ion chelators such as EDTA or metal binding compounds, such as dicarboxylates and thiols [147]. Zinc independent MBLI's have been recently reported to indirectly chelate zinc by mimicking the interaction of the β-lactam substrates [148]. Finding a suitable MBLI is exacerbated by the structural and mechanistic differences within the three MBL subclasses, with only a few conserved motifs bearing zinc co-ordination residues [149]. Investigating metal ion chelators as possible MBLIs is an obvious choice and many studies have been conducted on picolinic derivatives [150], cyclam based chelators [151, 152], spiro-indoline-thiadiazole [153] and the natural product *Aspergillomarasmine* A [154], with outcomes of promising efficacies. Other reported MBLIs that have demonstrated potential and require further pre-clinical assessment include our comparators (Chapter four and five); nitrogen-based zinc chelator ZN148 [155] and pentadentate H_2dpa derivatives [156].

Chelators that are in the drug discovery pipeline (Figure 12) include, ANT2681 – pre-clinical, but ready for phase one clinical development [157], QPX722 – phase one clinical development [158]

and taniborbactam (VNRX-5113) – phase three clinical development [159]. ANT2681, is a thiazole, carboxylate derivative co-administered with meropenem that de-activates MBLs by interacting with their cluster of dinuclear zinc ions [160]. Both QPX722 and taniborbactam are boronic acid inhibitors, that have demonstrated superior activity in the preclinical evaluation with inhibition occurring against SBLs and MBLs [161]. However, currently still unknown is the activity spectrum of the boronates and their mechanism of action [162].



- Each stage output is the input of the next one.
- The system works like a pipeline, each phase feeding the following one with backups in prevention of program failures.
- Individual pipelines represent therapeutic concepts. Failed stages are not replaced by backups when there are no more appropriate molecules available, on target liabilities appear, compound does not prove therapeutic efficacy, or strategic decisions are applied.
- Costs and timelines represent the values for unique iterations of the respective phases.

Figure 12: The drug discovery process [163] (open access).

Our research group has previously studied the antimicrobial activity of various chelators in combination with commercially available antibiotics such as, meropenem. These chelators include 1,4,7- triazacyclononane-1,4,7-triacetic acid (NOTA) [151] di-(2-picolyl) amine (DPA) and *N,N,N′,N*-tetrakis (2-pyridymethyl) ethylenediamine (TPEN) [150] that have proven to be

efficacious in reducing the MIC *in vitro*. However, NOTA has shown poor bioavailability *in vivo* (Chapter 3). Studies have shown that increasing the lipophilicity of NOTA through the coupling of N-methylated amino acids, helped increase its bioavailability [117, 150, 164]. Thus, the coupling of a chelator to known β-lactam antibiotics has formed the premise of this study, with the hypothesis that the β-lactam will provide greater lipophilicity and specificity that will facilitate the transportation of the chelator to the MBL active site. This will then allow the chelating moiety to inhibit the MBL, allowing the β-lactam antibiotic to elicit its function on the bacterium. As a result, antibiotic efficacy is restored, bioavailability and pharmacokinetics is improved, and the pathogen is eliminated. Therefore, we anticipate that this novel approach will hold great promise for the effective and complete inhibition of, MBL harbouring pathogens.

The subsequent experimental chapters of this thesis will document our progress towards meeting our goal of establishing metal ion chelators that target antimicrobial resistance. One of the primary *in vitro* experiments conducted to assess the potential of an antimicrobial agent, is the antimicrobial susceptibility test. Section five provides an overview of the types of antimicrobial tests that will be carried out in this study. These tests generate the MIC and although referred to as "preliminary screening tools", they are fundamental in assessing if a compound possesses antimicrobial activity.

1.5. Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of a therapeutic drug can be determined by various antimicrobial susceptibility tests (AST). The MIC can be determined for a single drug or a combination of drugs [165, 166]. Synergy tests are used for combination studies with the ultimate goal of establishing the antimicrobial profile of the drugs. Two or more drugs are said to act synergistically when the combined effect is far better than the sum of the individual drug effect. These experiments can also identify additive and antagonistic interactions. Synergy testing *in vitro*

can be performed using the E-test, multiple-combination bactericidal antimicrobial testing (MCBT), checkerboard assay, time-kill study [167], as well as the serum bactericidal titre and critical inhibitory concentration technique, among others [168]. For this dissertation, we will focus on the checkerboard assay (which incorporates the broth microdilution technique), the serum bactericidal titre, and the time-kill study.

1.5.1. Checkerboard method

This technique utilizes serial two-fold dilutions to evaluate the potency of drug combinations at clinically achievable concentrations (Figure 13). The combinations are said to be synergistic, additive, or antagonistic (Figure 14) through calculation of the fractional inhibitory concentration index (FICI) derived from the generated MICs [168]. One of the few limitations to this method is that it requires many reagents for each antimicrobial combination to be tested and that only two combinations can be tested at a time [169]. However, this method aligned with our research goal as we did not investigate more than two combinations at a time (Figure 13).

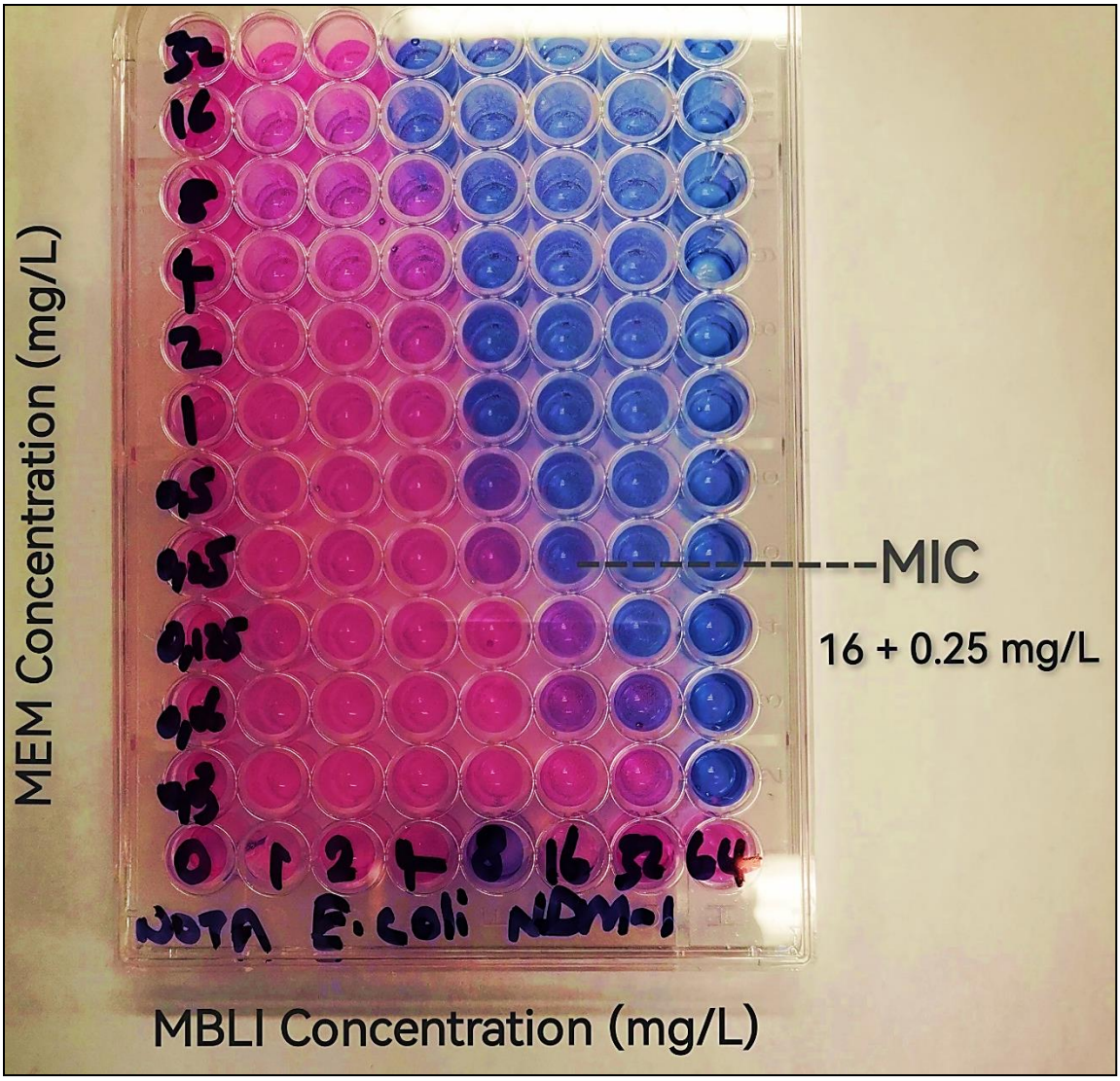


Figure 13: An example of the layout for a checkerboard assay. The antibiotic, MEM (meropenem) concentration is read from the ordinate of the microtiter plate. The MBLI concentration, in this case NOTA is read from the abscissa. Resazurin dye is used to visualize the inhibition and turbidity of the wells. Inhibition is noted by blue wells, absence of inhibition is noted by pink wells and purple wells note partial inhibition. The MIC is represented as the best combination of MBLI + antibiotic.

$$\mathbf{FIC} = \left(\frac{\mathbf{MIC\ drug\ A\ in\ combination}}{\mathbf{MIC\ drug\ A\ alone}}\right) + \left(\frac{\mathbf{MIC\ drug\ B\ in\ combination}}{\mathbf{MIC\ drug\ \ B\ alone}}\right)$$

Example:

Antimicrobial agent	MIC alone	MIC in combination
A	32	4
B	8	1

$$FIC = \left(\frac{4}{32}\right) + \left(\frac{1}{8}\right) = 0.25 \rightarrow Synergy$$

FIC Value	Interpretation
≤ 0.5	Synergy
>0.5 - 1	Additive
1 - 4	Indifference
>4	Antagonism

Figure 14: The Fractional Inhibitory Concentration Index used to assess the MIC generated from the checkerboard assay, adapted from literature [169].

1.5.2. MCBT method

This test can evaluate antimicrobial combinations consisting of two – four drugs and is designed to utilize blood concentrations that fall within an acceptable pharmacological range. In addition, the concentration used per experiment, largely depends on the amount of serum obtained from a patient. This technique is thus subjective and utilizes a fixed antimicrobial combination, whereas the checkerboard assay investigates multiple drug combinations [170].

1.5.3. Time-kill curve assay

The minimum bactericidal concentration (MBC) determines the drug concentration that can achieve 99.9% of bacterial killing over a 24-hour period. The time-kill assay is thus a derivative of the MBC [171]. The time-kill assay incorporates the principle of MCBT however, bactericidal activity is monitored over 24–48-hour periods instead of a single time point. Establishing the rate of bacterial killing is more relevant than the optimal killing concentration at a single time point, therefore, time-kill assays are preferred over the checkerboard assay in predicting patient outcome [172]. Although there is no true gold standard for synergy testing, time-kill assays is the primary comparator [167] in literature [173, 174].

1.5.4. Etest method

When examining the effects of a single drug, the Etest method functions using the principle of diffusion. This occurs when an antimicrobial strip is impregnated onto solid agar containing a bacterial lawn and continuously delivers a specified concentration gradient of antibiotic. After overnight incubation, the elliptical point of the zone (correlating to no-growth) that interacts with the strip is considered the MIC [176]. When two antimicrobials are studied, each antimicrobial strip is placed in a perpendicular position, with the intersection generating the combined MIC,

whilst the MIC alone is obtained from each of the strips. Synergy is then calculated from the FICI [173].

Another option is to place a strip containing drug one onto a bacterial lawn and removing it after an hour. Thereafter, another strip containing drug two is placed in the same position as the first strip to represent synergy. Individual antibiotic strips can be placed on the same plate at a different positions to generate the MIC of each antibiotic alone [174]. The FICI is therefore defined as synergistic when there is a $\geq$ three dilution reduction in the MIC, additive when the reduction is between two to three dilutions, indifference when two dilutions reduce the MIC and antagonistic when there is an increase in the MIC by three dilutions [174].

1.5.5. Serum bactericidal titre (SBT)

The serum bactericidal titre method predicts the pharmacokinetic (PK) properties of the antimicrobial in question, by assessing the drug elimination rate and determining the level of protein binding exhibited [177]. The method uses serum to determine the peak and trough antimicrobial concentrations, in combination or alone. The serum is serially diluted, and a standardized inoculum is added [178]. The SBT is established by the highest dilution of serum that correlates to the 99.9% bacterial killing. The SBT, the free drug concentration in serum, together with the minimum bactericidal concentration of the test drug in Mueller-Hinton broth, provide information on the drug interaction. The shortfall of this method is that there are technical difficulties in measuring the free drug concentration in serum [168].

1.5.6. Critical inhibitory concentration (CIC)

The critical inhibitory concentration is used to predict the synergistic effect that can occur *in vivo* [179]. Experimentally, this is achieved by using the agar pour plate technique to isolate the

microorganism. Thereafter, holes are punctured into the agar medium to create wells that are then filled with the antimicrobial combination at graded concentrations using various concentration ratios. Post incubation, the square distance between the zone of inhibition and the well is recorded. This data is plotted against the respective concentration, the resulting interception of the straight line generates the CIC [168]. Increased bacterial killing is noted by a reduced CIC [179].

Investigating the cytotoxicity of a drug, is crucial in the pre-clinical evaluation to determine if the test drug is safe to administer at the selected dose. This requires the use of various cell viability assays as tools to screen these test compounds. The next section will provide some insight into cytotoxicity testing, focusing on the two most common assays used in research.

1.6. Cytotoxicity

The term cell viability defines the quantity of functional cells in a population unhindered by effects of drugs [180]. A drug is said to be cytotoxic if it kills the cell, however if it causes cell growth inhibition then it is cytostatic [181]. When evaluating potential MBLI candidates it is important to ascertain if the candidate possesses toxic or haemolytic effects as it can halt the research progress and prevent further testing of the compound. Another factor to consider is the application of quantitative high-throughput screening, in which, a broad range of serial MBLI concentrations are tested [182] to generate results that mitigate negative and false positive outcomes as well as promote increased sensitivity towards cytotoxicity [183]. There are many assays that can be used to measure cytotoxicity by determining cell viability and these include, the lactate dehydrogenase assay (LDH) (colorimetric), the MTT (3-[4,5-

dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) metabolic cell proliferation assay (colorimetric), Tryphan blue assay (dye exclusion), alamarBlue assay (fluorimetric), and the ATP assay (luminometric) among others [184]. We will focus on the MTT assay and the LDH assay as it is the experimental methods of choice, used to assess the cytotoxicity of the BP compounds herein.

1.6.1. The MTT assay

The MTT assay is based on the principle whereby live cells can convert MTT, a water-soluble yellow tetrazolium dye, to formazan, a purple product insoluble in aqueous solution [185]. This process is facilitated by mitochondrial oxidoreductase enzymes dependent on NAD(p)H [181]. Mitochondrial activity by viable cells is directly related to the amount of formazan crystals produced. Dead cells have reduced metabolic activity indicated by a decline in MTT reduction, whilst proliferating cells show an increased rate of MTT reduction [186]. Formazan crystals can be solubilized in a detergent sodium dodecyl sulfate or a dimethyl sulfoxide solution (DMSO), this solution can then be measured spectrophotometrically between 540 and 720 nm [181, 187].

1.6.2. The LDH assay

LDH is found within the cell membrane and is a soluble enzyme of the cytoplasm. When the cell is damaged as a result of apoptosis (cell lysis occurring gradually 48 hours) or necrosis (rapid cell death occurring in $\leq$2 hours) [188], LDH leaks out of the cell into the culture medium, catalysing the conversion of lactate to pyruvate through the reduction of NAD^+ to NADH [181]. Using the NADH generated, diaphorase reduces resazurin to resorufin (Figure 15) [189]. The lactate dehydrogenase release assay is the most frequently used cell markers of toxicity [188].

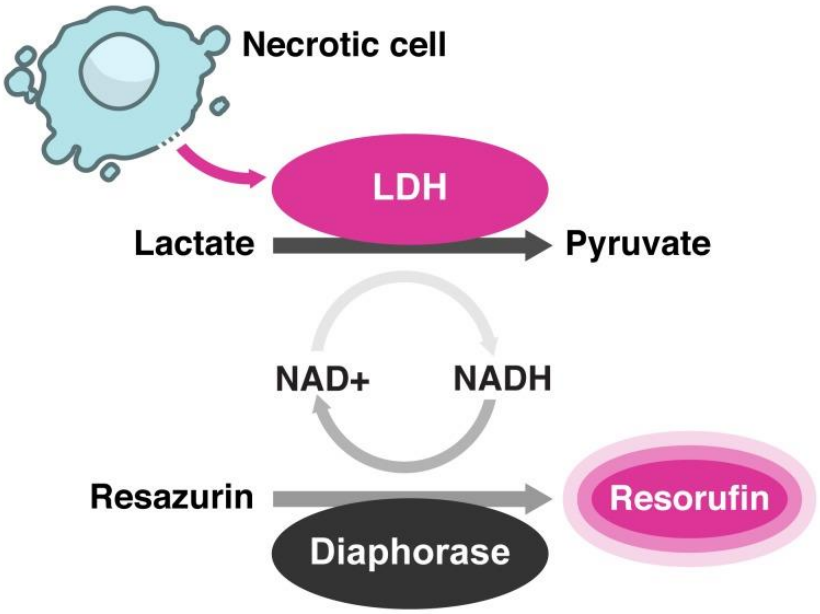


Figure 15: A diagrammatic representation of the chemical changes that occurs during the LDH release assay[189] (open access).

When LDH is leaked out of dead cells into the culture medium, it results in the catalysis of lactate to pyruvate. NADH is generated as a by-product of this reaction, but will reduce resazurin to resorufin, in the presence of diaphorase.

The interaction of β-lactamases to substrates such as antibiotics, is usually the first step in a reaction that leads to antibiotic deactivation. Therefore, it is important to understand how the MBLs (harboured in bacteria) behave when encountering an antibiotic. The next section, focuses on the intricacy of:

- The parameters governing the functioning of enzymes
- The interaction of MBLIs with β-lactamases and the effects exhibited on the catalytic activity of antibiotics

1.7. Enzyme Kinetics Characterization and Determination of Enzyme Properties

Enzymes are catalysts that decrease the reaction's activation energy and facilitate the conversion of substrates into products [190]. Addition of an enzyme to a substrate, leads to the occurrence of 3 distinct kinetic stages, pre-, post-, and steady state. Generally, the steady state is studied as this is where the rate of product formation is constant and occurs within seconds compared to the pre-steady state which only lasts for microseconds. Steady state kinetics often employs the Michaelis-Menten model [191]. The Michaelis-Menten kinetics model explains the reaction's enzymatic rate and how it depends on the enzyme and substrate concentration [192]. A substrate (S) binds to an enzyme (E) forming an enzyme-substrate complex (ES), which reacts via a rate limiting step, resulting in product (P) formation and release of the enzyme [193].

$$\mathbf{E + S \leftrightarrow ES \leftrightarrow E + P} \qquad \text{(eq.1)}$$

From the Michaelis-Menten model, there are two fundamental concepts Vmax and Km [194]. Vmax is the maximum reaction rate, in which all the enzyme's active sites are saturated with substrates. The Michaelis-Menten constant (Km) represents the substrate concentration corresponding to a reaction rate half of Vmax. This gives rise to the following equation, where V_i is the initial reaction rate affected by the substrate concentration, S [195].

$$\mathbf{Vi} = \frac{\mathbf{Vmax\,[S]}}{\mathbf{Km + [S]}} \qquad \text{(eq. 2)}$$

Enzymes are potent catalysts that can be represented by the constant K_{cat}, which refers to the turnover rate and is determined by the quantity of substrate molecules that can be turned into product using a one enzyme molecule, per unit of time [196]. The Michaelis-Menten graph is a

substrate concentration *versus* reaction rate graph, which follows a hyperbolic shape and indicates the rapid linear reaction rate increase when the substrate concentration increases. Thereafter the rate reach a plateau and increasing the concentration of substrate is ineffective on the reaction rate (Vmax) [197]. For our experiments the Lineweaver-Burk plot [191] was chosen, as a straight-line graph is achieved in which the y-intercept is equal to the Vmax and therefore provides a better plot for the analysis of enzyme assays using the inverse of the Michaelis-Menten equation (equation 3) [198].

$$\frac{1}{Vi} = \left[\frac{Km}{Vmax}\right]\frac{1}{[S]} + \frac{1}{Vmax} \qquad \text{(eq. 3)}$$

The affinity of the inhibitor for an enzyme (Ki) can be determined using equation 4, in which a low Ki is indicative of high MBL potency and will be used in our experiments with the Cheng-Prusoff [199] equation (5) to determine the level of inhibition exhibited by each MBLI investigated.

$$\frac{1}{Km} = \frac{1}{Km+\frac{[I]}{Ki}} \qquad \text{(eq. 4)}$$

$$Ki = \frac{IC_{50}}{\left(1+\frac{[S]}{Km}\right)} \qquad \text{(eq.5)}$$

Fluorescent quenching is an important analytical tool that will be used to determine the binding exhibited [200] between MBLIs and MBLs. By studying the quenching properties, a deduction can be made on the strength of the MBLI to bind to the different sites available on the metallo-β-lactamase. Coupling these results with the enzyme kinetic information and drug susceptibility profile, the *in vitro* efficacy of the MBLI can be determined.

1.8. Fluorescence quenching of MBLs

When a molecule absorbs light at a specific wavelength, it is excited from the electronic ground state to an excited state. Whilst in the excited state the molecule can transition between vibrational levels. The absorbed energy is released upon returning to the ground state, which results in light emission [201-203]. This is called fluorescence, and the molecules involved in this process are referred to as fluorophores [204]. The light emitted is of a longer wavelength and lower intensity compared to the excitation light (stokes-shift) [205].

Biological molecules are also capable of displaying intrinsic protein fluorescence. The aromatic amino acid residues, tryptophan, phenylaniline and tyrosine primarily facilitate this. Tryptophan produces the best fluorescence and can be monitored using an excitation wavelength of 295 nm. This wavelength is specific and does not allow for tyrosine absorption since tyrosine is easily quenched during emission [206].

The metallo β-lactamase NDM, is known to possess tryptophan residues. Such as Trp-93, which is a non-active site residue involved in maintaining the structure of NDM [207]. A decrease in the emitted fluorescence due to the introduction of an MBLI (BP), results in fluorescence quenching and provides us with more information on the efficacy of the MBLI and the binding that occurs between this protein-ligand interaction [208].

The Stern-Volmer model will be used, allowing for the fluorescence lifetime of the excited molecule to be studied and for the calculation of the quenching rate constant [209], equation 6. The number of tryptophan binding sites present on NDM (equation 7) is to be determined.

$$\frac{\mathbf{F0}}{\mathbf{F}} = \mathbf{1} + \mathbf{Ksv}\,[\mathbf{Q}] \qquad \text{(eq. 6)}$$

F0 represents the fluorescence intensity in the absence of a quencher (Q) and F, in the presence of a quencher. Ksv represents the Stern Volmer constant (quenching rate constant + lifetime of fluorophore), which is a measure of the accessibility of the fluorophore to the quencher.

$$\mathbf{Log}\left(\frac{\mathbf{F0-F}}{\mathbf{F}}\right) = \mathbf{Log\ Ka + n\ log[Q]} \qquad \text{(eq. 7)}$$

Ka is the association constant and n is the number of binding sites available for ligand interactions.

1.9. *In vivo* efficacy studies

Animal studies are an integral part of our study and are important in evaluating many drug candidates. This done to assess the drug candidate's full potential, subsection 1.9.1. details the necessity of these studies and their scientific contribution in determining the efficacy of a drug. Whilst subsections 1.9.2 – 1.9.3 provide a closer insight into the study design of an animal trial.

1.9.1. The importance of using animal models

The importance of pharmacokinetic studies is highlighted by the concept that many drugs have less favourable efficacies *in vitro*, however, *in vivo,* they are more favourable due to better bioavailability, tissue distribution and drug elimination rate [210]. A classic example of this is Prontosil rubrum, which possessed no antibacterial activity *in vitro* but effectively treated mice infected with *Pneumococcus* sp. [211]. On the other hand, many other promising compounds show promising *in vitro* activities, but once tested *in vivo*, it fails due to a variety of reasons [212]. The use of animal models thus has the following advantages [213]:

- The ability to mimic the human biological system

- Currently the best alternative to study a drug's efficacy before advancing to clinical trials on humans
- Results generated are faster and reproducible with high statistical significance of individual drug parameters that can be optimised according to the predicted therapeutic efficacy

In vitro tests such as the MIC indicate the potency of a drug at a single point in time but do not provide information on dosing and post-antibiotic effects. Therefore, antimicrobial therapy requires both *in vitro* and *in vivo* examination to conclude on the rate of efficacy exhibited by a drug [214].

Another important consideration is that antimicrobial agents of differing chemical class do not behave the same. Whilst fluoroquinolones and aminoglycosides enhance killing by increasing the drug concentration, β-lactams exhibit enhanced killing due to longer drug exposure times [215]. One of the benefits of using small rodents as animal models, is the faster elimination rate, which makes it ideal for infection studies [216, 217].

1.9.2. Selecting animal models

Many animal models exist for antimicrobial therapy and have been described in depth by Zak and Sande [218]. However, using rats and mice for experimental trials is the preferred animal choice due to easy handling and cheaper purchase costs [219]. In bacterial strains that are less virulent, animals are either immunocompromised by adding an adjuvant such as mucin [220, 221] or choosing to infect the animal with a higher inoculum density [219]. Infection of the thigh is generally done using a mouse model [222, 223] however, rats have also been studied [224]. Various animal infection models for pneumonia have been described, such as using mice, rats and

rabbits, in which many infection routes can be selected [225]. In a meningitis model, rabbits are the animals of choice[226] whilst in the peritonitis model either mice [227] or rats [228] are used.

1.9.3. The thigh infection model

Our studies will focus on a murine thigh infection model (chapters three to five), since multiple studies have reported it to be the most common infection model that has proven successful for CFU enumeration [229]. The thigh infection model was first described in 1953 [230] and has been further modified by other scientists over the years [216, 224, 231-233]. The thigh infection in neutropenic mice is initiated by a 0.1 ml inoculum injection containing 10^5-10^8 bacterial density. About an hour or two later antibiotics are administered, the efficacy of each antibiotic dose (once-off or multiple) is determined by sacrificing the animal, excising the thigh, thereafter, homogenizing it for bacterial count measurements. Non-neutropenic mice may also be used to study the effect elicited by leucocytes [234].

The research presented herein, specifically in chapters three to five, adopts the murine thigh infection model for analysis of the test compounds. This type of infection model has been tailored to the specific needs of our study. It successfully allows for initiation of infection and subsequent treatment, with visible results in a short period of time. In addition, the infection model and study design provide a good indication of key pharmacokinetic parameters that can be differentiated by comparison to the control groups of meropenem monotherapy and the placebo effect. This will aid us in ascertaining the full potential of the test compounds.

Research focused on drug discovery for antimicrobial therapy calls for the use of high throughput approaches to deliver fast and accurate results for the quantitation of drugs, in biological matrices. Liquid chromatography coupled with tandem mass spectrometry, is a bioanalytical technique that

meets this criterion in pre-clinical testing of new drugs and will be used to achieve the *in vivo* pharmacokinetic and pharmacodynamic profile of the MBLIs under investigation.

1.10. The use of LC-MS/MS in therapeutic drug monitoring

The coupling of specific chromatographic techniques such as liquid chromatography (LC) to mass spectrometry (MS) has been greatly favoured due to the high sensitivity of MS [235]. In MS, 10^{-12} concentrations of compounds can be detected in complex biological samples [236]. MS is based on the principle of ion separation according to the analytes mass-to-charge (m/z) ratio observed and the quantitation of these ions [236]. There are three main components to MS, the ion source, the mass analyser and lastly the detector[237]. The incorporation of an electron spray ionisation (ESI) interface to LC-MS, by Fenn in 1980 [238], has emerged as an important tool for the detection of biological molecules in biochemistry [235]. Although ESI is also known as "soft ionization" and it is the most utilized ion source, it is limited to molecules that are highly polar (Figure 15) [239]. Non-polar molecules such as lipids, display poor ionisation with this method. Therefore, two alternative approaches to ESI exist, atmospheric pressure chemical ionization (APCI) (Figure 15) and atmospheric pressure photoionization [235]. Whilst ionisation begins at the source, it is the mass analyser which is primarily responsible for the quantification of the analyte. Therefore, selection of an analyser is based on the analyte's properties post ionization as well as the experiment being conducted [240]. There are many mass analysers available such as the quadrupole analyser, time of flight (TOF) analyser, the ion trap, and a quadrupole time of flight (QTOF) analyser (a hybrid). Operation of the mass analyser can occur using MS or tandem mass spectrometry (MS/MS). Using MS, detection of a single product ion is generated. Whilst in the MS/MS, molecular fragments are generated from the break-down of precursor ions. The latter is preferred in pharmacology [241].

Previously, immunoassays were used to measure the interactions of drugs, however, this assay was limited by cross-reactivity with other metabolites. To curb this problem, investments were made to acquire LC-MS/MS instruments as a therapeutic drug monitoring tool. LC-MS/MS has since gained popularity due to the large amount of pharmacokinetic information that can be retrieved on several drugs in a single run [235]. In addition, novel or unknown compounds can be identified in matrix by identifying chemical properties, analyte concentration, and the speed and time of analysis, among other parameters [242]. These characteristics enable the quantitation of drugs and the generation of various pharmacokinetic data, which aids in the research and development of new antimicrobial compounds [243].

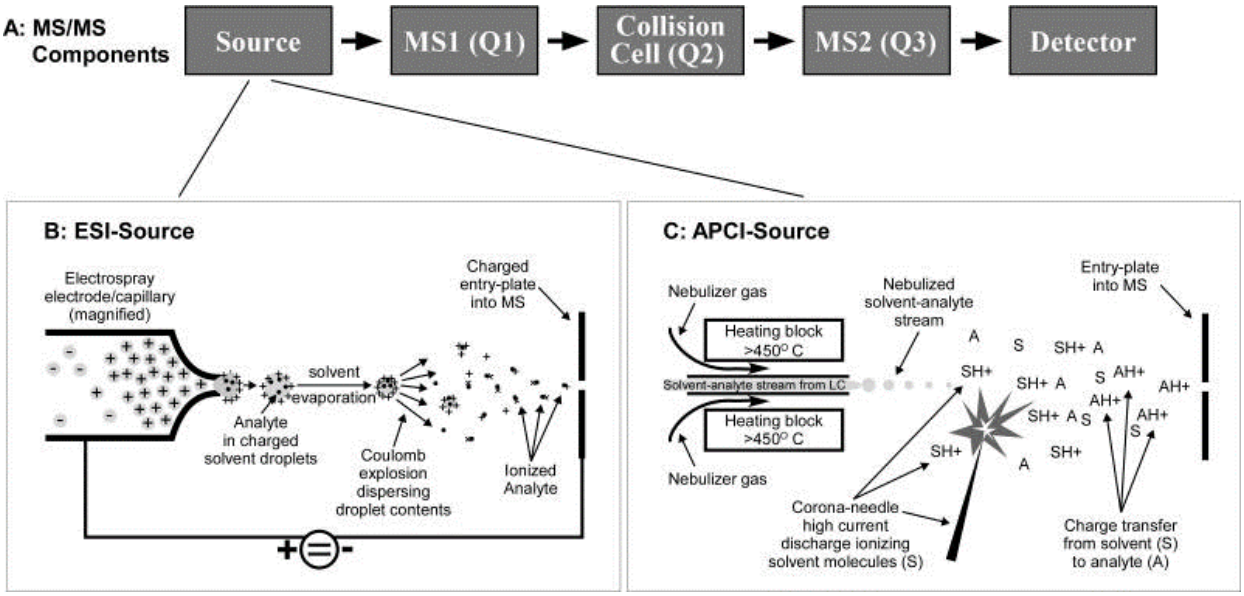


Figure 16: Tandem mass spectrometry components (A) adapted from Grebe and Singh [244], and the principle of ESI (B)[245] and APCI (C) [246] used in LC-MS/MS (open access).

1.11. Research synopsis

Current pharmaceutical research is largely focused on combination therapy as a highly efficacious treatment strategy in targeting infections caused by CRE's, thus reducing the burden of AMR [149]. This strategy is based on the mechanism of combining a novel β-lactamase inhibitor with a β-lactam. The combination of these two chemical molecules results in a therapeutic agent that elicits two functions [247]:

1. Inactivating the β-lactamase enzyme
2. Inhibiting the growth of the target bacteria by restoring the potency of the β-lactam

Combination therapy has proved excellent in targeting bacteria producing serine β-lactamases , as evidenced by the current clinically available combinations [104, 248]. However, research towards combination therapy against metallo-β-lactamase producing bacteria, has been ongoing. Although many candidates have shown promise in the pre-clinical evaluation, none has successfully made it through clinical trials yet [249]. Therefore, this study evaluated combination therapy using meropenem and potential novel metallo-β-lactamase inhibitors (NOTA, BP- 1, 6, 10 and 14) synthesized and characterized at the Catalysis and Peptide Research Unit, UKZN. The following aims and objectives formed the cornerstone for this study:

1.12. Aim and Objectives:

1.12.1 Aim

To determine the potency of the BP inhibitors by investigating the binding specificity to metallo-β-lactamase producing bacteria, the subsequent neutralization of the enzyme and the re-sensitization of the pathogens to meropenem, without any cytotoxic effects.

1.12.2. Specific objectives

1.12.2.1. To establish what advances have been made in finding a suitable metallo-β-lactamase inhibitor, from 2018 to 2019 (Chapter two), for the purpose of this thesis, it was updated to 2022.

1.12.2.1.1. By performing a thorough a literature search on patents that were filed during this period, that identify as a metallo-β-lactamase candidate by demonstrating scientific competency.

1.12.2.1.2. Summarizing the main findings from each patent critically and providing the readers with an update of the current research stans.

1.12.2.1.3. Highlighting the benefits of combination therapy and informing the readers in the field that no metallo-β-lactamase inhibitor is available clinically. However, there has been advances in the research and development towards one.

1.12.2.2 To determine if the metallo-β-lactamase inhibitors (BP compounds, common name given to our class of novel compounds), can safely restore the efficacy of meropenem to therapeutic concentrations (Chapters four and five).

1.12.2.2.1. By investigating the minimum inhibitory concentration of the BP compounds and meropenem alone and in combination therapy against a panel of ≥ seven metallo-β-lactamase producing *Enterobacterales* and through studying the time-kill kinetics of *Klebsiella pneumoniae* NDM.

1.12.2.2.2. Calculating the level of synergy exhibited by BP and meropenem.

1.12.2.2.3. Assessing the safety profile of the BP compounds by conducting cytotoxicity experiments.

1.12.2.3. To study the kinetic properties of inhibition, utilizing the BP compounds and determining if these compounds bind specifically to the metallo-β-lactamase only (Chapter four and five).

1.12.2.3.1. By determining the kinetic properties of NDM-1 and VIM-2 and the inhibitory properties of the BP compounds on these enzymes.

1.12.2.3.2. Investigating the fluorescent quenching properties of the BP compounds against NDM-1 and VIM-2.

1.12.2.3.3. Examining if the binding specificity of the BP compounds is exclusive to metallo-β-lactamases.

1.12.2.4. To conduct an *in vivo* efficacy study on the BP compounds co-administered with meropenem and establish if the potency of meropenem is restored, in a murine infection model:

1.12.2.4.1. By observing if there is a visual reduction in the colony forming units per millilitre (cfu/ml), during and after an 8-hour treatment of BP + meropenem (Chapters four and five).

1.12.2.4.2. Developing a rapid and sensitive LC-MS/MS method for the detection of the novel BP compounds (Chapters four and five).

1.12.2.4.3. Determining the concentration of the drug in plasma and thereby concluding on the pharmacokinetic properties of the drug using LC-MS/MS (Chapters three, four and five).

1.12.2.4.4. Establishing which BP compound produced the best *in vivo* results and if it is comparable to the *in vitro* data (Chapter six).

1.13. Outline of thesis

Chapter one provides a brief background and research synopsis of the study. The aims and objectives of this study is also stipulated in this chapter

Chapter two is presented as a review paper, focusing on metallo-β-lactamase inhibitors patented during 2018-2019. This review encompasses a comprehensive overview of the potential displayed by metallo-β-lactamase inhibitory candidates for admission into clinical trials. This chapter also includes a brief update on new patents filed between 2020 to 2022.

Chapter three is the secondary manuscript of this thesis, detailing the drug susceptibility profile of metallo-β-lactamase producers against inhibitor; NOTA, utilizing the checkerboard and time-kill assays. The *in vivo* efficacy of NOTA is also included in this manuscript.

Chapter four is presented as the third manuscript in which the inhibitor BP1, is studied. Prioritized experiments are antimicrobial susceptibility testing, metallo-β-lactamase inhibition, cytotoxicity, inhibitor specificity, fluorescence quenching and animal studies. In this chapter, the potency of the novel BP1 inhibitor against metallo-β-lactamases are measured and interpreted.

Chapter five is the fourth manuscript, descriptive of the *further three* novel metallo-β-lactamase inhibitors i.e., BP6, 10 and 14. BP10, co-administered with meropenem against *Klebsiella pneumoniae* NDM-1, in a murine infection model. In this manuscript, BP10 in combination with meropenem is evaluated against a larger panel of bacteria (n = 21).

Chapter six summarizes the findings of this study. In addition, concluding remarks governing the limitations of the study are provided. Moreover, recommendations for future experiments, are highlighted.

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Chapter two: Manuscript one

A 2018-2019 patent review of metallo-β-lactamase inhibitors

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Abstract:

Antibiotic resistance caused by β-lactamase expressing bacteria poses a concern given its global dissemination and proliferation. The emergence of metallo-β-lactamases is an indefinite health threat towards which current antibiotics have limited clinical efficacy. One solution is to develop metallo-β-lactamase inhibitors (MBLIs) capable of restoring the activity of β-lactam drugs. This review focuses on potential metallo-β-lactamase inhibitors that have been patented during the period of 2018-2019. The aim is to provide insight into the diverse class of compounds which exhibit a synergistic inhibitory effect on carbapenem resistant bacteria, when co-administered with a beta-lactam antibiotic. The treatment strategy, of creating a broad-spectrum β-lactamase inhibitor, is beneficial to the health sector as well as rural communities. Unfortunately, most of the inhibitors lack published data from both *in vitro* and *in vivo* evaluation, thus preventing an expert opinion on the likelihood to progress as candidates for clinical trials. From this report, the bismuth complexes, pyridinyl-nicotinamide derived sugars and thiazole sulphonamide derivatives, portray

promising properties for further advancement. Since there is currently no FDA approved MBLI, there remains an urgent need for the development of these combination treatment strategies.

Keywords: β-lactamase inhibitors, carbapenem-resistant *Enterobacterales,* metallo-β-lactamase inhibitors, serine β-lactamase inhibitors

1. Introduction

β-lactam antibiotics are the most widely prescribed class of antimicrobial drugs around the world [1]. The therapeutic benefits offered by this class of antimicrobials include accessibility, safety and a unique mechanism of action against specific prokaryotic cell structures [2], making it the ideal antimicrobial drug of choice. However, with increased consumption, inappropriate prescribing practices and misuse of β-lactams to treat minor infections, β-lactam resistance has escalated to uncontrollable rates [3]. Gram negative bacteria are the main protagonists responsible for the rise in resistance, facilitated by genes carried on plasmids or other mobile genetic elements, which confer resistance to β-lactam drugs [4]. Carbapenems are β-lactam drugs that are often referred to as the last line of defense in the treatment of bacterial infections [4]. However, the emergence of carbapenem-resistant *Enterobacterales* (CRE), along with carbapenem-resistant *Acinetobacter baumannii* (CRAB) and carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) have emerged as urgent threats, requiring immediate intervention, as stated by the World Health Organization [5].

The mechanism of action of β-lactams involve the inhibition of the penicillin binding proteins (PBPs), which are essential for the synthesis of the bacterial cell wall [6]. The expression of resistant genes harbored by bacteria results in the production of β-lactamases that hydrolyze the β-lactam ring of the antibiotic, prior to the drug eliciting its function [7]. This is the primary

mechanism of resistance in gram negative bacteria against β-lactams [8], other mechanisms include; alterations in the structure of penicillin binding proteins [9,10], mutations or loss in porin expression [11] and the presence of efflux pumps [4,12].

The Ambler classification system [13] categorizes β-lactamases into four distinct classes (A, B, C and D), according to the homology of their amino acid sequences [8]. Classes A, B and D are referred to as serine β-lactamases (SBL) [14], since they utilize serine for hydrolysis at the active site [15]. Class B carbapenamases use zinc ions at the active hydrolysis site and are therefore known as metallo-β-lactamases (MBL) [8,16]. Currently in the β-lactam family of drugs, monobactams (aztreonam) are the only constituents not hydrolyzed by MBLs [17]. Three MBL subclasses exist, B1, B2 and B3 based on the homology of the primary amino acid sequences, as well as the active site occupancy of either one or two zinc ions [6,14]. Although MBLs display < 25% amino acid conservation [18], a common characteristic among MBLs is the presence of an αβ/βα sandwich fold in the protein structure [19]. In subclass B1, one zinc ion (Zn1) is bound to three histidine residues (H116, H118 and H196), the second zinc ion (Zn2) is bound to three different residues (Asp120, Cys221, and H263) [18]. Subclass B2 shares the same residues as the Zn2 binding site of B1, however, the Zn1 binding site differs by a single residue (N116, H118 and H196) [18]. The amino acid residues present in B3 Zn1 (H/Q116, H118 and H196) and Zn2 (Asp120, H121 and H263) are variably distinct compared to the other MBL subclasses [18]. Among B1 MBLs the amino acid residues present in both binding sites are conserved and therefore provides as a method of distinguishing B1 MBLs from B2 and B3 [19].

CRE's producing metallo-β-lactamases are clinically catastrophic and are often termed as "nightmare bacteria"[20], due to treatment difficulty and wide dissemination [17]. These MBLs include the New Delhi MBL (NDM) [21], Verona Integrase MBL (VIM) [22] and Imipenemase

MBL (IMP) [15,23], all belonging to the B1 subclass. Therefore, subclass B1, is of clinical importance and contains the largest number of members [18]. Subclass B2 contains the least number of members and includes producers such as, *Serratia fonticola* Sfh-I [24], *Aeromonas sobria* ImiS [25] and Ae*romonas hydrophila* CphA [26]. Whilst, *Chryseobacterium meningosepticum* GOB-1 [27] and *Stenotrophomonas (Xanthomonas) maltophilia* L1 [28] are members of subclass B3.

The two current approaches to combat resistance mediated by β-lactamases revolves around:

1. The development of novel therapeutic agents that are designed to be enzymatically stable β-lactams [8,29].

2. Inhibitors that offer protection to the enzymatically labile β-lactams [8,29].

The latter approach is a more popular strategy that has been employed by scientists. Another fairly new strategy described by Papp-Wallace and Bonomo, (2016) is the use of β-lactam enhancers (BLEs), which describes a new class of antimicrobial drugs [16]. Although they are co-administered with β-lactams, BLEs target a different PBP as compared to β-lactams, thus ensuring bactericidal activity is achieved synergistically [30]. Zidebactam, is an example of a bicyclo-acyl hydrazide that functions as a BLE, and has successfully completed phase two clinical trials, and is set to enter phase three trials[31]. Another targeting strategy is the use of prodrugs as in the example of cefditoren pivoxil that is an orally absorbed prodrug hydrolysed in the intestine to generate the active cephalosporin antibiotic, cefditoren [32].

The currently approved FDA serine β-lactamase inhibitors (SBLI) on the market are clavulanic acid [33], sulbactam [34], tazobactam [35] [first generation β-lactamase inhibitors (BLIs) [17], avibactam and vaborbactam [36]. While a plethora of SBLI's exist and are commercially available

for treatment in combination with a β-lactam antibiotic, there has yet to be an MBLI approved for clinical use [37]. SBLIs elicit no inhibitory effect on MBLs [2,30]. According to the WHO report on new antimicrobials in the current pipeline (2018-2019), mainly SBLIs have made it to and completed clinical trials [38].

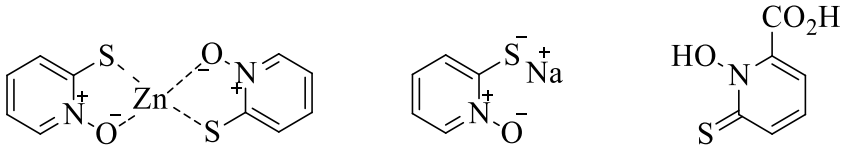
Although promising MBLI candidates such as, Aspergillomarasmine A [39], (N,N,N0,N0-Tetrakis(2-pyridylmethyl)ethylenediamine(TPEN), di-(2-picolyl)amine (DPA) [40] and the ANT431 scaffold [41] have been reported, none has advanced to clinical trials, nor is there an FDA approved inhibitor that concurrently targets both SBLs and MBLs [38,42]. Although, Hecker and co-workers[43] lately reported an inhibitor, QPX7728 from the boronic acid class, which has demonstrated pre-clinical activity against SBLs and MBLs. With the given antibiotic crisis and the growing need for MBLI's to contribute to treatment options, this review aims to shed light on the patented research and development of potential MBLIs that have shown significant promise during the period of 2018-2019. For this review the half maximal inhibitory concentration (IC_{50}), has a dual definition, and refers to both a measure of cytotoxicity on biological cells, as well as drug potency via MBL inhibition.

2. Patents filed in 2018-2019 reporting compounds with MBLI activity according to the chemical class and its respective biological activity

2.1.1-Hydroxypyridine-2 (1H)-thiones inhibitors

Zinc pyrithione (**1**) was initially isolated from the Chinese herbal roots of *Polyalthia nemoralis* [44], with reports indicating its use as an anti-fouling biocide agent [45,46]. Sham *et al*,[47] have investigated the effect of 1-Hydroxypyridine-2 (1H)-thione containing compounds such as compound **2** on VIM-2 expressing bacteria, using an array of biochemical procedures. These

authors went on to investigate compound **3** against VIM-2, which was first reported as an intermediate in the synthesis of dipeptides by Muthyala *et al*. [48]. From a single-dose inhibition study; L-captopril, compound **2** (sodium pyrithione salt) and compound **3** produced excellent biochemical inhibition (98%, 93% and 98%, respectively) against VIM-2 producing bacteria. Although compound **1** displays structural similarity to compound **3** (identical anions), it exhibited poor inhibition (15%). It is important to note that compound **2** had an IC_{50} of 0.91 μM, an inhibitory constant (Ki) of 0.22 μM, as well as a ligand efficiency (LE) of 1.15, indicating it is a promising antimicrobial inhibitor. In addition, observations from the cell viability assay indicate that compound **2** alone, displayed more than 89% growth inhibition of eukaryotic cells. Therefore, indicating unsuitability as a BLI for combination therapy. Observations from the same assay showed compound **3**, restoring the efficacy of amoxicillin against *E. coli* strains harboring VIM-2. In addition, a therapeutic index of 880 was produced with little cytotoxicity (data not shown). Therefore, indicating promise as a potential MBLI for compound **3**.



1 **2** **3**

Inhibition = 15% **Inhibition = 93%** **Inhibition = 98%**

IC_{50}[a] **IC_{50} = 0.91 μM** **IC_{50} = 0.27 μM**

Inhibition = % inhibition using 50 μM compound against VIM^{-2}

[a]No IC_{50} result were reported

Figure 1: Hydroxypyride-2 (1H)thiones with IC_{50} values against VIM-2 from US20180369217A1 (MICs were not reported) [47].

2.2.Diazabicyclooctanes (DBO) inhibitors

Avibactam, first of the DBO series of SBL inhibitors was approved in 2015 in combination with a 3rd generation cephalosporin, ceftazidime. Avibactam alone has poor antibacterial activity; however, it has broad-spectrum activity against class A, C and some D *β*-lactamases. A recent study by, Levy *et al.*,[49] reported that a derivative of avibactam without a carboxamide group (compound **5**) displayed no activity towards PBP2. These authors investigated the enzyme inhibition and drug susceptibility profile of the synthesized compounds; however, the enzyme inhibition experiments did not include MBLs. Notably, upon introduction of an endocyclic double bond to afford compound **6**, the activity was restored to the same level as that of avibactam. Compound **6** was then used as a scaffold for the development of additional DBOs. This subsequently, resulted in the development of compounds **7** and **8**, which demonstrated good biochemical activity (IC_{50} of 0.01 and 0.03 μM respectively) against PBP2. The efficiency of compound **8** is possibly due to the pKa:s and zwitter-ionic nature of the compound. This is important for entry into the bacterial cell wall (which has a transverse electric field) and facilitating the uptake of compound **8,** through porin channels [30].

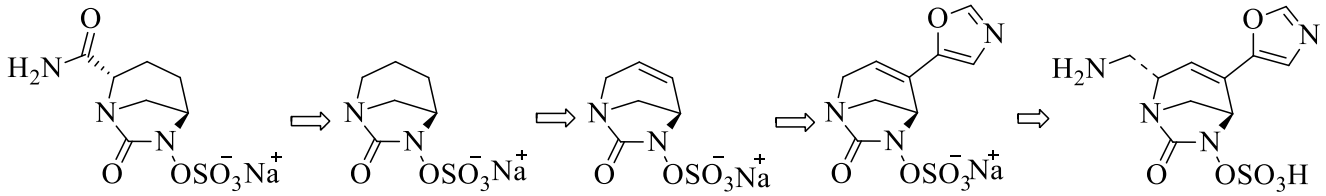
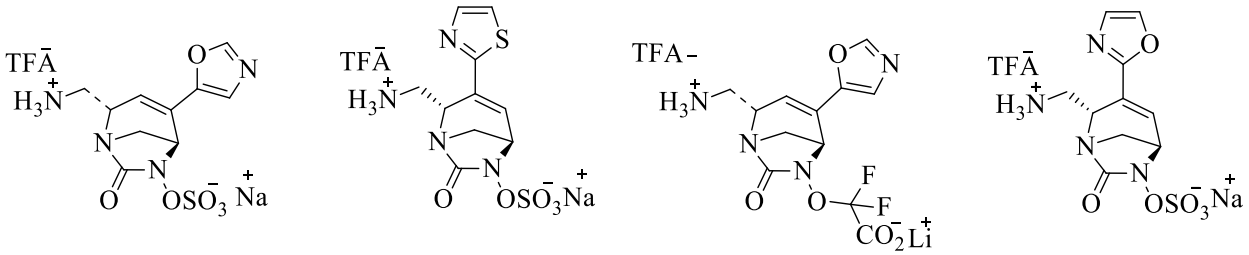


Figure 2: Biochemical IC_{50} of Avibactam with its DBO derivatives against PBP2 [49].

Caravano *et al.*,[50] reported the synthesis of compounds **6** and **8** in 2016. Caravano *et al.*, [50-53] developed the work of Levy from compound **6** into several DBO derivatives, and this work have been reported in four patents [50-53]. Bacterial strains were selected for testing based on the

multiple SBLs and MBLs expressed by each bacterium. Compounds **8** and **9** (Figure 3) were exemplary and showed the best activity across a wide spectrum of bacteria. Whilst compounds **10** and **11** showed good activity, they were however tested against fewer bacteria. The minimum inhibitory concentration (MIC) of all these compounds in the presence of 4 mg/L ceftazidime ranged from 0.125 – 0.5 mg/L. The results reported by these authors are extremely promising as it implies SBLs (TEM, SHV, KPC and OXA), ESBLs (CTX-M15) as well as MBLs (NDM, VIM and IMP) can be targeted by these compounds using a reduced amount of antibiotic/inhibitor drug concentration ratio.



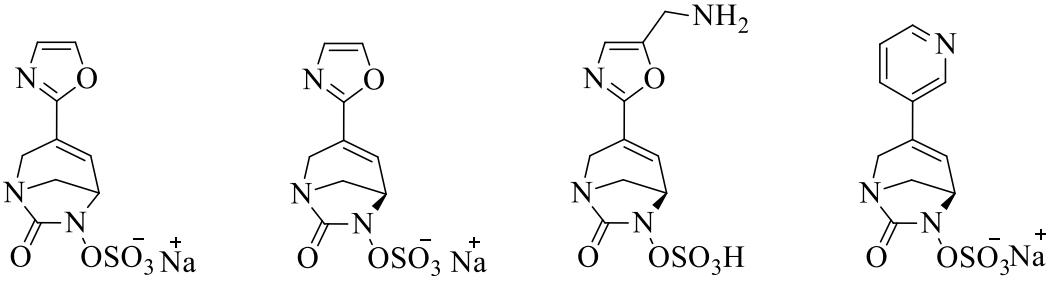
8 **9** **10** **11**

MIC: <0.25+4 **MIC: <0.25+4** **MIC: <0.25-16+4** **MIC: <0.25+4**

MIC (mg/L) = MBLI + Ceftazidime (4 mg/L)

Figure 3: DBO active compounds against IMP-29, NDM-1, VIM-1, VIM-2, and VIM-4 MBLs from WO2016156348A1[50].

Further investigation of the DBO derivatives by Barbion *et al.*,[51] afforded 44 other compounds, with only four (compounds **12-15**), showing activity against different MBLs. These compounds included compound **12** in its enantiomerically pure form, i.e., compound **13**, that was obtained via preparative chiral resolution of a synthetic intermediate. This activity was demonstrated alone (MIC: 0.5 - 4 mg/L) as well as synergistically (MIC: ≤ 0.125 – 0.25 mg/L), when administered with 4 mg/L of ceftazidime.



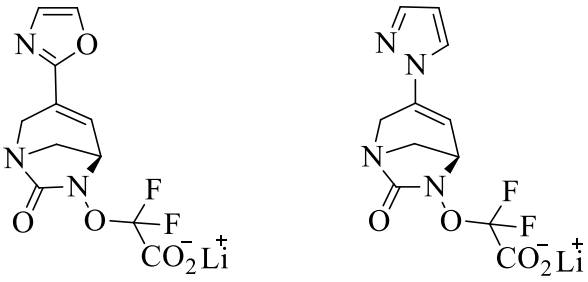
12 **13** **14** **15**

MIC: <0.25+4 **MIC: <0.25+4** **MIC: <0.25+4** **MIC: <0.25+4**

MIC (mg/L) = MBLI+ Ceftazidime (4 mg/L)

Figure 4: DBO active compounds against IMP8, VIM-1 and VIM-4 MBLs from WO2016177862A1[51].

Lithium salt derivatives (compounds **16** and **17**), were prepared in the report by Barbion *et al.*,[51]. However, synergistic testing of these compounds with ceftazidime included other examples (compounds **12**–**15**, Figure 4), but excluded compounds **16** and **17**. Bonnard *et al*.,[52] further evaluated compounds **16** and **17** that demonstrated excellent activity with amoxicillin, ceftazidime or cefixime antibiotics yielding MIC values in most cases of $\leq$ 0.25 mg/L antibiotic with 8 mg/L of compound **16** or **17** against several bacteria harboring MBLs.



16 **17**

MIC: 8+<0.25 **MIC: 8+<0.25**

MIC (mg/L): MBLI + Antibiotic
Antibiotics = Amoxicillin, ceftazidime or cefixime

Figure 5: DBO lithium salts active against MBLs from WO2016177862A1 [51].

Bonnard *et al.* [52] further prepared prodrugs of compound **16** to furnish derivatives **18-24**. When prodrugs **18-23** were intraduodenally administered to rats at a dose of 20 mg/kg, the hydrolyzed form (compound **16)** in plasma showed bioavailability in the range of 2 - 60%. Compound **21-23** showed promising bioavailability of 49-60% which is important to afford low inhibitor MICs of 8 mg/L and low antibiotic MICs of $\leq$ 0.25 mg/L [52].

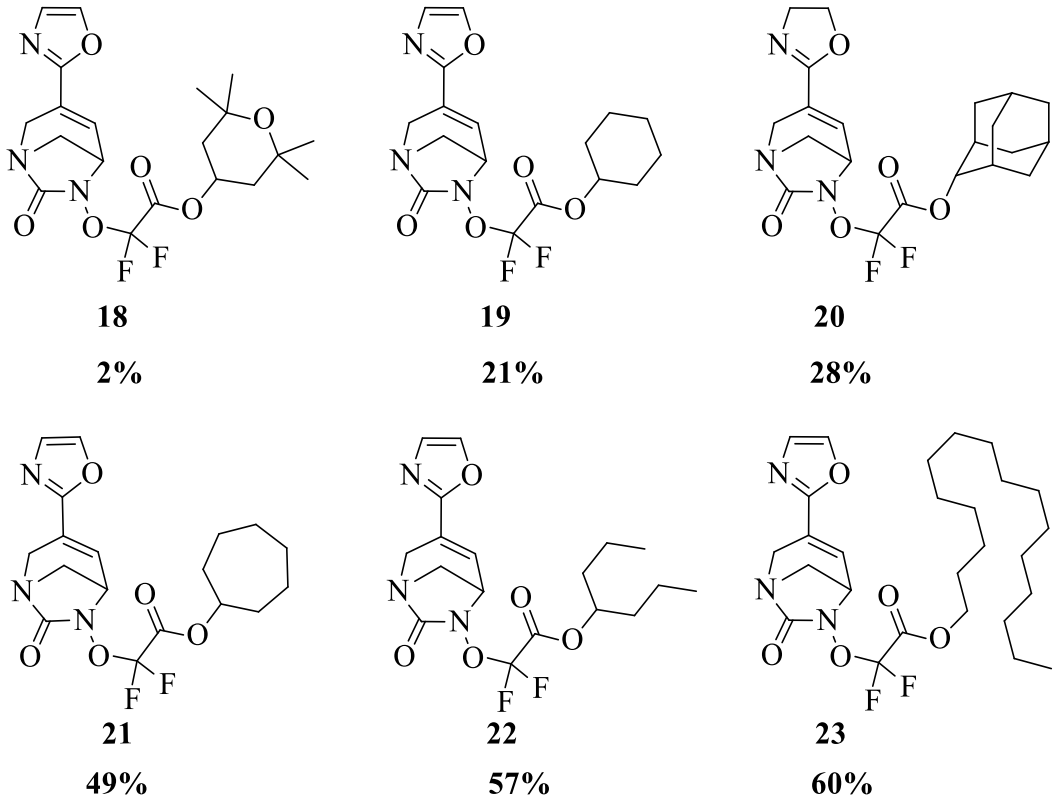
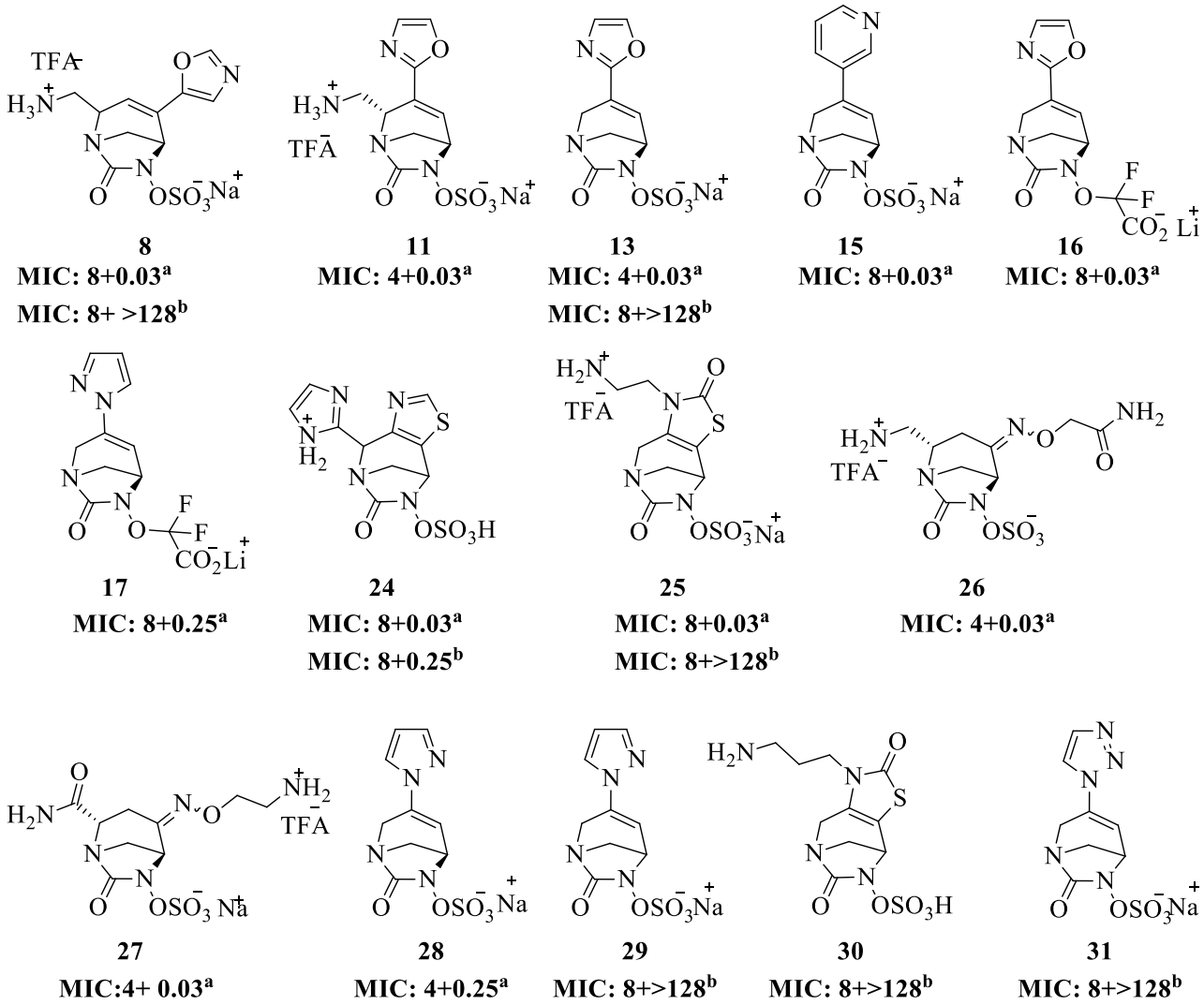


Figure 6: Prodrug of compound 16 with the respective bioavailability obtained in plasma after intraduodenal administration in rats from WO2018141986A1[52].

Bonnard *et al*.,[53] further discussed the synthesis and biological activity of the 14 examples depicted in Figure 7. These examples include compounds **8**, **11**, **13**, **15**, **16**, and **17** which the group had reported before. Drug susceptibility testing was conducted using a fixed concentration of either 4 or 8 mg/L of each inhibitor in combination with various antibiotics. All compounds were tested

at a concentration of 4 or 8 mg/L against *E. coil* strains expressing MBLs. At the administered concentration, low meropenem MICs of $\leq$ 0.03 mg/L were produced for compound **24** - **28**. However, these compounds failed to demonstrate similar activity when tested against different strains of bacteria. It is worth noting that compound **24** was the only compound showing good activity against *A. baumannii* expressing either NDM-1, VIM-1 or IMP-2 MBLs. This compound was able to restore the activity of several antibiotics, generating an MIC of 0.25+4 mg/L. However, all other compounds (**8**, **13**, **25** and **29-31**) investigated displayed an MIC >128+8 mg/L against *A. baumannii*. Time-kill kinetic studies for *Pseudomonas aeruginosa* harboring VIM-2 against ciprofloxacin or amikacin (1 mg/L) and compound **8** (16 mg/L) were also investigated. Time-kill kinetic studies showed that the co-administration of ciprofloxacin with compound **8** reduced bacterial growth by at least 3 $\log_{10}$ units. In addition, synergy was demonstrated between compound **8** and meropenem. Further investigation of compound **8**, is therefore required to determine its efficacy in an *in vivo* infection model, before it can be considered as a potential MBLI.



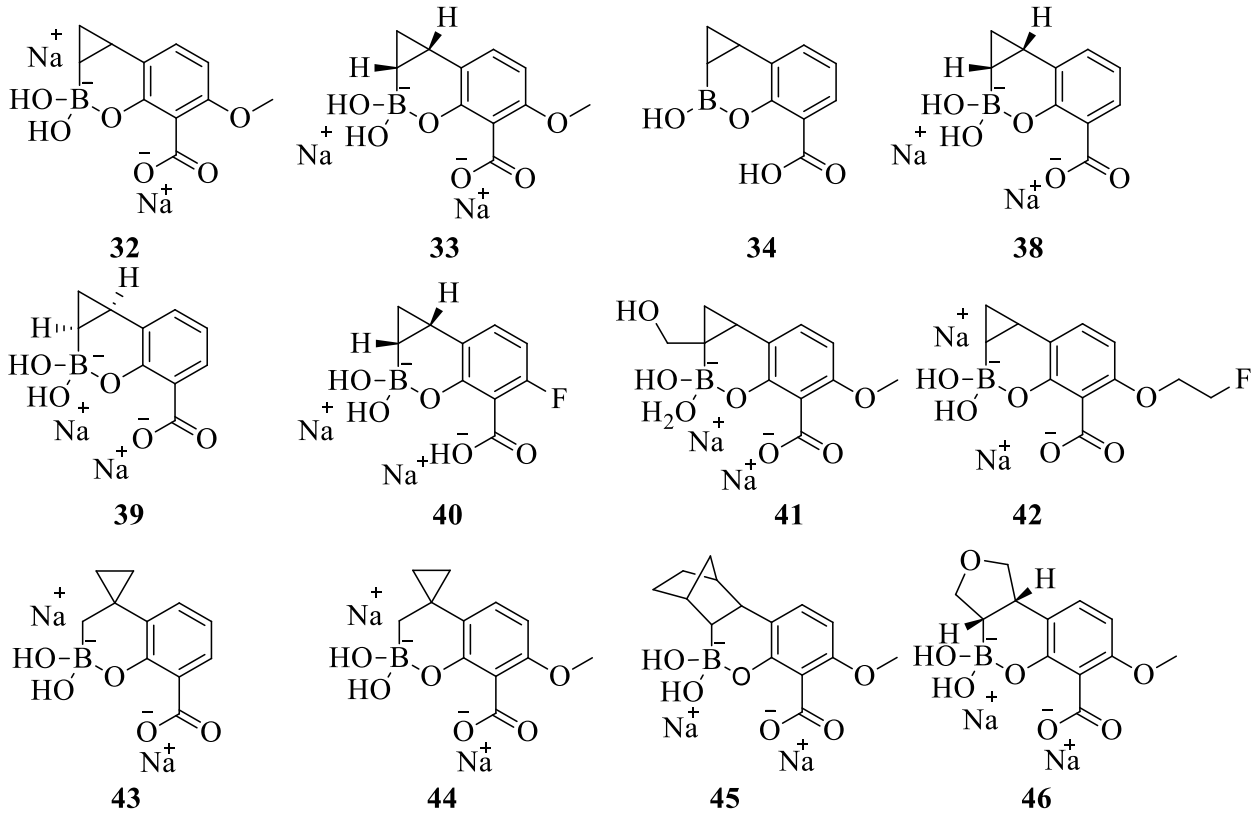
MIC (mg/L): MBLI+Meropenem
All MICs reported using [a]*E. Coli* (NDM-1+) and/or [b]*A. Baumannii* (NDM-1, VIM-1 or IMP-2)

Figure 7: MBL active compounds from WO2018060484A1 [53].

2.3.Boronic acid inhibitors

Hecker *et al*.,[54], reported a patent in which the synthesis of 24 boronic acid derivatives, exhibiting (lactamase inhibition) therapeutic benefits was demonstrated. The authors focused on SBLs with the addition of two bacterial strains harboring either NDM-1 or VIM-1 MBLs. Of the

24 compounds tested, only half of the compounds, **32-43,** displayed activity against both NDM-1 and VIM-1 with an MIC of $\leq$ 5 mg/L in the presence of a sub-inhibitory concentrations of 1.0 mg/L biapenem. Hence these compounds are effective inhibitors of NDM-1 and VIM-1. As for biapenem, MICs alone, were previously determined to be in the range of 16–32 mg/L for the same MBL expressing bacteria. However, a recent publication of this work [43], revealed more precise MIC results for some compounds (Figure 8). Furthermore, compound **37** (also known as QPX7728**,** not evaluated for biological activity in this patent) demonstrated superior activity against MBLs and SBLs. A meropenem MIC_{90} of 2 mg/L in the presence of 8 mg/L of compound 37 was observed. The inhibitor's potency was also determined, with VIM-1 and NDM-1 exhibiting the best inhibition. Additional experiments were also conducted with compound 37, which included pharmacokinetics, toxicology, protease inhibition and murine infection efficacy models. Compound 37 was observed to be bound *via* tetrahedron boron to NDM-1, VIM-2 and two other SBL enzymes by co-crystallization experiments. Given the excellent pre-clinical results and simultaneous inhibition of MBLs and SBLs, compound **37** presents as an ideal candidate for clinical trials experimentation and has currently advanced to late-stage pre-clinical development to support the IND filing.



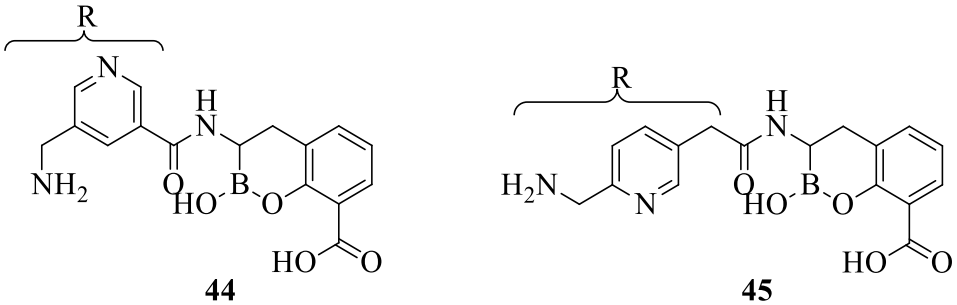
MIC: ≤5+1 (for all compounds)

MIC (mg/L) = MBLI + Biapenem

Figure 8: NDM-1 and VIM-1 active compounds from a recent publication and the filed US10294249B2 patent (IC_{50} results were not reported for MBLs)[43,54].

The patent filed by Wu *et al.,*[55], claims the synthesis of cyclic boronic acid derivatives that possess efficacy as inhibitors of both SBLs and MBLs, as well as Ambler class C β-lactamases. The synthesis of 16 examples were reported with variation on the amide carbonyl group as depicted by the R-group shown in Figure 9. However, only the biological activity of compounds **44** and **45** were reported which showed moderate activity against VIM-2. A crystal structure of example 44 bound to VIM-2 was reported by Krajnc *et al.,*[56] depicting the bicyclic borate inhibiting the MBL via a tetrahedral boron bound to the enzyme and thus forming a tricyclic borate. However, no further experiments were reported by Krajnc *et al.,*[56]. The broth microdilution technique was employed by Wu *et al.,*[55] to determine the MIC's of the inhibitors in the presence of a fixed

amount of ceftazidime or cefotaxime. In addition, the IC_{50} was also determined for each compound. The β-lactamases included in this study were SHV-5 (SBL), CTXM-15 (ESBL), KPC-2 (SBL) VIM-2 (MBL), P99+ (CLASS C) and OXA-23 (SBL). All the enzymes tested against compound **44** and **45** produced a biochemical IC_{50} of < 0.10 μM. Compound **44** and **45** showed similar results for VIM-2, P99+ and OXA-23, producing a MIC within the range of 2-16 mg/L. However, for SHV-5, CTXM-15 and KPC-2, compound **44** produced a much lower MIC of < 1 mg/L, indicating suitability as an inhibitor for SBLs



44
IC_{50}: < 0.10 μM
MIC: 2-16 + 16

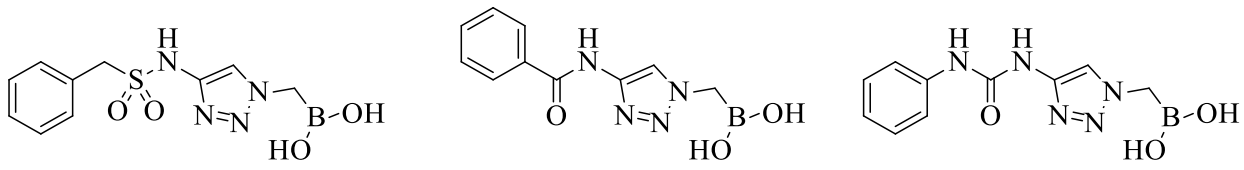
45
IC_{50}: < 0.10 μM
MIC: 2-16 + 16

MIC (mg/L) = MBLI + ceftizidime

Figure 9: Compounds tested against MBLs from US20190048027A1 [55].

The patent filed by Bonomo *et al.*, [57] reported the synthesis and biological activity of five boronic acid derivatives. However, only three derivatives, depicted in Figure 10, were active against MBLs. The IC_{50} of the compounds were conducted with 50 μM nitrocefin as substrate in a biochemical assay of IMP-1, NDM-1 VIM-2 and VIM-4 MBLs. The compounds **46**, **47** and **48** resulted in an IC_{50} of 51, 55 and 39 μM respectively, against VIM-2. However, these compounds were inactive against IMP-1, NDM-1 and VIM-4 with IC_{50} values > 100 μM. Although these initial data are discouraging, further experiments, such as drug susceptibility testing, could be conducted

for compounds **46**, **47** and **48** to determine their antimicrobial effectivity on VIM-2 expressing bacteria.



46 **47** **48**

IC_{50}: 51 μM **IC_{50}: 55 μM** **IC_{50}: 39 μM**

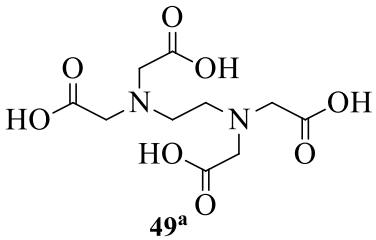
IC_{50} of compound record in 50 μM nitrocefin

Figure 10: Compounds active against VIM-2 from US20180256614A1 (MICs not reported)[57].

2.4.Bismuth(III) derived inhibitors

In the search towards lactamase inhibitors, known zinc (II) chelators such as EDTA (**49[a]**)[58], DL captopril (**50[a]**) [59] and NOTA [60] have been screened as potential MBL inhibitors. These chelators on their own suffer from poor selectivity and low *in-vivo* efficacy [61] hence, they are being used as a skeleton structure in the development of other derivatives. Sun *et al*., [62] used a number of zinc chelators to prepare bismuth(III) complexes and evaluated their MBL inhibition potential. Complexes **49-52** were selected from the study and showed good activity against NDM-1 and VIM-2 (Figure 11). Evidence from X-ray crystallography showed that an ion exchange reaction of Bi(III) and two Zn(II) ions at the active site of the enzyme resulted in cell death (from c → b, Figure 12)[63]. An extensive number of biological tests were then conducted, which included *in vitro* assays as well as *in vivo* experiments. Three MBLS were investigated; NDM-1, IMP-4 and VIM-2, however, the authors only reported the IC_{50} for NDM-1 and VIM-2. The bismuth complexes exhibited zero to minimal cytotoxicity on human hepatocyte cell lines (MIHA) even at concentrations as high as 100 μM. Compounds **49, 50**, Colloidal Bismuth Subcitrate (CBS,

51), **52** and $Bi(NO_3)_3$ exhibited MICs within the range of 0.5 – 4 mg/L in the presence of 32 μg/ml of meropenem for NDM-1^+, VIM-1^+ and IMP-4^+. Synergism was achieved for all MBLs tested against CBS (**51**), compound **52** and $Bi(NO_3)_3$. The *in vivo* experiment showed that CBS (**51**), when co-administered with meropenem increased the survival rate of infected mice by 50% in comparison to meropenem alone (25%) [62,63]. These findings suggest that CBS holds strong potential as a candidate for entry into clinical trials. However, further drug susceptibility testing should be considered, with experimental focus on utilizing reduced meropenem concentrations for combination therapy. This will provide a clearer indication on the efficacy of the bismuth complexes.

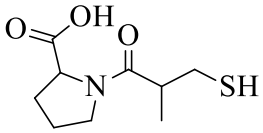


49[a]
EDTA

Bi (**49**[a]) = **49**

MIC: 0.5+32[b]

IC_{50} = 3.9 μM[g]

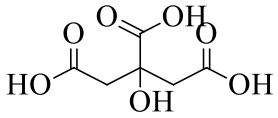


50[a]

$Bi(\mathbf{50}^{a})_2$ = **50**

MIC: 0.5+32[b]

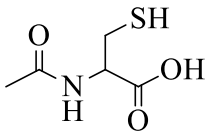
IC_{50} = 6.2 μM[h]



51[a]

$Bi_2(\mathbf{51}^{a})_2$ = **51**

MIC: 2+32[b]
MIC: 4+32[c]
MIC: 0.5+32[d]
MIC: 0.5+32[e]
MIC: 4+32[f]
IC_{50} = 5.83 μM[g]
IC_{50} = 44.5 μM[h]



52[a]

$Bi(\mathbf{52}^{a})_3$ = **52**

MIC: 0.5+32[b]
MIC: 1+32[c]
MIC: 0.5+32[d]
MIC: 0.5+32[e]
MIC: 2+32[f]
IC_{50} = 17.7 μM[g]

MIC (mg/L) = MBLI mg/L+ 32 (mg/L) of Ampicillin

[a]Ligand used, [b]*E. coli* (NDM-1^+), [c]*K. pneumoniae* (NDM-1^+), [d]*C. freundii* (NDM-1^+) ,[e]*E. coli*(VIM-2^+) and [f]*E. coli* (IMP-4^+), [g]Freshly prepared Zn_2-NDM-1 and [h]Freshly prepared Zn_2-VIM-1

Figure 11: Ligands with their bismuth complexes displaying promising activity against MBLs from US20180085335A1[62].

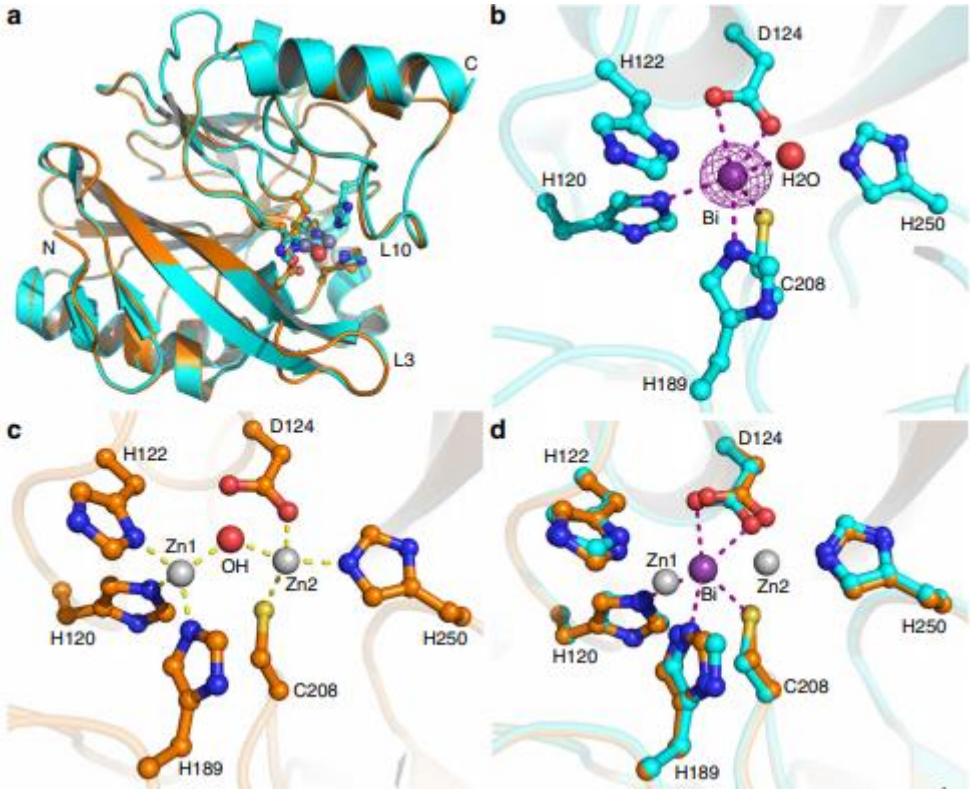


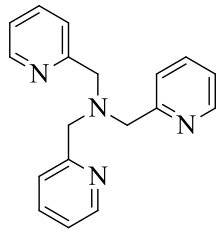
Figure 12: (a) X-ray structure superimposition of Bi-bound NDM-1 (cyan) with native Zn bound NDM-1 (orange) (b) X-ray structure of Bi-bound NDM-1 in the active site (c) X-ray structure of active site of NDM-1, (d) Comparing NDM-1 active site with native Zn (grey) with Bi-bound (purple) NDM-1 (PDB code: 3Q6X)[63]. Image obtained through the Creative Commons Attribute 4.0 International License. http://creativecommons.org/licenses/by/4.0/.

2.5.Pyridinyl-nicotinamide sugars as inhibitors

Rongved *et al.,*[64] reported MBLs inhibitors that comprise of zinc chelator moieties (one or two) covalently bound *via* a linker to hydrophilic moieties (one, two or three). The zinc chelators were mostly N, N,N-tris(2-pyridylmethyl) amine (TPA) (**53**) or N,N,N",N"- tetra(2-pyridyl-methyl)-ethylene diamine (TPEN) (**54**) derivatives while the hydrophilic moieties were sugars, carboxylic acids, amines, alcohols and boronic acids derivatives. The use of metal chelators such as EDTA against MBLs has been effective *in vitro,* however, *in vivo* studies show that EDTA easily passes the eukaryotic cell membrane and is cytotoxic to the host. In addition, they have a high affinity for soft metal ions such as zinc, iron and calcium which are found in several different enzymes in a

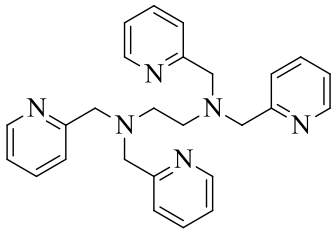
biological system which results in poor selectivity with high cytotoxicity. Rongved *et al.*,[64] performed extensive *in vitro* and *in vivo* experimental procedures. Results revealed that TPA (**53**) derivatives linked to hydrophilic moieties (compounds **55** and **56**) reduce the cellular permeability to the chelator (from high to low Log_{10} P value), hence reduced cytotoxicity (IC_{50} increased from 10 to >1000 μM) was observed whilst the activity remained constant (Figure 13).

Compounds **56** and **57** administered at a fixed dose of 25 mg/L resulted in a reduced meropenem MIC of $\leq$ 0.03 - 1 mg/L across 36 MBL producing bacteria. Upon conducting the checkerboard assay to determine the MIC of the drugs in combination, compound **56** was found to be active towards bacteria producing MBLs (NDM and VIM), ESBLs (CTX-M) and SBLs (SHV, TEM and OXA). The activity of compound **56** was achieved at a concentration of 12.5 and 25 mg/L, whilst restoring the efficacy of meropenem to lower concentrations of $\leq$ 0.06 and 0.12 mg/L. Cytotoxicity experiments indicated that the compounds lacked hemolytic activity at concentrations below 500 μM. During storage periods, compound **56** maintained its activity and was more stable than compound **57** and was therefore utilized for subsequent experiments. The safety of compound **56** was further conveyed by the *in vitro* lack of interaction with zinc-containing human enzymes. Compound **56** was also assessed for its *in vivo* efficacy with meropenem against a strain of *Klebsiella pneumoniae* expressing NDM-1, and resulted in an impressive 3 log_{10} reduction of bacterial colony forming units/ml. This compound (**56)** did not exhibit a significant log_{10} reduction when administered on its own. Therefore, indicating strong potential for combination therapy against MBLs. In addition, these results for compound **56** show great potential for clinical trials.

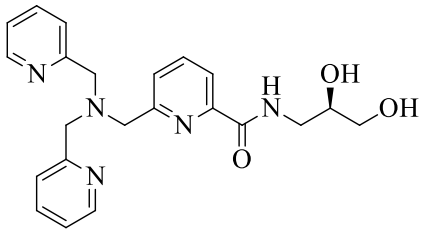


53
TPA

Log P = 2.7
HepG2 IC_{50} = 10 μM
MIC: 50+1 (VIM-2)
MIC: 50+0.125 (NDM-1)

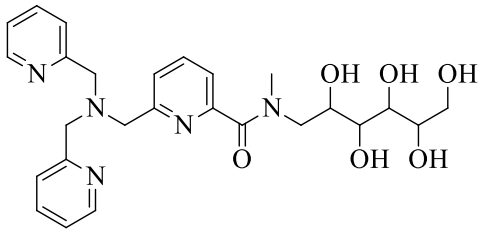


54
TPEN[a]



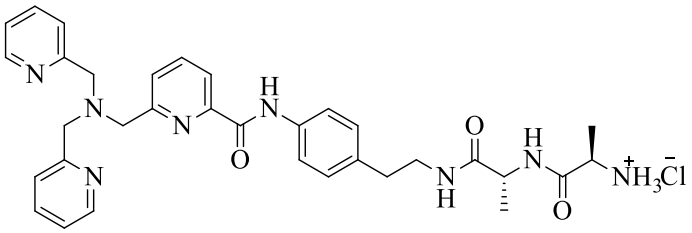
55

Log P = 0.79
HepG2 IC_{50} = 130.6 μM
MIC: 50+2 mg/L (VIM-2)
MIC: 50+0.125 (NDM-1)



56

Log P = -0.59
HepG2 IC_{50} >1000 μM
MIC: 12.5+≤0.0625 (VIM-2)
MIC: 25+(≤0.03−1.0) (NDM-1)



57

HepG2 IC_{50} >1000 μM
MIC: 25+≤1.0 (VIM-2)
MIC: 25+0.125 (NDM-1)

MIC (mg/L): MBLI+Meropenem (VIM-2/NDM-1)
[a]No MIC were reported

Figure 13: Pyridinyl-nicotinamide sugars active against MBLs from WO2018033719A1 [64].

2.6. Pyrroles and thienopyrroles

Panduwawala *et al.*[65], reported the synthesis of 29 compounds which were comprised of pyrroles, imidazoles and indoles derivatives. All these compounds' biological activity was evaluated against four MBLs (NDM-1, VIM-1-, VIM-2 and IMP-1). Pyrroles derivatives **58-63** showed the best activity against all four MBL, with a pIC_{50} value of $\geq$ 6 (Figure 14). Drug susceptibility testing was only reported for compound **58**. In which 4 mg/L of this compound was co-administered with meropenem, against 14 bacteria expressing either NDM or VIM. Compound **53** displayed good potency and reduced the MIC of meropenem by 2-4 folds. Acceptable MICs

of; 4 mg/L MBLI + 0.5 mg/L meropenem *for E. coli* (NDM) and 4 mg/L MBLI + 0.5 mg/L meropenem for *K. pneumoniae* (VIM), *A.baumannii* (VIM) and *C. freundii* (NDM), were produced. The next step for the authors should be to conduct cytotoxicity experiments and if the compound is found to be non-toxic, animal studies on the bioavailability and efficacy of the compounds should follow. This is due to the good *in vitro* data obtained, and the potential this compound displays for advancement into clinical trials.

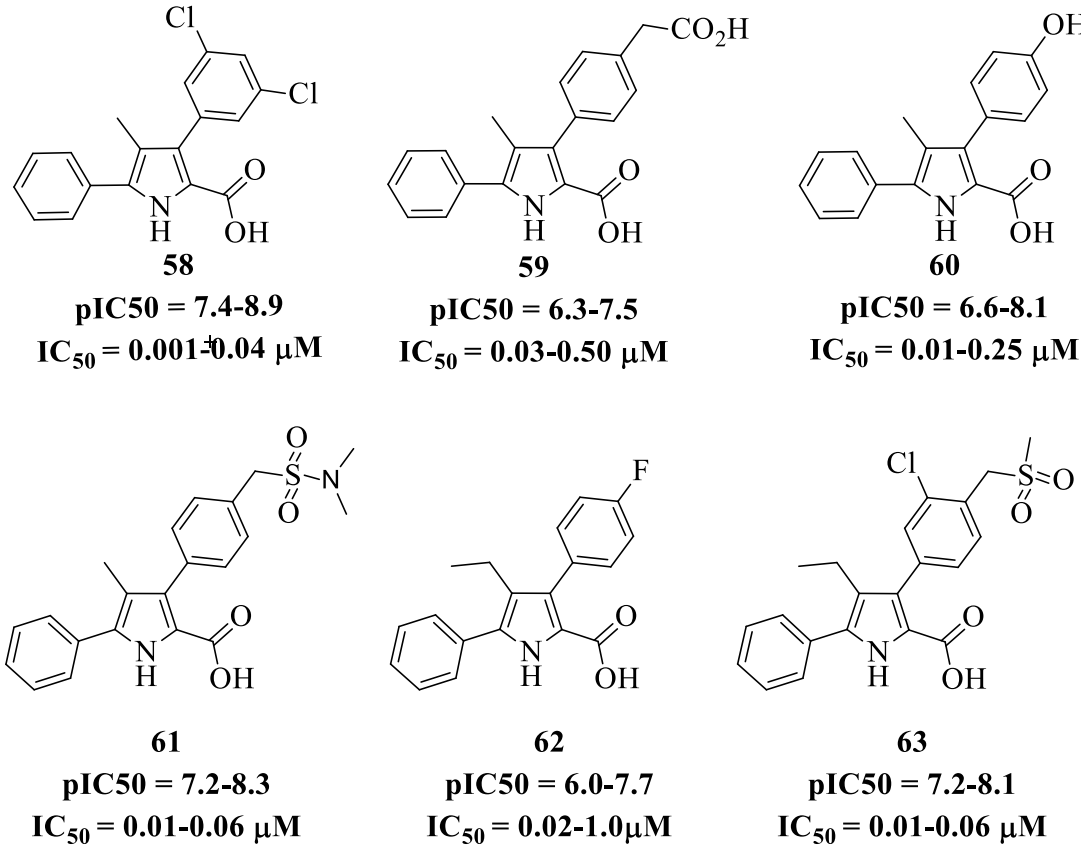
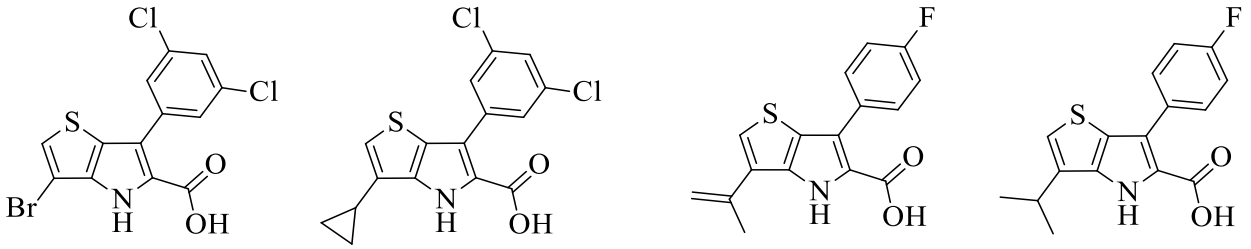


Figure 14: Most biologically active compounds against MBLs from WO2018215799A1[65]

Gising *et al.,*[66] (from the same group as Panduwawala *et al.*[65] before), reported the synthesis of 43 thienopyrroles and pyrrolopyrazoles derivatives. From the synthesized compounds, 14 selected compounds were evaluated for biological activity against four MBLs enzymes (NDM-1,

VIM-1-, VIM-2 and IMP-1). Compounds **63-66** (Figure 15) were the most active with pIC_{50} values greater than 6. The MICs of these compounds against several bacterial harboring MBLs were reported as a log 2 difference between meropenem activity alone and activity with 2 mg/L of MBLI + meropenem. At a concentration of 2 mg/L MBLI, a 1-2-fold decrease in meropenem MIC was observed. Increasing the MBLI drug concentration from 2 mg/L to 4 mg/L did not have a significant effect in reducing the meropenem MIC, however, increasing the MBLI drug concentration to 8 mg/L had a significant effect of a 3-fold decrease in MIC for compounds **64** and **66**. Further testing should be conducted on compounds **63-66** to ascertain the toxicity of these compounds as well as the efficacy in an animal model since these compounds show good promise of restoring the potency of meropenem.



63
pIC_{50} = 6.0-7.4
IC_{50} = 0.04-1.0 μM

64
pIC_{50} = 6.9-8.3
IC_{50} = 0.04-1.0 μM

65
pIC_{50} = 6.2-7.4
IC_{50} = 0.04-1.0 μM

66
pIC_{50} = 6.2-7.4
IC_{50} = 0.04-1.0 μM

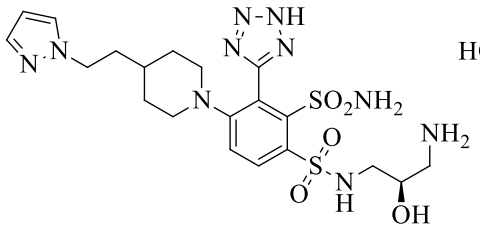
MBL tested: NDM-1, VIM-1, VIM-2 and IMP-1

Figure 15: Most biologically active compounds against MBLs from WO2018215800A1[66].

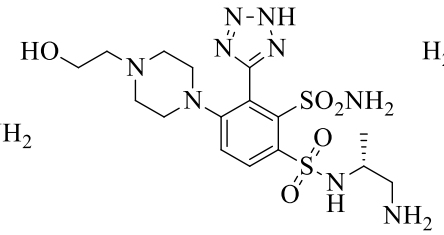
2.7. 2H-tetrazol-5-yl sulfonamides and sulfones as well as, thiazole sulfonamide derivatives

Pasternak *et al.,*[67], reported the synthesis of 209 1H- and 2H-tetrazol-5-yl sulfonamides and sulfone thienopyrroles derivatives. All 209 derivatives were evaluated for biological activity against four MBLs enzymes (NDM-1, VIM-1-, VIM-2 and IMP-1). Compounds **67-72** displayed

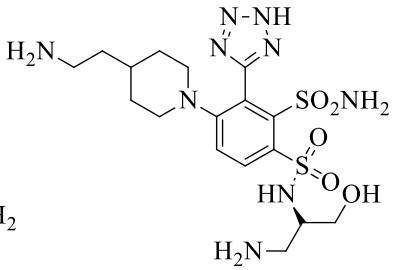
IC_{50} results of $\leq$ 0.5 μM (Figure 16). Furthermore, the synergistic concentration of the derivatives was determined as the lowest concentration of derivatives with 1 mg/L of imipenem that was required to inhibit 95% of the growth of the bacteria (MITC95). Three bacterial species were included: *E. coli* (NDM-1)*, S. marcescens* (IMP-1) and *K. pneumoniae* (VIM-1). The MITC95 result showed that the best activity was for compounds **73-75**. Efflux pump experiments were conducted on transformed mutants (without efflux pumps) and wild type bacteria (with efflux pumps) both expressing MBLs. Results indicate that presence or absence of an efflux pump does not have a large effect on efflux resistance, as bacteria were still able to exhibit resistance due to the expression of MBL enzymes. This finding also indicates that the production of MBLs is the main mechanism of resistance elicited by most of these bacteria. Efflux pump ratios predicting the fold difference between efflux positive and efflux negative strains, were mainly 1.00 - 4.00. These ratios indicate the diminished rate at which the MBLI is subject to efflux, and thus convey the efficacy of the MBLI to reach the target site.



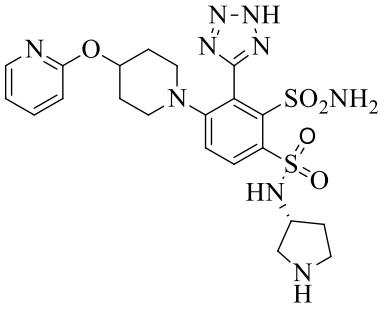
67
IC_{50} = 0.06-0.27 μM
MITC95 = (3.13-6.25) +1.0



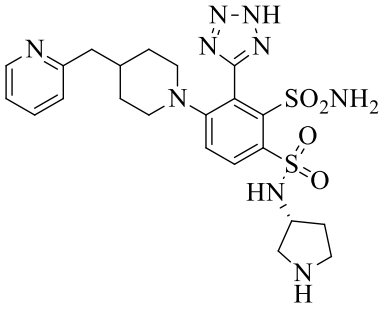
68
IC_{50} = 0.06-0.32 μM
MITC95 = (1.56-6.25) +1.0



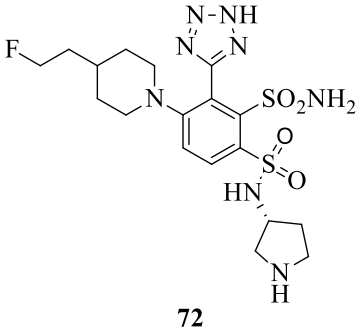
69
IC_{50} = 0.03-0.49 μM
MITC95 = 0.78 +1.0



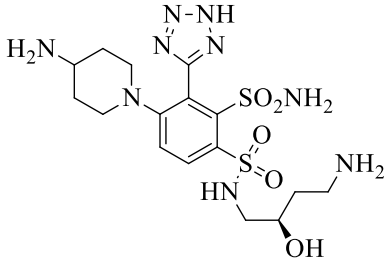
70
IC_{50} = 0.06-0.47 μM
MITC95 =(3.13-6.25) +1.0



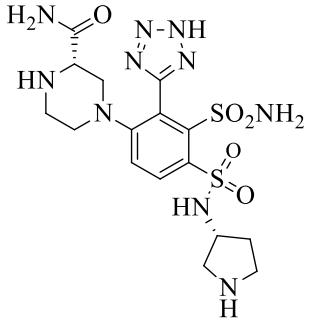
71
IC_{50} = 0.06-0.46 μM
MITC95 =(3.13-6.25) +1.0



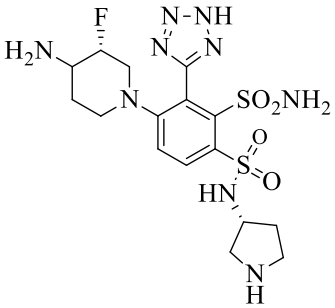
72
IC_{50} = 0.06-0.49 μM
MITC95 = (1.56-3.13) +1.0



73
IC_{50} = 0.17-26.54 μM
MITC95 = (0.20-0.39) +1.0



74
IC_{50} = 0.14-2.86 μM
MITC95 = (0.20-0.78) +1.0



75
IC_{50} = 0.18-4.27 μM
MITC95 = (0.10-0.39) +1.0

MBL tested (IC_{50}): NDM-1, VIM-1, VIM-2 and IMP-1
Bacteria Strain tested (MITC95): *E. Coil* (NDM-1), *S. marcensces* (IMP-1) and *K. pneumoniae* (VIM-1)

Figure 16: Most biologically active compounds against MBLs from WO2019018186A1[67].

Pasternak *et al.*,[68] filed another patent that included 49 more 1H- and 2H-tetrazol-5-yl sulfonamide and sulfone thienopyrroles derivatives. From these compounds **76-79** demonstrated the best IC_{50} activity values while compounds **80** and **81** had the best MITC95 activities (Figure

17). Experiments investigating cytotoxicity are required before the compound's full potential can be determined.

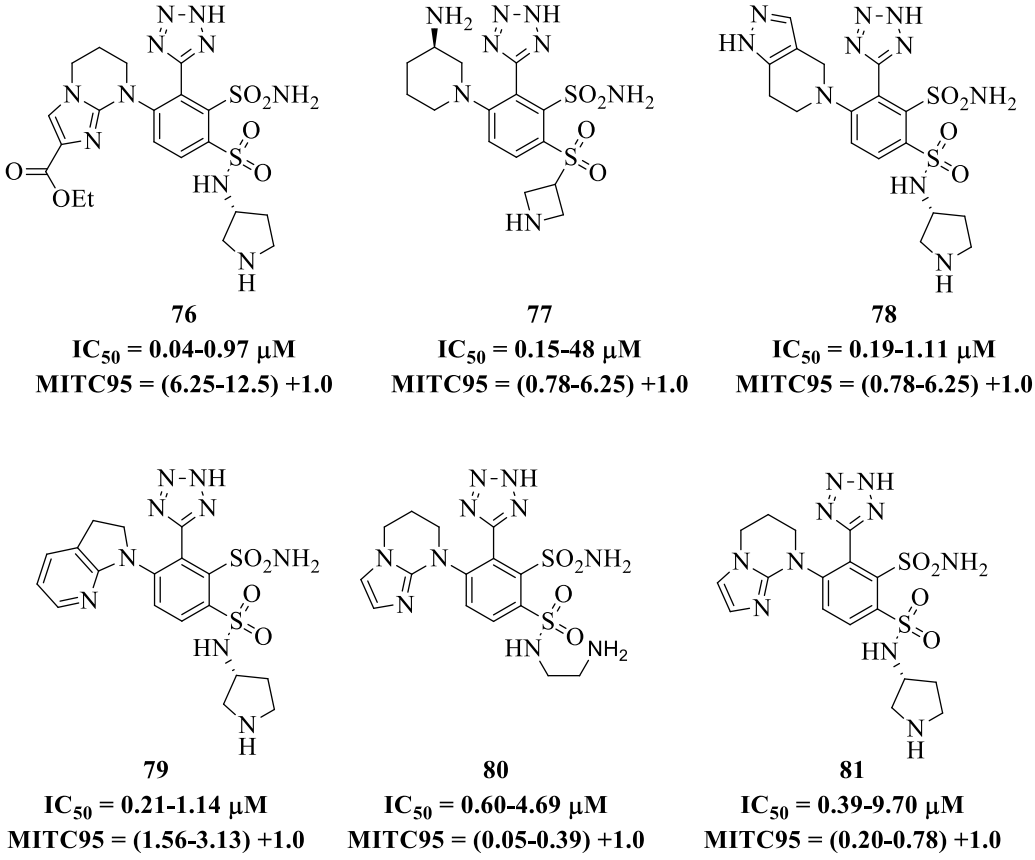
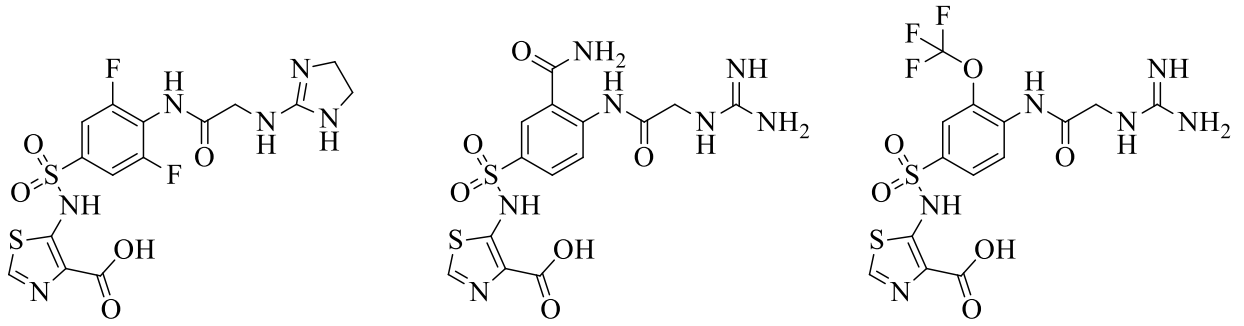


Figure 17: Most biologically active compounds against MBLs from WO2019135920A1[68].

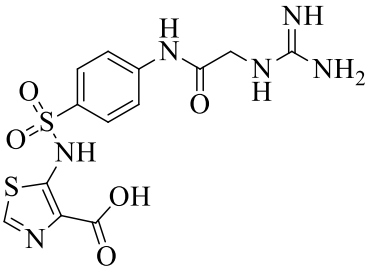
Davies *et al.,* [69] prepared 46 thiazole sulphonamides derivatives and investigated the biological activity of these compounds by conducting numerous experiments. The binding affinity (Ki) of all 46 derivatives against four MBLs, NDM-1, VIM-1, VIM-2 and IPM-1 were reported and converted to IC_{50} data using to Cheng Prusoff equation (Figure 18). It is important to note that exemplary compounds **82-87** possessed the strongest binding to all four MBLs. However, the MIC results of these compounds were not promising enough to pursue further.



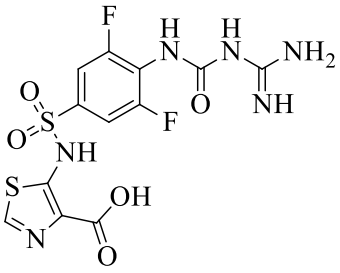
82
$IC_{50} \leq 40$ μM

83
$IC_{50} \leq 40$ μM

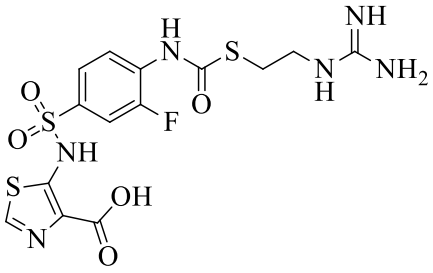
84
$IC_{50} \leq 40$ μM



85
$IC_{50} \leq 40$ μM



86
$IC_{50} \leq 40$ μM



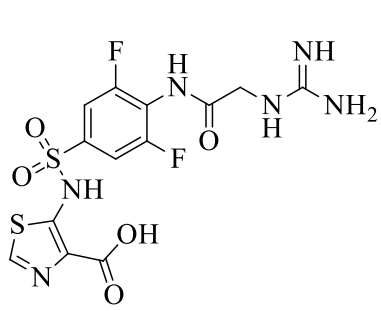
87
$IC_{50} \leq 100$ μM

MBL tested: NDM-1, VIM-1, VIM-2 and IMP-1

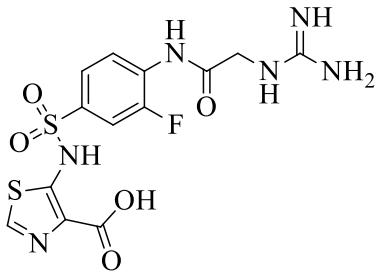
Figure 18: Most active compounds against MBLs from WO2019016393A1[69].

The best MIC result was observed with compounds **88-94** which demonstrated excellent activity against the 8 out of 10 MBL-producing strains tested, with a reduced meropenem MIC in the range of ≤ 2 mg/L in the presence of 8 mg/L of MBLI (Figure 19). Compounds **88** and **91** were highly stable in human plasma, with the compounds maintaining 87% and 100% stability after 5 hours of MBLI introduction, respectively. This should be compared to a structurally similar derivative of **88** and **91** where the guanidine group is replaced by a primary amine (structures not shown), where 0% material remained after 5 hours and 29% after 2 hours, respectively. A pharmacokinetic study on male Swiss albino mice was conducted using 1mg/kg of compound **91** and its amine derivative. The results indicate that compound **91** achieves a 100-fold higher plasma concentration and exposure time compared to an amine derivative of **91**, as it is cleared 50-fold slower from the blood

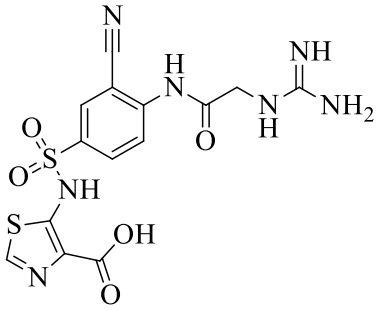
as compared to the amine derivative. An *in vivo* efficacy study on mice infected with *K. pneumoniae* (NDM-1 and SHV-11 producing) was also conducted with 30 mg/kg meropenem alone and 30 mg/kg of compound **88** + 30 mg/kg of meropenem. No significant difference was observed in the bacterial growth when meropenem alone was administered, however, combination therapy with compound **88** and meropenem, yielded a 1.6 $\log_{10}$ reduction in bacterial growth.



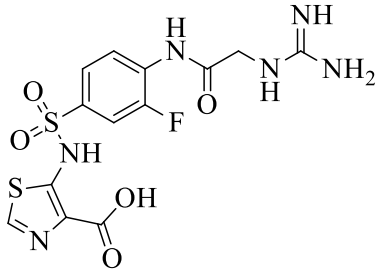
88



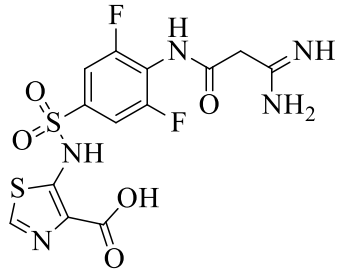
89



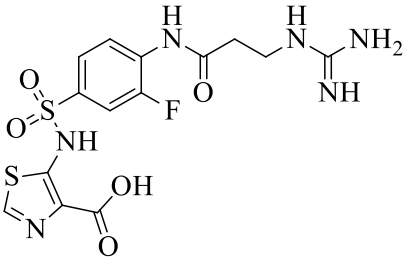
90



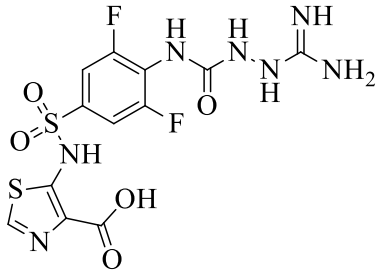
91



92



93

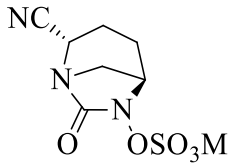


94

MIC = 8 + ≤ 2 (For 8/10 bacterial species tested)
MIC (mg/L) = MBLI + Meropenem
MIC tested against 10 MBL expressing bacteria species

Figure 19:Most biologically active compounds against MBL bacterial species from WO2019016393A1[69].

The SBLI WCK4234 (**95**) (Figure 20) was also assessed for restoring the efficacy on meropenem; as expected, it did not exhibit a significant effect in reducing the meropenem MIC against MBL producing bacteria. On the contrary, triple combination therapy using meropenem, 4 mg/L WCK4234 and 8 mg/L of compound **94** against two NDM-1 expressing *K. pneumoniae* strains, proved effective. The triple combination therapy resulted in a reduced meropenem MIC of 0.5 mg/L. The work represented in this patent is unique in that it had investigated triple combination drug susceptibility tests. It is recommended that the authors present the cytotoxicity data and thereafter advance to an *in vivo* efficacy study, using triple combination therapy. This will promote a higher log_{10} reduction in the bacterial load and thus make this treatment strategy an ideal candidate for clinical trials, provided the three drugs do not display unfavorable drug-drug interactions.



M = H/Na

WCK4234

95

Figure 20: SBLI WCK4234

3. Conclusion

The need for more effective SBLIs has been largely met as evidenced by the large number of newly approved candidates. The formidable challenge, however, remains with the dire need for a suitable MBLI to enter the market. Several *in-vitro* studies have demonstrated that zinc chelation at the active site of MBLs is a key target for inhibition, as a result, good activity has been observed from EDTA, captopril, TPA and TPEN. These chemical compounds are known to possess strong zinc chelating properties. However, many chelators are non-specific and demonstrate poor *in vivo*

efficacy, this is primarily due to many zinc enzymes encountered in a biological system. This review presents possible strategies to overcome this inefficiency. For example, bismuth chelated with EDTA has shown reduced cytotoxicity compared to EDTA alone. Another strategy utilized is the covalent linkage of a chelator to hydrophilic moieties that is chemically selective for MBLs. A third strategy is the use of triple combination therapy, which includes a carbapenem antibiotic, SBLI and MBLI. Despite the increased research in this area, NDM-1 and VIM-1 have still managed to disseminate globally [15,70], causing a catastrophic health concern. On the contrary, scientists as portrayed by patent literature, have risen to the occasion with the synthesis of various chemical derivatives capable of MBL inhibition. However, most BLIs patented in 2018 and 2019 have been described as a continuation of current chemical scaffolds, with little focus on exploring completely new pharmacophores. Interestingly, increased focus has been given to the simultaneous targeting of SBLs and MBLs as an innovative strategy. Although many chemical compounds have been claimed as MBLIs, evaluation of the compounds' biological activity has been limited to a minimal number of *in vitro* tests, with the exception being the bismuth complexes and pyridinyl-nicotinamide sugars. The lack of comprehensive biological testing implies the compounds are still in the early stages of drug development and far from entry into clinical trials, with their broad-spectrum activity towards MBLs being questionable. Biological evaluation that offers more information on the pharmacokinetics, pharmacodynamics, efficacy, and safety of these inhibitory compounds needs to be made public. It is also important to note that many of the experimental procedures of the claimed inhibitors, except for 1H- and 2H-tetrazol-5-yl sulfonamides and sulfone thienopyrrole derivatives, do not include their effects on other resistance mechanisms such as permeability barriers and efflux pumps. Therefore, additional *in vitro* and *in vivo* experiments are currently needed to produce a substantial body of information on the efficient targeting of MBLIs.

This could be due to the scope of the individual patent applications; however, such information will hopefully become available in subsequent publications.

4. Expert opinion

According to literature, 13 patents have been filed between 2018 - 2019, with some patents being listed as a continuation or update of previous work. One of the key findings from this review, is that non-beta-lactam scaffolds, could serve as potential MBLIs. Furthermore, some chemical entities could be utilized for therapy against bacteria carrying both SBL and MBL genes. Inhibitors that are based on metal ion chelation are increasingly being modified chemically to increase targeting to the zinc ions in bacterial β-lactamases; further studies are needed to tell if this strategy can reduce premature chelation before the target is encountered *in vivo*. Another positive outcome relates to the promising new chemical matter, i.e., the bismuth complexes, pyridinyl-nicotinamide derived sugars, boronic acid and thiazole sulphonamide derivatives. Whether these inhibitory compounds pass the phases of clinical trials is still to be determined. What is clear is that antibiotic resistance caused by MBL will continue to increase and that researchers therefore should be encouraged to delve into further research on MBLIs as well as other treatment strategies that have the capacity to target β-lactamases, carbapenem-resistant *Enterobacterales*, or both and thus neutralize carbapenem resistance. The primary treatment strategy of creating a broad-spectrum BLI, will prove useful to the health sector, especially in communities that have a high disease burden, as well as house clinicians lacking insight to resistance patterns initiated by the various β-lactamase genes in circulation.

While many inhibitory compounds that exhibit activity towards MBLs and SBLs have been described, further evaluation of these compounds is still warranted. A formidable challenge that remains is the synthesis of potential MBLI compounds, in terms of the high cost and level of

expertise versus the low product yield. Furthermore, most of these compounds do not pass *in vitro* and *in vivo* experimental evaluation and must be discontinued for reasons such as cytotoxicity, high MICs, or low bioavailability. However, such thorough biological evaluation is expensive, requires proper equipment and infrastructure as well as efficiently trained lab technicians/technologists, and therefore poses as a limitation to many developing countries. This is one of the primary reasons that most MBLIs have not made it to clinical trials yet.

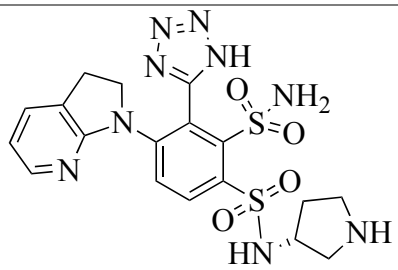
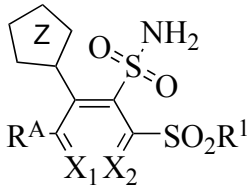
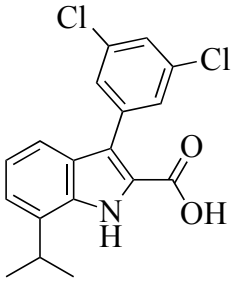
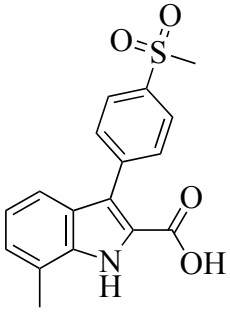
Global efforts, such as the Enable program within the European Union's Innovative Medicines Initiative program and the US National Strategy for Combating Antibiotic-Resistant Bacteria, source innovative ideas globally, put up detailed quality criteria, gathers drug discovery expertise as well as the required infrastructure and, are therefore highly valuable initiatives to increase productivity in this area. Finally, better clinical practice should not be forgotten! Assistance and constant improvement by antibiotic stewardship programs can be boosted in all countries to ensure the continued efficacy of antibiotics and prevention of antibiotic resistance to both old and new drugs!

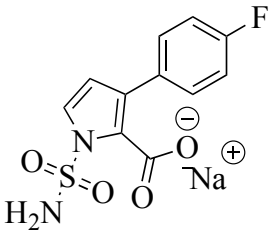
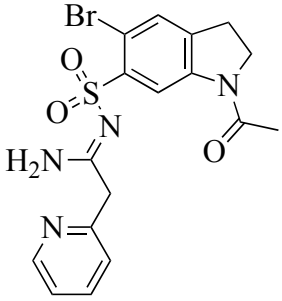
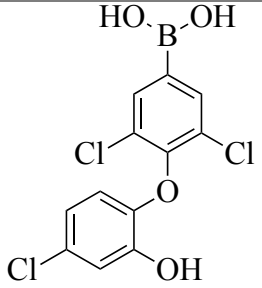
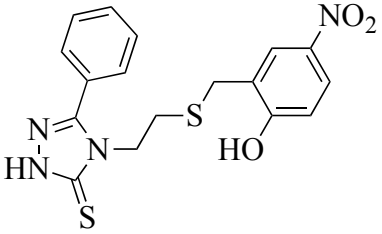
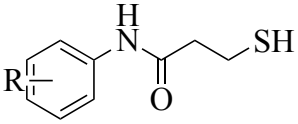
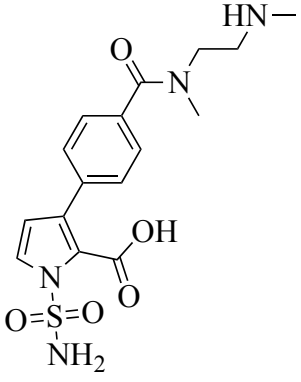
5. Update on MBL patents since 2019

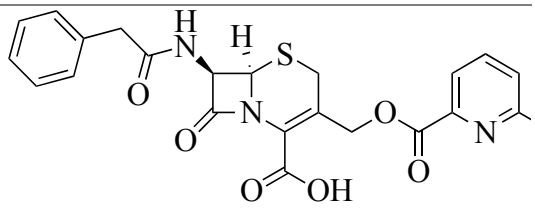
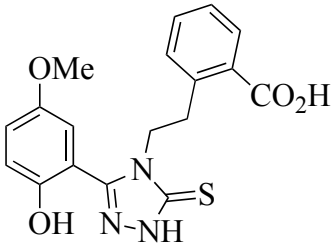
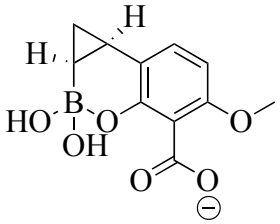
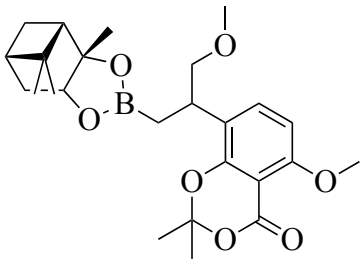
For this thesis, a brief update in a list format on the patents that have been filed since 2019 to present have been included in Table 1 below.

Table 1: MBLI patents (that included biological activity) filed since 2019

Authors/ Inventors	Patent Number	MBLI Classification	MBLI Structure

Pasternak *et al*., [71]	US20200375987A1	1H and 2H - tetrazol - 5 - yl sulfonamide and sulfone compounds	
Bennet *et al*.,[72]	US10544130B2	1H- 2H -tetrazol- 5 -y l sulfonamide class	 Z= tetrazolyl X_1= N or CH X_2= N or CH
Gising *et al.*, [73]	US20210137886A1	Thienopyrroles and pyrrolopyrazoles	
Panduwawala *et al.*, [74]	US20210137884A1	Pyrroles, imidazoles and indoles derivatives	Expanded on **58** refer to Figure 14
Brem *et al*., [75]	US20200375946A1	Indole carboxylate derivatives	

Cooper *et al.*, [76]	US20210230115A1	-1sulfamoyl - 1 H - pyrrole carboxylic acid	
Becker *et al.*, [77]	US10626087B2	Indoline and tetrahydroquinoline sulfonyl inhibitors	
Lee *et al.*,[78]	US20210154216A1	Dichlorophenylboronic acid	
Hernandez et al., [79]	US20220000841A1	1,2,4 - triazole - 3 - thione moiety	
Hartmann *et al.*, [80]	WO2021191 21A1	N-Phenyl-3-mercaptopropanamide	
Wilkinson *et al.*, [81]	WO2021099 79A1	1-Aminosulfonyl-2-carboxypyrrole derivatives	

Martin *et al*., [82]	WO2020204 71A1	Cephalosporin prodrugs	
Docquier *et al.*, [83]	WO2020099 645 A1	Substituted 4,5-dihydro-1H-1,2,4-triazole thiones	
Hecker *et al.*, [84]	US11180512 B2	Boronic acid derivatives	
Reddy et al., [85]	US20210361 682A1	Boronic acid derivatives	

Research towards finding a potential MBLI is ongoing, with 15 patents reported since 2019 (to the best of our knowledge). Pasternak's, Hecker's and Brem's research groups seem to be in the frontline of leading the synthesis and evaluation of derivatives in their respective classes. It is noteworthy to mention that to date no research group has reported β-lactam-metallo-β-lactamase inhibitors attached to a cyclic chelator, as the novel compounds we report in the subsequent chapters of this thesis.

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Declaration of interest

The authors declare no conflict of interest

Author contribution

NR performed the literature searches, wrote and prepared the manuscript. MS co-wrote the manuscript and drew all chemical compounds. The remaining are supervisors who conceptualized the topic, guided the writing and preparation of the manuscript.

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Chapter three: Manuscript two

The *in vitro* and *in vivo* potential of metal-chelating agents as metallo-beta-lactamase inhibitors against carbapenem-resistant *Enterobacterales.*

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Abstract

The recent surge in β-lactamase resistance has created superbugs which pose a current and significant threat to public healthcare. This has created an urgent need to keep pace with the discovery of inhibitors that can inactivate these β-lactamase producers. In this study, the *in vitro* and *in vivo* activity of 1,4,7-triazacyclononane-1,4,7 triacetic acid (NOTA) - a potential metallo-β-lactamase inhibitor was evaluated in combination with meropenem against metallo-β-lactamase producing bacteria. Time-kill studies showed that NOTA restored the efficacy of meropenem against all bacterial strains tested. A murine infection model was then used to study the *in vivo* pharmacokinetics and efficacy of this metal chelator. The co-administration of NOTA and meropenem (100 mg/kg.bw each) resulted in a significant decrease in the colony-forming units of *K. pneumoniae* NDM-1 over an eight-hour treatment period (> 3 $\log_{10}$ units). The findings suggest that chelators, such as NOTA, hold strong potential for use as a metallo-β-lactamase inhibitor in treating carbapenem-resistant *Enterobacterale* infections.

Keywords

NOTA, Carbapenem-resistant *Enterobacterales,* infection modeling, combination therapy

1. Introduction

The β-lactam antibiotics are a group of bactericidal drugs that share a chemical structure and are characterized by the presence of the β-lactam ring [1, 2]. Penicillins, cephalosporins, carbapenems, penems and monobactams are examples of the currently available β-lactam antibiotics in clinical use [1]. These antibiotics are the most widely used antimicrobial agents, with a long history of success in the treatment and management of infectious diseases [1]. β-lactamases hydrolyze the β-lactam ring through a series of reactions which ultimately lead to the destruction of the antibiotic and subsequent loss of activity [3]. β-lactamase enzymes are categorized as either serine β-Lactamases or metallo-β-lactamases based on their structural configuration and mode of action [4]. The former has a serine residue at its active site, while the latter has zinc ions [4]. Metallo-β-lactamases (MBLs) require zinc ions to catalyze the hydrolysis of β-lactam antibiotics, this involves the binding of the zinc ion to the active site of the β-lactamase enzyme. As a result, the metallo-β-lactamase enzyme is truncated, and the efficacy of the β-lactam drug is significantly reduced [5].

The broad-spectrum of β-lactam's make them suitable for treating a wide range of clinically relevant infections caused by gram-positive and gram-negative bacteria [6]. The physicochemical factors associated with the structures of β-lactam's influence their antimicrobial spectrum of activity [7]. Their lipophilicity favors activity against Gram-positive bacteria, whereas their hydrophilicity favors action against Gram-negative bacteria [1]. One of the most important classes of β-lactam antibiotics are carbapenems. They are resistant to hydrolysis by most lactamases and

can act as slow substrates or inhibitors of β-lactamases in some cases [8]. Their mechanism of action involves the use of porin channels to penetrate the bacteria's outer membrane and target penicillin binding proteins. Allowing for broad antibacterial activity against a wide range of Gram-positive and Gram-negative pathogens [9]. However, resistance to carbapenems has increased significantly and created a worldwide health concern [10]. Carbapenem-resistant *Enterobacterales* (CRE) infections have recently risen in public healthcare settings, particularly due to infections caused by *Klebsiella pneumoniae*, creating a significant cause for concern [11, 12].

The synthesis of β-lactamase inhibitors capable of overcoming resistance to β-lactam drugs is a thriving area of research [13]. Serine β-lactamases have been successfully inhibited by clinically available β-lactamase inhibitors, including clavulanic acid, avibactam, sulbactam, and tazobactam [14]. However, metallo-β-lactamase inhibitors are not yet clinically available, posing a significant worldwide health concern in the fight against antibiotic resistance [15]. Many successful attempts have been made to discover metal-chelating agents that function as metallo-β-lactamase inhibitors against metallo-β-lactamase (MBL) producing bacteria. These examples include; Aspergillomarasmine A [16], Dipicolinic acid [17], pyridine 2,4-dicarboxylic acid [18], 1,4,7-triazacyclononane-1,4,7 triacetic acid (NOTA) [19], 2,2′,2″,2‴-(1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetic acid (DOTA) [20], and diethylenetriaminepentaacetic acid (DTPA) [21]. The strong affinity of these chelators for metal ions has been exploited for their ability to bind zinc [22], which is an essential requirement for MBL action [4].

The aim of this study is to evaluate the *in vitro* and *vivo* efficacy of the combination of meropenem- a carbapenem, and NOTA- a metallo-β-lactamase inhibitor against MBL-producing carbapenem-resistant *Enterobacterales* (CREs). We expand on our previous study which reported the *in vitro*

activity of NOTA [19] to report the time-kill kinetics and the *in vivo* efficacy of the combination in a murine infection model.

2. Materials and Methods

2.1. Ethical Statement

All animal study experiments were approved by the Institutional Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal (UKZN) (approval reference: AREC/013/016D for NOTA PK study and AREC/081/015D for NOTA *in vivo* efficacy study). All animal experiments were carried out in accordance with the ARRIVE guidelines.

2.2. Bacterial source

Well characterized metallo-β-lactamase producers belonging to the order of *Enterobacterales* were purchased from Patrice Nordmann at the Institut National de la Santé et de la Recherche Médicale (U914), Paris, France [23] The bacterial strains used in this study were *Escherichia coli* NDM-1, *Escherichia coli* IMP-1, and *Enterobacter cloacae* NDM-1. *Klebsiella pneumoniae* NDM-1 was acquired from from Hartford Hospital, USA [24]. Bacterial stock solutions were preserved in Trypticase soy broth supplemented with 10 % glycerol and 4mm glass beads, stored at -80ºC. *Escherichia coli* ATCC 25922 was used as a carbapenem susceptible control.

2.3. Preparation and storage of antibiotics and inhibitors

Meropenem was obtained from Sigma-Aldrich (Schnelldorf, Germany) and NOTA from Macrocyclics (Texas, United States of America). Distilled water was used for preparing the meropenem stock solution (32 mg/L), while phosphate-buffered saline (PBS) was used for NOTA (128 mg/L). The stock solution of meropenem was stored at -80ºC.

2.4. Susceptibility testing of meropenem co-administered with NOTA

The checkerboard method under the guidelines of the Clinical and Laboratory Standards Institute (CLSI), was used to determine the susceptibility profile of meropenem alone and in combination with NOTA [25, 26]. In brief, two-fold dilutions of meropenem (32 – 0.03 mg/L) with NOTA (64 – 1 mg/L) were prepared in Mueller Hinton Broth (MHB). A 0.5 McFarland-standardized bacterial inoculum was added to each well, to a final volume of 100 µL, and plates were incubated at 35 °C for 18-20 h. The checkerboard assays were performed in triplicate. The minimum inhibitory concentration (MIC) was recorded as the lowest antibiotic-inhibitor concentration that showed no visible growth (Table 1). We have previously reported the cytotoxic potential, where the drug was found to be non-cytotoxic at the concentrations used in this study [19].

2.5. Time- kill assay of meropenem co-administered with NOTA against CREs

An initial inoculum density of 10^7cfu/ml (colony forming units) of each test organism was added to Eppendorf tubes containing MHB and meropenem at graded concentrations of MIC, 1*MIC, 2*MIC, 4*MIC, 8*MIC, and 16*MIC. The chelator was then added at the minimum bactericidal concentration (MBC) of 8 mg/L (MBC determined from the co-administration with meropenem). Meropenem, and bacterial growth control groups were included in the time-kill kinetics study. The meropenem control group consisted of the MIC of meropenem alone against the test organism used (32 mg/L for *E. cloacae* NDM-1 and 16 mg/L for *E. coli* IMP-1) except for *K. pneumoniae* NDM-1 and *E. coli* NDM-1 where only clinically achievable concentrations were used (32 mg/L). Aliquots of 100 µl in duplicate were removed for count determination at 0, 2, 4, 6, 8, 10 and 24 hours. Viable counts were determined by the serial dilution method and plated on Mueller Hinton agar (MHA). These plates were incubated at 35ºC for 24 h, and plate counts were done after 24 h

of incubation. Values for each time point were generated from the mean ± SD values of the duplicate cfu/ml count from a single experiment (Figures 1-4).

2.6. Pharmacokinetic (PK) study of meropenem and NOTA

Male Balb/c mice (average weight 26 ± 2 g) were obtained from the Biomedical Resource Unit (UKZN, Durban, South Africa) and housed under standard conditions, in an air-conditioned room with a 12 h light/dark cycle and were given *ad libitum* access to food and water. In the pilot PK study animals were dosed with 10 mg/kg.b.w of meropenem and 10 mg/kg.b.w of NOTA via intraperitoneal injection, to determine if the resulting plasma concentrations were below cytotoxic levels. The animals were euthanized at 0, 5, 15, 30, 45, 60, 90, and 120-min post-dosing (n=3 per time point). This allowed for a single dose plasma time-concentration curve of each drug to be generated. At the time of termination, approximately 0.5 - 0.7 ml of blood was collected into heparinized micro-tubes for plasma-drug concentration analyses. Blood plasma was separated by centrifugation at 10000 rpm for 10 minutes and analyzed using liquid chromatography-mass spectrometry (LC-MS/MS).

2.7. Sample preparation for the LC-MS/MS analysis of meropenem and NOTA

During sample preparation, 100 µl of the biological sample was spiked with 20 µl of internal standard (IS) and vortexed for 1 min, after which 880 µl of MeOH was added to extract target analytes and to induce the precipitation of proteins. The mixture was then vortexed for 1 min, followed by centrifugation at 13000 g for 15 min at 4ºC. The supernatants were filtered through an SPE cartridge [DSC-18 (50mg)]. The filtrate was then collected into auto-sampler vials and vortexed briefly before injecting into the LC-MS/MS system. The calibration samples were prepared using the same procedure.

The liquid chromatography (LC) system was an Agilent technology 1100 (Agilent, Germany) series coupled to a Bruker QTOF-II (Bruker Daltonics, Bremen, Germany) with electrospray ionization (ESI) source and a time-of-flight mass spectrometry (TOF-MS) mass analyzer (Bruker Daltonics, Bremen, Germany). Chromatographic separation was achieved using an Ascentis Express RP-Amide column (5cm x 2.1 mm; 2.7 µm particle size) (Supelco, Sigma-Aldrich, Germany). Mobile phase A was millipore water (0.1% v/v FA), and mobile phase B was methanol (0.1% v/v FA), with a flow rate of 0.4 ml/min and column compartment set to room temperature. A gradient method was used to increase chromatographic separation from 70% A to 30% B, with a sample injection volume of 5µl and a total run time of 10 mins. The MS acquisition parameters were positive ion polarity; endplate offset - 500 V; capillary voltage -5000 V; nebulizer - 1.8 bar; dry gas flow rate - 8 l/min; dry heater temperature - 180 ºC; scan range was from m/z 100 - 500; collision cell radiofrequency was 500 Vpp; collision energies were 1eV for, NOTA, meropenem, and ampicillin (internal standard). Data Analysis 4.0 SP 5 (Bruker Daltonics) was used to further process the data.

2.8. *In vivo* murine thigh infection modelling of CREs and inhibitor administration

A thigh infection protocol was performed as described by Michail *et al.* [27]. Briefly, six-week-old, specific pathogen-free, male Bagg inbred albino c-strain (BALB/c) mice weighing 20-25 g (n=40), were rendered neutropenic (neutrophils <100/mm^3) by pretreatment with cyclophosphamide, intraperitoneally (IP) at 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to infection. The right thigh was infected using a 100ul intramuscular injection containing 10^7-10^8 cfu/ml of *K. pneumoniae* NDM-1. This procedure was done two hours prior to treatment with the meropenem + NOTA (100 mg/kg.b.w each) combination, based on dose extrapolation from the PK study. The mice were randomly separated into two groups, the infected control, and the treated

group. In keeping with the criteria of reducing animal numbers, as outlined in the ARRIVE guidelines [28], a meropenem-only control group was not included. This was due to the well-documented *in vivo* pharmacokinetic profile of meropenem [29]; additionally, meropenem administered alone is ineffective against the metallo-β-lactamase producing bacteria. Mice were humanely euthanized, by isoflurane overdose, at 2h, 4h, 6h, and 8h post-treatment. The right thigh muscle was then aseptically removed and homogenized in 5ml of PBS. Homogenates were serially diluted eight times and plated onto antibiotic-free Mueller-Hinton agar plates for each dilution and incubated at 35^{o}C for 24h. Following the incubation period, the plates were assessed for growth and enumerated. The titer was then expressed as $\log_{10}$ cfu/ml for each thigh muscle.

2.9. Statistical analyses

Experimental data generated from the time-kill kinetic study were analyzed using GraphPad Prism version 5.0 (GraphPad Inc., San Diego, CA, USA). The bacterial density was represented using $\log_{10}$ cfu/ml and plotted against each bacterium's time in hours. The kill rate was determined at different time intervals using a linear regression model. A comparative analysis of the kill rate of meropenem in combination with the β-lactamase inhibitor was assessed using the two-way analysis of variance (ANOVA).

3. Results and Discussion

From the checkerboard assay, NOTA alone did not produce any inhibition at concentrations up to 64 mg/L. When used in combination with meropenem, inhibition was observed with a NOTA concentration of 4 mg/L and 8 mg/L. The MIC of meropenem alone and in combination with NOTA against carbapenem-resistant *Enterobacterales,* was also determined. The results (Table 1)

show that *E. coli* NDM-1, *K. pneumoniae* NDM-1 were highly resistant to meropenem while, *E. coli* IMP-1, and *E. cloacae* NDM-1 were moderately resistant. NOTA achieved excellent activity in restoring the efficacy of meropenem at a concentration as low as 0.06 mg/L for all microorganisms, except *K. pneumoniae* NDM-1 and E. *coli* NDM-1, for which the MICs were 0.125 mg/L. These findings demonstrate that the metallo-β-lactamase-producing *Enterobacterales* are susceptible to inactivation by metal chelators *viz.* NOTA. This is in line with the previous report where NOTA was able to restore the efficacy of meropenem against *E. coli* NDM-1 and *E. cloacae* NDM-1 [19].

Table 1: The MICs of meropenem only, and in combination with NOTA (n=3)

Reference strain identification	**Organism**	**Minimum Inhibitory Concentration (mg/L)**		
		[a]	**[b]**	**[a+ b][1]**
AUS 271	***Escherichia coli* NDM-1**	**128**	**> 256**	**0.125+4**
USA 449	***Klebsiella pneumoniae* NDM-1**	**128**	**> 256**	**0.125+4**
JAP	***Escherichia coli* IMP-1**	**16**	**> 256**	**0.06+4**
IR 386	***Enterobacter cloacae* NDM-1**	**32**	**> 256**	**0.06+4**

N.B.: a = minimum concentration (mg/L) of meropenem alone; b = minimum concentration (mg/L) of NOTA alone; [1]= minimum concentration (mg/L) of meropenem and NOTA that resulted in inhibition.

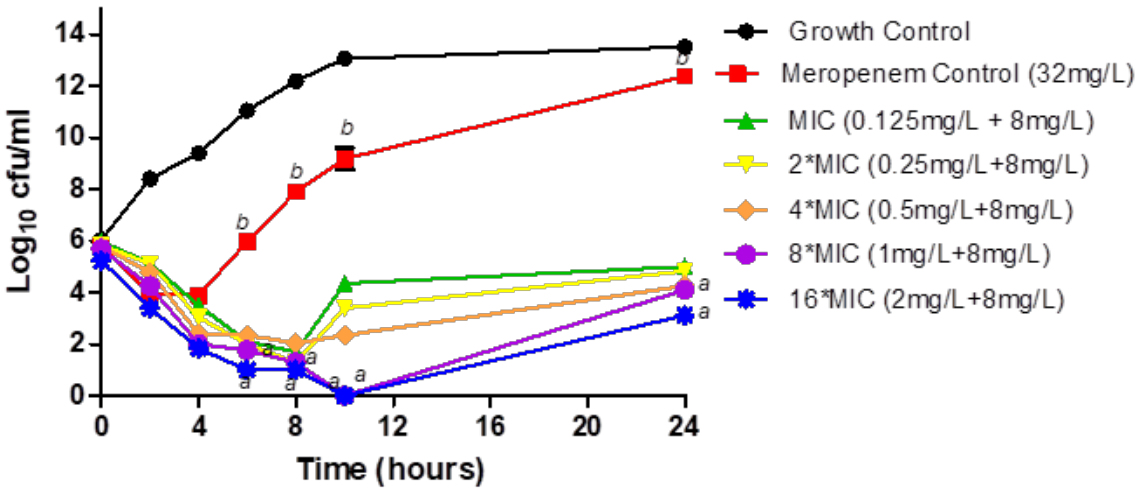

Figure 1: *E. coli* NDM-1 treated with multiple meropenem concentrations and a fixed NOTA concentration of 8 mg/L, representing the different MICs used to achieve a bactericidal effect. Combination with NOTA shows a significant difference ($P < 0.05$) over the killing effect of meropenem alone (meropenem control). Symbol a indicates a significant increase in the rate of kill as compared to b. Data is represented as a mean ± SD.

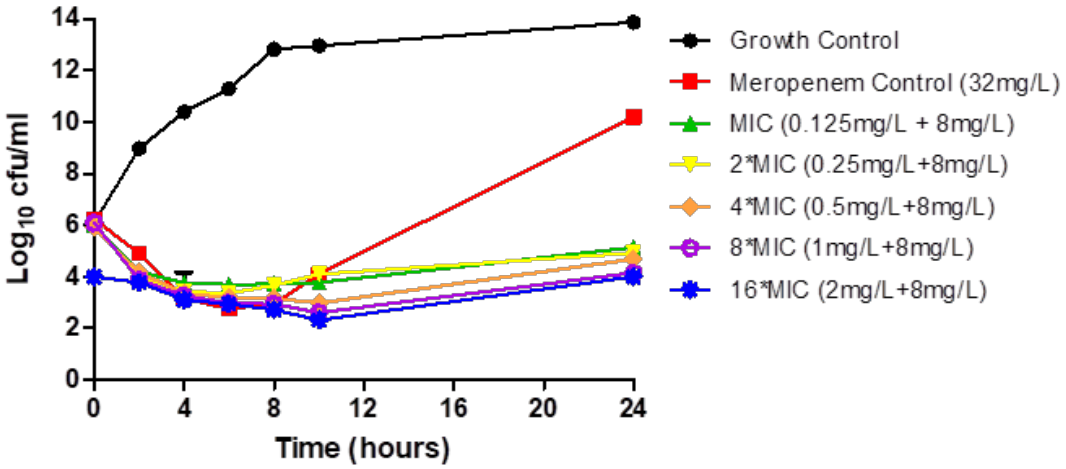

Figure 2: K. *pneumoniae* NDM-1 treated with multiple meropenem concentrations and a fixed NOTA concentration of 8 mg/L, representing the bactericidal effect achieved at different MICs. There is no significant difference ($P < 0.05$) between the treatment groups. Data is represented as a mean ± SD.

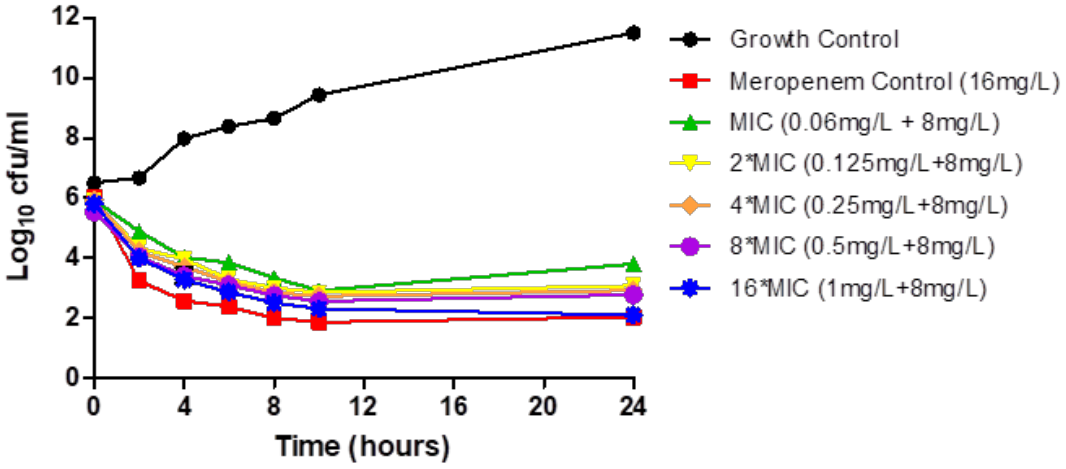

Figure 3: E. *coli* IMP-1 treated with multiple meropenem concentrations and a fixed NOTA concentration of 8 mg/L. These curves represent similarity in the bactericidal effect achieved when using reduced meropenem concentrations compared to the high MIC of meropenem alone (meropenem control). There is no significant difference ($P < 0.05$) between the treatment groups. Data is represented as a mean ± SD.

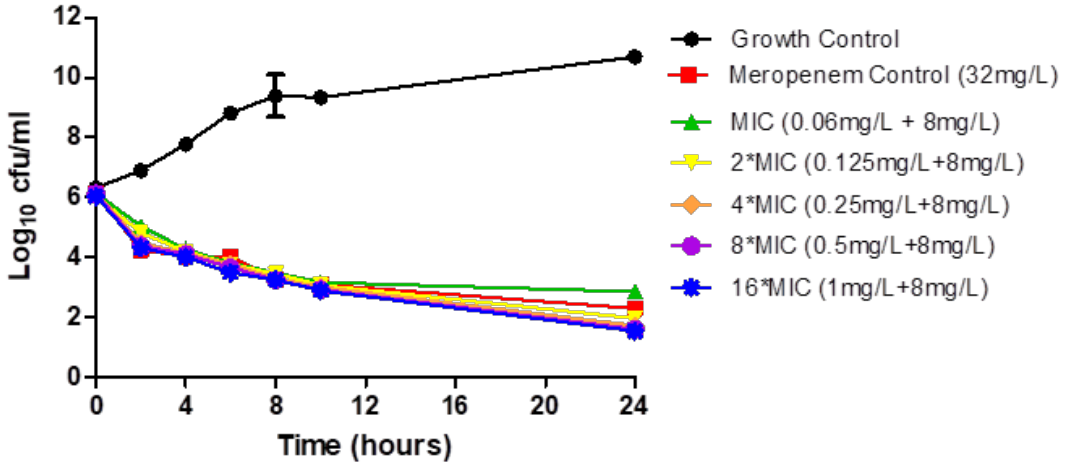

Figure 4: E. *cloacae* NDM-1 treated with multiple meropenem concentrations and a fixed NOTA concentration of 8 mg/L. These curves represent similarity in the bactericidal effect achieved when using lower meropenem concentrations compared to the high MIC of meropenem alone (meropenem control). There is no significant difference ($P < 0.05$) between the treatment groups. Data is represented as a mean ± SD.

The bactericidal activity of meropenem when co-administered with NOTA over 24 hours are presented in Figures 1-4. *E. coli* NDM-1 and *K. pneumoniae* NDM-1 (Figures 1 and 2, respectively), displayed similar outcomes showing bacterial re-growth at 10 hours post-treatment, this could be attributed to the low chelator concentration selected. However, in Figures 1-4, a visible decrease in the cfu/ml count was observed for all bacteria investigated when meropenem concentrations of 0.125 – 2 mg/L when used in combination with NOTA. The inhibitory effect of the combination of meropenem + NOTA against *E. coli* NDM-1 and *K. pneumoniae* NDM-1 showed no significant difference.

The action of meropenem is time-dependent [30, 31], showing that meropenem alone possesses bactericidal activity in the early logarithmic growth phase up to 4 hours in *E. coli* NDM-1, thereafter a growth relapse was observed (Figure 1). This same pattern was also observed in our study with respect to *K. pneumoniae* NDM-1 (Figure 2). Therefore, meropenem administered alone at a concentration of 32 mg/L cannot inhibit bacterial growth. This trend could be attributed to the presence of resistant mutants within the bacterial colony [32]. However, meropenem, when administered alone, displayed similar bactericidal activity against *E. coli* IMP-1 (9.47 $\log_{10}$ at 16 mg/L) and *E. cloacae* NDM-1 (8.4 $\log_{10}$ at 32 mg/L) compared to the investigated combinations, as presented in Figure 3 and 4, respectively.

Meanwhile, in *E. coli* NDM-1, the meropenem-inhibitor combination of 8*MIC, and 16*MIC extended the killing rate to 10 hours before a weaker effect was noticed (Figure 1). Thus, not only did the inhibitors restore the efficacy of meropenem, while also prolonging the duration of its action. This prolonged action was also observed for *K. pneumoniae* NDM-1, which maintained bactericidal activity until 10 hours before bacterial regrowth was observed. *E. coli* IMP-1 was susceptible to meropenem when co-administered with the inhibitor, showing an impressive

bactericidal effect of 8.44 log_{10} and 9.08 log_{10} at 0.125 mg/L of meropenem when co-administered with NOTA, respectively (Figures 3); the same was observed in *E. cloacae* NDM-1 which was 8.77 log_{10} at 0.125 mg/L of meropenem co-administered with NOTA (Figure 4).

The data presented in Table 1 reflects that, meropenem co-administered with NOTA in a two-fold serial dilution, resulted in a 10-fold reduction in meropenem MIC against E. *cloacae* NDM-1 compared to its meropenem only MIC (32 mg/L), 9-fold reduction in meropenem MIC against E.*coli* IMP-1 (MIC of meropenem administered alone is 16 mg/L) and an 11-fold decrease in the MIC of meropenem against E. *coli* NDM-1 and K. *pneumoniae* NDM-1 when compared to their MICs with meropenem only (128 mg/L).

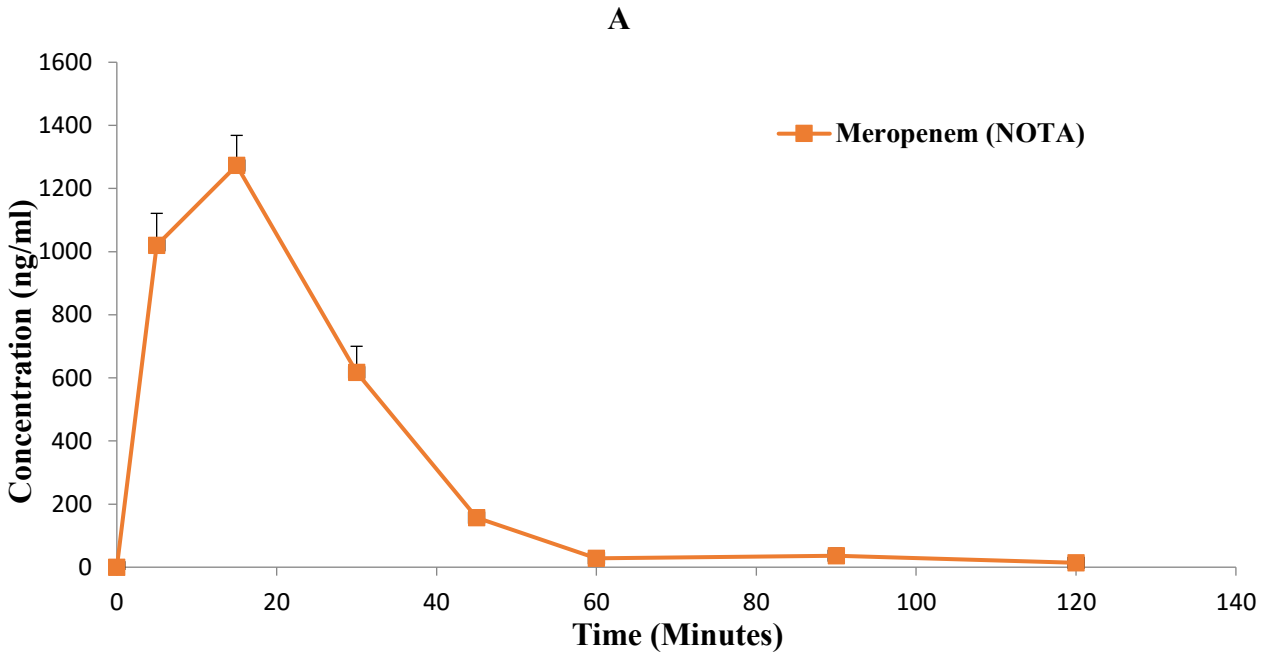

Figure 5A: Concentrations of meropenem in plasma, following a single 10 mg kg−1 intraperitoneal dose of meropenem and NOTA (data are represented as mean ± SD, n= 3). NOTA was above the limit of detection (10 $ngml^{-1}$) but lower than the limit of quantification (100 $ngml^{-1}$). Data is represented as a mean ± SD.

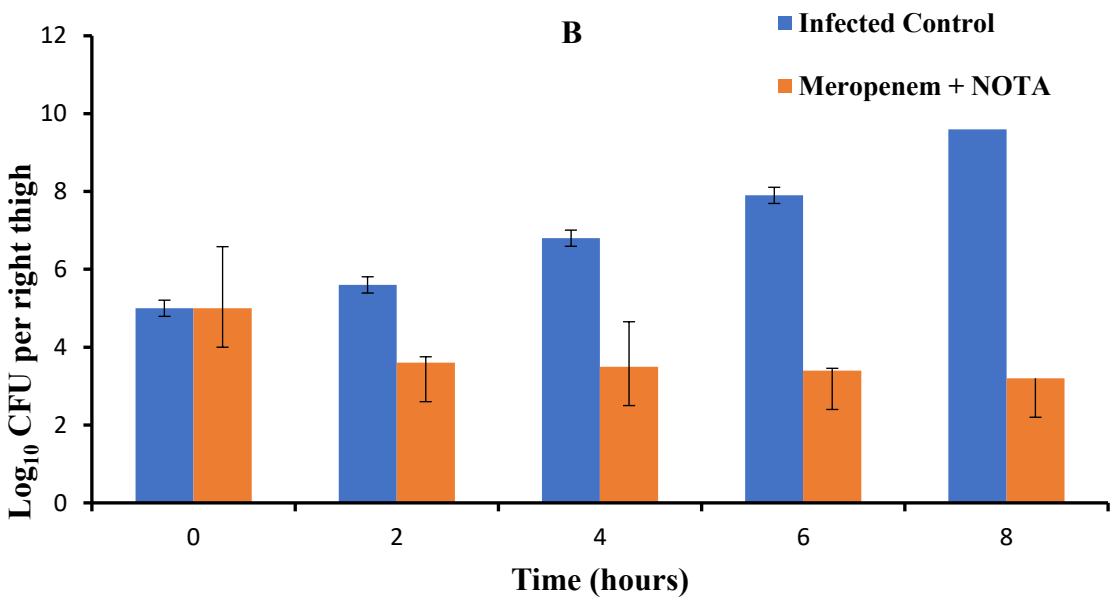

Figure 5B: *In vivo* efficacy of NOTA (100 mg/kg.b.w) when co-administered with meropenem (100 mg/kg.b.w) in a murine thigh infection model with *K. pneumoniae* NDM-1 (data are represented as mean ± SD, n= 3). The Student T-test revealed a significant difference ($P = 0.0031$) between the infected control and the meropenem+ NOTA treated group. Data is represented as a mean ± SD.

NOTA was below the limit of quantification in the PK study (Figure 5A) however, it was detected at lower concentrations. This warranted a safe and effective dose (NOTA is non-haemolytic up to a concentration of 500 mg/L *in vitro*) of 100 mg.kg.b.w used in the *in vivo* efficacy study. The results presented in Figure 5B, shows that NOTA was able to restore the potency of meropenem by significantly reducing the cfu/ml count of *K. pneumoniae* NDM-1 infected animals by > 3 $\log_{10}$ units, when compared to the infected control highlighting the potential of NOTA as a potent MBLI.

4. Additional Notes

This study also screened 1,4,7- tris (2-picolinyl)-1,4,7-triazacyclononane (NO3PY), it differs from NOTA in that it has picolyl arms that replace the pendant carboxylic acid groups (Figure 5).

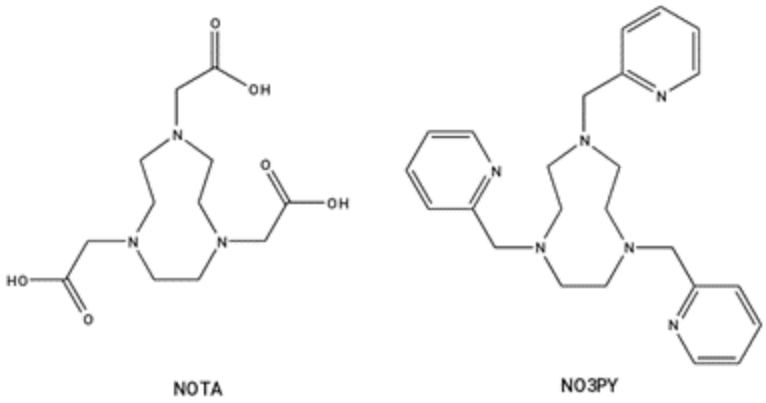

Figure 5: Molecular structure of chelators NOTA and NO3PY

Thus far, the use of NO3PY has been limited to radiochemistry [33] and has not been investigated for its antibacterial effect or synergistic properties, in combination with meropenem. In this study, NO3PY was evaluated as a potential MBLI, it was synthesized and characterized (all spectral data matched that reported in literature [33]) in our laboratory. NO3PY generated excellent *in vitro* results (Figure S1-S4) by restoring meropenem's MIC to 0.06 mg/L at a concentration of 4 mg/L. In the *in vivo* pharmacokinetics study, NO3PY had poor bioavailability (below the limit of detection). The derivatization of NO3PY with a carrier biomolecule could serve to transport the chelator to the target site leading to the enhancement of its bioavailability.

5. Conclusion

This study showed that the co-administration of NOTA *in vitro* improved the efficacy of meropenem against *K. pneumoniae* NDM-1, *E. coli* NDM-1, *E. coli* IMP-1, and *E. cloacae* NDM-1, demonstrating the potential use of these compounds as MBLIs. However, further preclinical development is needed to improve the pharmacokinetic properties of these agents to increase their bioavailability and tissue distribution. This study suggests that metal chelators, such as NOTA, hold promise as potential MBLIs given the rise of antimicrobial resistance and the lack of safe and effective therapeutic interventions.

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Chapter four: Manuscript three

The *in vitro* and *in vivo* development of a β-lactam-metallo-β-lactamase inhibitor: targeting carbapenem-resistant *Enterobacterales*

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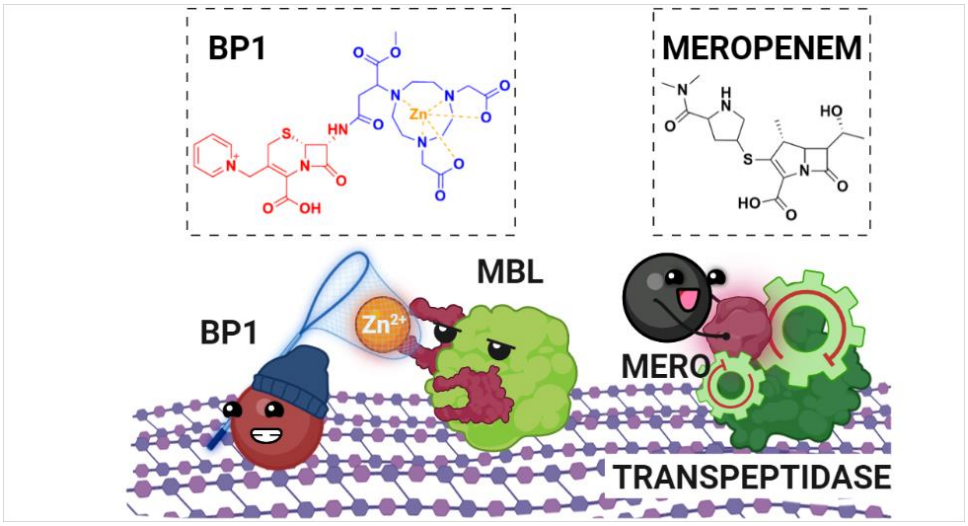


Abstract

β-lactams are the most prescribed class of antibiotics due to their potent, broad-spectrum antimicrobial activities. However, alarming rates of antimicrobial resistance now threaten the clinical relevance of these drugs, especially for the carbapenem-resistant *Enterobacterales* (CRE) expressing the metallo-β-lactamases (MBLs). Antimicrobial agents specifically targeting these enzymes to restore the efficacy of last resort β-lactam drugs, i.e., carbapenems, are desperately needed. Herein, we present a cyclic zinc chelator covalently attached to a β-lactam scaffold (cephalosporin) i.e. BP1. Observations from *in vitro* assays (with seven MBL expressing bacteria from different geographies) have indicated that BP1 restored the efficacy of meropenem to $\leq$ 0.5 mg/L, with sterilizing activity occurring from 8 hours post-inoculation. Furthermore, BP1 was non-toxic against human hepatocarcinoma cells (IC_{50} > 1000 mg/L) and did not inhibit the human zinc-containing enzyme glyoxylase II up to 500 μM. Enzyme inhibition studies and molecular docking of BP1 with NDM-1 and VIM-2 shed light on BP1's mode of action. In *Klebsiella pneumoniae* NDM infected mice, BP1 co-administered with meropenem was efficacious in reducing the bacterial load by > 3 $\log_{10}$ units' post-infection. The findings herein propose a favourable therapeutic combination strategy that restores the activity of the carbapenem antibiotic

class and complements the few MBL inhibitors under development, with the ultimate goal of curbing antimicrobial resistance.

1. Introduction

The emergence of resistance to the β-lactam (BL) antibiotics, i.e., the most important class and last resort antimicrobials (due to their low toxicity and broad spectrum activity), arises when bacteria produce β-lactamase enzymes that destroy the β-lactam scaffold thereby reducing drug activity.[1] The World Health Organisation has stated that the highest concern for resistant bacteria is the carbapenem-resistant *Enterobacterales*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*.[2] For the past four decades, β-lactamase inhibitors have effectively neutralized the effects of this resistance.[3] However, bacteria have evolved resistance mechanisms such as the carbapenemases, with various modes of action; these are divided into two groups according to their active sites: (i) serine carbapenemases (Ambler classes A, C, and D) and (ii) metallo-β-lactamases (MBL, class B). Currently, our clinical arsenal lacks MBL inhibitors to fortify public health against resistant infections.[3-7]

Combating resistance to lactam antibiotics has been accomplished by co-administering the BL antibiotic with a suicide BL such as clavulanic acid, tazobactam, or sulbactam.[4] However, these regimens are currently only effective for serine β-lactamases and still pose significant challenges for treating multi-drug resistant bacteria.[2] Of recent concern are the MBLs; these enzymes rely on a zinc ion chelate residue to coordinate the BL and deliver a water molecule to facilitate the hydrolysis of the drug.[8] Unlike serine carbapenemases, MBLs are not inhibited by irreversible binding to sacrificial BLs.[3] There is an urgent need for MBL inhibitors, which are now being labelled as an unattended global threat.[6] The impact of COVID-19 has overwhelmed hospitals

worldwide, providing cannon fodder for the issue at hand and creating an environment for the upsurge of antimicrobial resistance.[9-11]

The two main strategies that have emerged to combat resistance due to the bacterial production of novel lactamases are (1) the discovery of new BL antibiotics (more resistant to β-lactamase hydrolysis) and (2) the use of β-lactamase inhibitors. However, despite the success of strategy (2) in combating resistance to serine carbapenemases, none of the six FDA-approved inhibitors target the inhibition of MBLs.

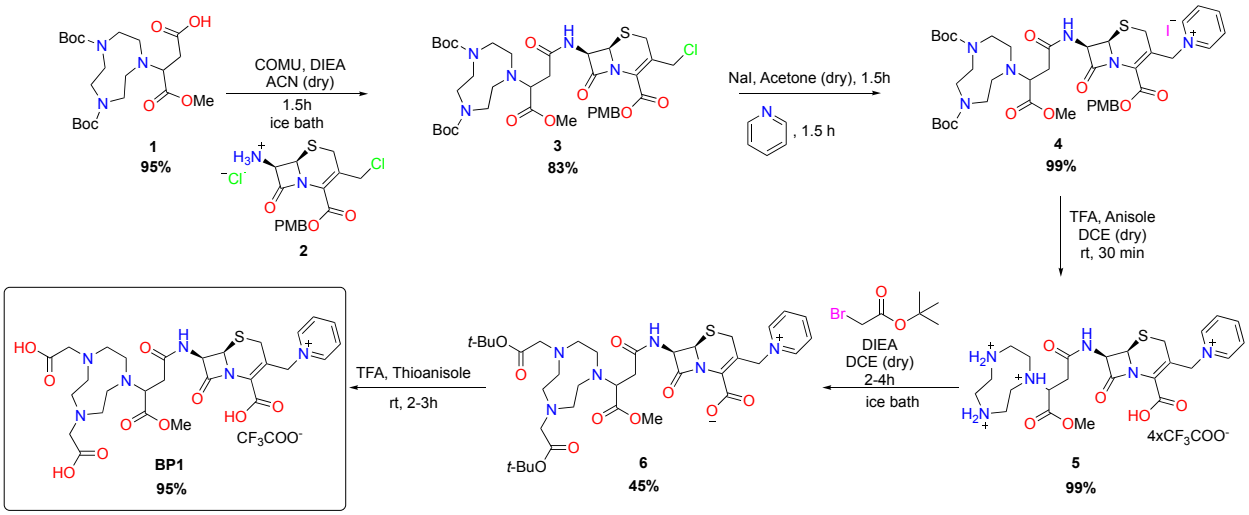
A promising method to target this mode of action is via the use of metal ion chelators that remove the essential zinc ions from the active site of the MBL enzyme.[5] Several scaffolds including N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN)[12] and analogues[13, 14] thereof, thiosemicarbazone[15, 16], 1,4,7,10-tetraazacyclododecane (DOTA) analogues[17, 18], 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA)[18], 2-picolinic acid, phosphonates[19] rhodanine, thioenolates[20], Magnalol[21], tris-picolylamines[22], *N*-acylhydrazone[23], 2,6-pyridine dicarboxylates (H2dpa's)[24], as well as 1,2,4-triazole-3-thione[25], have shown good activity against the New Delhi metallo β-lactamase (NDM-1) and the Verona integron-encoded MBL (VIM-2) expressing bacteria, partially restoring meropenem activity. In many cases, these chelators have been coupled with dipeptide vectors that mimic the bacterial cell wall sequence. However, these have also been shown to possess eukaryotic cytotoxicity.[19] Despite the ubiquity of metal chelators, the off-target activity often accompanies potent chelators leading to unwanted physiological effects.[26] In the context of β-lactamase inhibitors, compounds bearing cyclic boronates such as taniborbactam [27], and QPX7728 [28] display good activity to type B β-lactamases. These compounds are currently in phase III and phase I clinical trials, respectively. However, their mechanism of inhibition is still not completely understood.

An area gaining traction is the employment of BL conjugates or vectors as prodrugs and inhibitors, forming a relatively stable enzyme-hydrolyzed product complex to target the MBL.[21, 29, 30] In 2015, Harris *et al*.[31] and Lee *et al*.[32] emphasized the need for β-lactam-β-lactamase inhibitors for infections caused by extended-spectrum β-lactamase producers. To the best of our knowledge, this is the first report of such a β-lactamase inhibitor targeting the MBL. In this study, our novel approach was to covalently attach a well-binding metal/zinc chelator to a β-lactam scaffold (cephalosporin) to give both compounds an equal chance of reaching the target bacterial cell wall-producing enzymes, thereby providing a lethal duet of high specificity to eradicate the bacterial infection. We report the synthesis and preclinical development of an MBL inhibitor (BP1) with promising *in vitro* and *in vivo* efficacy.

2. **Materials and Methods**

2.1. Synthesis of BP1

4-Methoxy-1,4,7-triazacyclononane-butanoic acid analogue (**1**) was coupled with a commercially available cephalosporin, 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid p-methoxybenzyl ester hydrochloride (**2**) using the peptide coupling agent COMU in the presence of diisopropylethylamine to produce product (**3**) in 83% yield in its racemic form.



Scheme 1: Synthetic route of BP1

The leaving group at the 3-position of the cephalosporin was then subjected to a pyridination to yield (**4**), which was isolated in 99% yield using supercritical fluid chromatography followed by a Boc deprotection to furnish compound (**5**). Thereafter the amines on the cyclononane were alkylated **(6),** and the protecting groups were subsequently removed using trifluoroacetic acid (TFA) to produce the de-protected final product (**7** aka BP1) in 95% yield as an off-white solid. All reactions were monitored and optimized using liquid chromatography-mass spectrometry (LC-MS), and products were fully characterized using standard methods.

2.2. Bacterial Source

Well-characterized CRE strains producing MBLs or SBLs were acquired from the Institut National de la Santé et de la Recherche Médicale (U914), Paris, France [33]. While *Klebsiella pneumoniae* NDM was obtained from Hartford Hospital, USA [34]. *E. coli* ATCC 25922 was employed as a carbapenem-susceptible control. All bacterial stock solutions were preserved in Trypticase soy broth supplemented with 10% glycerol and contained 4 mm glass beads at -80 °C.

2.3. Antimicrobial agents

Meropenem was obtained from Sigma-Aldrich (Schnelldorf, Germany), and BP1 was synthesized in our labs *vide supra* (see supporting information for experimental details). Meropenem was prepared in distilled water (m/v), and BP1 was prepared in 50% (m/v) DMSO. The final DMSO concentration was < 1.0 %. Antimicrobial stock solutions were stored at -80 °C.

2.4. Antimicrobial susceptibility testing

The drug susceptibility profile of meropenem, in combination with BP1, was conducted using the checkerboard assay. The assay was performed according to a previously described protocol [35] and as per CLSI antimicrobial susceptibility guidelines [36]. Briefly, twofold dilutions of meropenem with BP1 were made in Mueller Hinton Broth (MHB). A 0.5 McFarland-standardized bacterial inoculum was added to each well, generating a final volume of 100 µL, and plates were incubated at 35 °C for 18-20 h. The checkerboard assays were performed in triplicate. The MIC was determined as the lowest concentration at which no visible growth was present. The fractional inhibitory concentration index (FICI) was calculated from the equation of FICI = FIC_a (MIC of drug A in combination/MIC of drug A alone) + FIC_b (MIC of drug B in combination/MIC of drug B alone) [37]. The FICI was categorized as follows: synergistic, FICI <0.5; additive, 0.5< FICI <1; indifferent, >0.5 FICI <4; and antagonistic, FICI > 4 [38].

2.5. Effects of human serum

To study the effects of human serum on the MIC values, the above antimicrobial susceptibility testing protocol was adopted, however, the broth was prepared differently. MHB was prepared according to the manufacturer's instructions (Oxoid Ltd, ThermoFisher Scientific, United

Kingdom). Thereafter equal volumes of broth and 100% human serum were utilized to generate a medium that contained 50% human serum.

2.6. Time-kill kinetic study

Time-kill studies were performed according to previously published methods [39], including those described by CLSI document M26-A [36]. In summary, an overnight culture of *K. pneumoniae* NDM was diluted to approximately 10^6 cfu/mL. The prepared bacterial suspensions were added to wells containing a fixed dose of 32 mg/L BP1 and meropenem in concentrations of 0.5, 1, or 2 mg/L. Plates were incubated at 35 °C and 100 rpm shaking. A bacterial control without the addition of drugs and a meropenem-only control were included under similar conditions. Viability counts were performed at 0, 2, 4, 6, 8, and 24 h by sampling 0.1 mL and spreading onto Mueller Hinton agar (MHA). These plates were incubated at 35 °C for at least 18 h, followed by the enumeration of colony-forming units per millilitre (cfu/mL).

2.7. Cytotoxicity assay

2.7.1. Cell culture

Human hepatocarcinoma (HepG2) cells were cultured in 25 mL cell culture flasks using Eagle's minimum essentials medium (EMEM) supplemented with 10% fetal bovine serum, 1% pen-strep-fungizone, and 1% L-glutamine, maintained in a humidified incubator (37 °C, 5% CO_2) until approximately 80% confluent.

2.7.2 Methyl Thiazol Tetrazolium (MTT) Assay

The MTT assay was one of the methods used to determine *in vitro* cell viability of BP1 on HepG2 cells. HepG2 cells (15,000 cells/well) were seeded into a 96-well microtiter plate and allowed to adhere overnight (37 °C, 5% CO_2). Thereafter, the cells were incubated (37 °C, 5% CO_2) with a range of BP1 concentrations (0, 1, 8, 10, 50, 100, and 200 µg/mL) in triplicate for 6 h. After the 6 h incubation, the cells were washed with 0.1 M phosphate buffered saline (PBS) and incubated with MTT salt solution (5 mg/mL in 0.1 M PBS) and 100 µL CCM for 4 h (37 °C, 5% CO_2). The MTT salt solution was removed, and DMSO (100 µL/well) was added and incubated for 1 h. The optical density was measured using a spectrophotometer (Bio-Tek µQuant) at 570/690 nm. Results are expressed as % cell viability versus BP1 concentration (µg/mL).

2.7.3. Lactate Dehydrogenase (LDH) Assay

The LDH assay was used to assess membrane damage of HepG2 cells. Supernatant collected from the control and BP1 treated cells were centrifuged (400 xg, 24 °C, 10 minutes) and dispensed (100 µL/well) in triplicate into a 96-well microtiter plate. LDH reagent (100 µL, 11644793001, Sigma Aldrich) was added to each well. The plate was incubated for 30 minutes at room temperature in the dark. Absorbance was read using a spectrophotometer (Bio-Tek µQuant,) at 500 nm. Results are represented as relative fold change compared to untreated control.

2.8. Inhibition assay

A dose-dependent enzyme inhibition assay was performed using a Biotek PowerWave XS2(Biotek Instruments, Inc, USA) plate reader. NDM-1 and VIM-2 enzymes were purchased from RayBiotech (RayBiotech Life Inc, USA). Enzymes in the quantity of 1 nM (NDM-1) or 4.5 nM (VIM-2) were used in this study with a fixed nitrocefin concentration of either 120 µM (NDM-1)

or 50 μM (VIM-2) and varying BP1 concentrations ranging from 10-500 μM (NDM-1) and 1-400 μM (VIM-2) in 50 mM HEPES buffer supplemented with 100 μg/mL BSA and 10 μM $ZnCl_2$. Inhibition was measured at 482 nm at 25 °C. All assays were conducted in triplicate.

2.9. Binding specificity

To determine the binding specificity of the inhibitors to other zinc containing enzymes, glyoxylase (BioVision Inc, USA) was utilized to measure the level of specificity exhibited by BP1. The methodology has been previously described [22], with minor modifications in utilizing a temperature of 37 °C, measuring the absorbance at 520 nm, and the inclusion of positive controls; EDTA and TPEN (purchased from Merck KGaA, Germany).

2.10. Ethical statement

All animal experiments carried out in this study were approved by the institutional Animal Research Ethics Committee at the University of KwaZulu-Natal, with approval reference AREC/013/016D (for the pharmacokinetic study) and AREC/00002618/2021 (for the *in vivo* efficacy study). All sample sizes used in this study were estimated using G*Power Version 3.1.9.4.

2.11. *In vivo* single-dose pharmacokinetics

Female Bragg inbred albino c-strain (BALB/c) (6-8 weeks old) were purchased from the Biomedical Resource Unit (BRU). The animals were collected two weeks prior to the experimental procedures to allow for acclimatization. The animals were housed in well-ventilated cages, located in temperature and humidity-controlled housing units, with a 12-hour/ 12-hour light-dark cycle. Environmental enrichment, in the form of shredded paper and plastic tunnels, were added to the

cage. The animals were provided with commercially pelleted feed and clean water *ad libtium*. Experimental animals received a combination of meropenem (10 mg/kg.b.w) and BP1 (10 mg/kg.b.w) via intraperitoneal (IP) administration. Animals were then sacrificed periodically at 0, 5, 15, 30, 45-, 60-, 90-, and 120-minutes post-dosing (n=3) to determine the pharmacokinetics of meropenem and BP1, respectively. Blood was collected via cardiac puncture and stored at -80 °C until analysis.

2.12. *In vivo* efficacy

A murine thigh infection model described by Michail *et al.*[40] was established with minor modifications. Male BALB/c mice weighing 18-22 g (n=90) were used, and these animals were randomly assigned to treatment groups using simple randomisation. Each of the three groups constituted of n=30 mice, with n=6 mice receiving treatment every two hours. Prior to infection, mice were treated IP with 150 mg/kg cyclophosphamide on day one and 100 mg/kg on day four of the trial. This was done to induce neutropenia in the mice; neutropenia was confirmed by a neutrophil count of $<100/mm^3$. On day five of the trial, animals received a 0.1 mL inoculum containing 10^6-10^8 cfu/mL of *K. pneumoniae* NDM intramuscularly (i.m) into the right thigh of the mice. Meropenem monotherapy (M), BP1 + meropenem (BP1) combination therapy, or normal saline (S) was administered every two hours over an eight-hour treatment period. Mice were euthanized, by isoflurane overdose, at 2, 4, 6, and 8 h post-dosing. Our initial preclinical PK study showed that the drug concentration was sufficiently reduced after a 2hr period to allow for subsequent dosing without the risk of cytotoxicity. The right thigh muscle was then aseptically removed and homogenized in 5 mL of PBS. Homogenates were spread onto Mueller-Hinton agar and MacConkey agar plates, followed by incubation at 35 °C for 24 h and enumeration of the

cfu/thigh. (Since BP1 did not have activity towards MBL resistance on its own, we did not study the standalone effect of BP1 *in vivo* as it is not ethical to study the effect of a drug alone when it did not provide acceptable MICs. Our animal studies were done in accordance with ARRIVE guidelines and aimed to reduce the animal numbers utilized in this study.)

2.13. LC-MS/MS Quantification

A Shimadzu Nexera Series (Shimadzu Corporation, Kyoto, Japan) liquid chromatography system coupled with Shimadzu LCMS-8050 tandem mass spectrometer (Shimadzu, Kyoto, Japan) was used.

Chromatographic separation was achieved using a Shim-Pack Velox SP-C18 column (100 mm × 2.1 mm, 2.7 µm particle size) with a gradient mobile phase comprised of Millipore water (0.1% v/v TFA) (A) and acetonitrile (0.1% v/v TFA) (B). The gradient method started from 5.0 to 95.0% B in 3 minutes, then held at 50 % up to 7 minutes thereafter, it was brought back to 5 % at 7.1 minutes. The column equilibration time was 2.9 minutes with a flow rate of 0.4 mL/min and the column oven temperature was 40 ºC. The injection volume was 15 µL and the total run time of the method was 10 minutes. Quantitative and qualitative studies were conducted by using MRM mode *via* an ESI interface, with the following source parameters: nitrogen nebulizer gas flow of 3 L/min; heat gas 10 L/min and interface temperature of 300 °C. The precursor and product ions optimized were *m/z* 325.2→80.15 for BP1, *m/z* 384.50→68.25 for Meropenem and *m/z* 350.50→304.40 for ampicillin (IS). Results were analyzed using LabSolutions Insight LCMS. All data are expressed as a mean ± SD.

2.14. Computational methods

These methods have been detailed in the Appendices section of this thesis.

2.15. Statistical analyses

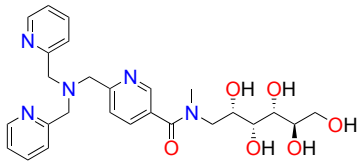
All analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Inc., San Diego, CA, USA). Linear and non-linear regression models were used to determine parameters from the enzyme inhibition and fluorescence quenching assays. The one- and two-way analysis of variance (ANOVA) was used to determine the bactericidal effect expressed in the time-kill and *in vivo* efficacy studies. In the time-kill assay, each experimental BP1 and meropenem dose, was assessed against meropenem monotherapy and the bacterial control. Whilst for the *in vivo* efficacy study, the three treatment groups; S (saline only), M (meropenem only), and BP1 combination therapy (BP1 and meropenem), were compared. Statistical significance was represented by a decrease in the p value ($p< 0.05$) and an increase in the F ratio.

3. Results and Discussion

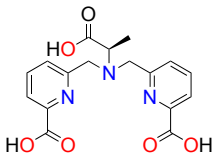
Our research group has previously demonstrated that metal chelating agents like NOTA, DOTA and DPA are potent MBL inhibitors, acting on MBL producing CREs.[18, 41] These compounds carried non detectable toxic effects at effective concentrations *in vitro*.[41, 42] This prompted us to further investigate the cyclic zinc chelator's capacity to inhibit MBLs when attached to a β-lactam moiety to improve the molecule's overall pharmacological properties. BP1[43] was synthesized in five steps through a procedure adapted from Dutta *et al.*[44].

A desirable β-lactamase inhibitor should simultaneously neutralize MBLs while preserving the activity of existing carbapenem antibiotics, thereby targeting antimicrobial resistance, and restoring the efficacy of BLs. BP1 demonstrated the potential to achieve this, as observed in the

in vitro experiments. BP1 restores the efficacy of meropenem as evidenced by the > 8-fold reduction in the MICs of the seven MBL expressing bacteria (from different geographies) used for this study (Table 1). These results suggest that meropenem can be administered at therapeutically acceptable concentrations defined herein as a meropenem MIC of < 2 mg/L coupled with a BP1 MIC of < 64 mg/L. More importantly, our MIC breakpoints concur with the CLSI guidelines, as well as the more stringent EUCAST recommendations. The pairing of BP1 with meropenem in combination therapy has the potential to be a favourable solution since both antimicrobials work synergistically to target the MBL and the pathogen (Table 1). Martin and co-workers [30] also attached a chelator to a cephalosporin as a potential MBLI, however, their BL acts as a prodrug and upon hydrolysis releases a zinc binding thiol moiety to the enzyme active site. Their MIC data indicates that relatively higher concentrations (> 32 mg/L) of their most potent compounds are required to re-sensitize the clinical isolates to meropenem, with the compounds having more activity towards bacteria expressing imipenemase (IMP) MBLs in comparison to VIM and NDM. [29, 30] It should be noted that BP1 is active against IMP, NDM and VIM MBLs at lower concentrations. When comparing our results to other recent chelators as potential MBLIs that are also based on nitrogen-bearing ligands, we came across the noteworthy contributions from Rongved *et al*. [22] where the MIC of meropenem reaches concentrations < 2 mg/L with 50 µM of their most potent compound i.e. ZN148 based on a tris-picolylamine ligand.



ZN148



H_3dpaa

Scheme 2: Examples of recent nitrogen-bearing chelators as promising MBLIs [22, 24]

BP1 utilizes a lower MBLI concentration (32 mg/L or 27 μM) whilst resulting in more efficacious meropenem MICs of < 0.5 mg/L. The most recent studies from another promising MBLI class of pentadentate-chelating N-O ligands i.e. H_2dpa derivatives [24] report similar activity as BP1. Although BP1 displays a broader spectrum of inhibition compared to the H_2dpa derivatives, both compounds share a similar synergistic effect with meropenem according to the FICI values of < 0.13.

Table 1: Antimicrobial susceptibility testing of BP1 and Meropenem across seven MBL harboring bacteria utilizing the broth microdilution assay

Bacterial reference	Bacterial strain	MBL produced	MIC (mg/L)			FICI
			MEM alone	BP 1 alone	BP 1 + MEM	
ATCC 25922	*E. coli*	N/A	0.06	N/A	0 + 0.06	N/A
AUS 271	*E. coli*	NDM-1	> 128	> 256	16 + 0.25	0.13
FEK	*E. coli*	NDM-4	128	> 256	16 + 0.5	0.07
JAP	*E. coli*	IMP-1	8	> 256	8 + 0.03	0.04
TWA	*E. coli*	IMP-8	4	> 256	8 + 0.25	0.09
IR 386	*E. cloacae*	NDM-1	32	> 256	16 + 0.125	0.07
KAR	*E. cloacae*	VIM-1	16	> 256	32 + 0.5	0.16
USA 449	*K. pneumoniae*	NDM	> 128	> 256	16 + 0.5	0.07

MEM=Meropenem
N/A = Not applicable
Synergy, FICI $\leq$ 0.5; additive 0.5-1; indifference, >0.5 FICI < 4; and antagonism, FICI $\geq$ 4
All assays were conducted in triplicate
Compound **(2)** i.e. the cephalosporin component of BP1 displayed no activity on its own or in combination with mereopenem
NOTA pre-complexed to zinc displayed no activity on its own or in combination with mereopenem
BP1 + Meropenem displayed no activity towards non MBL producing strains *S. marcescens* KPC-2 and *E. coli* 0XA-28

Serum had no considerable effect on the MIC of BP1+ meropenem combinations for the seven bacteria studied. To study the cytotoxicity of BP1, an extracellular LDH assay was undertaken. LDH leakage is a marker of cellular membrane damage. Employing HepG2 cell lines, LDH was significantly reduced (***$p<0.0001$) across the various evaluated BP1 concentrations (Figure 1).

Therefore, BP1 did not induce cell necrosis after 24 h exposure and was deemed safe to administer with a low potential for toxicity.

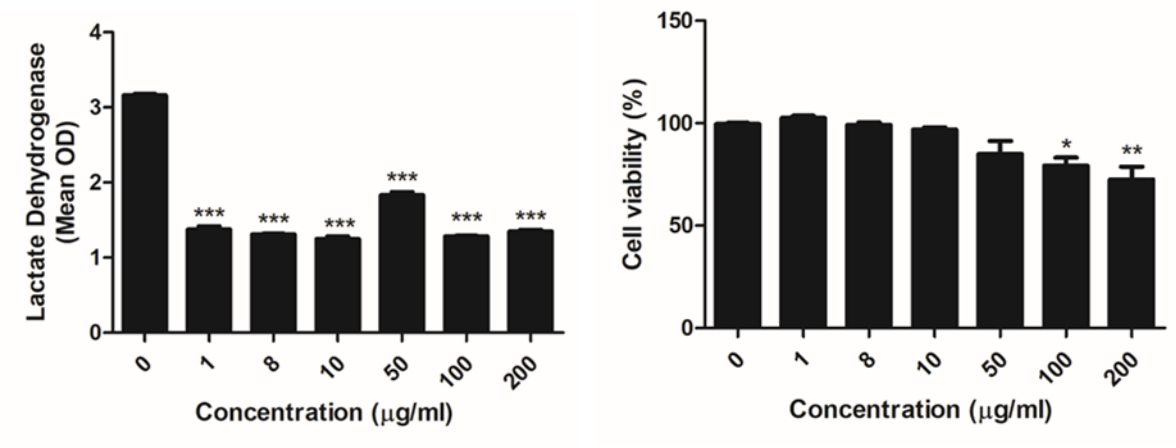
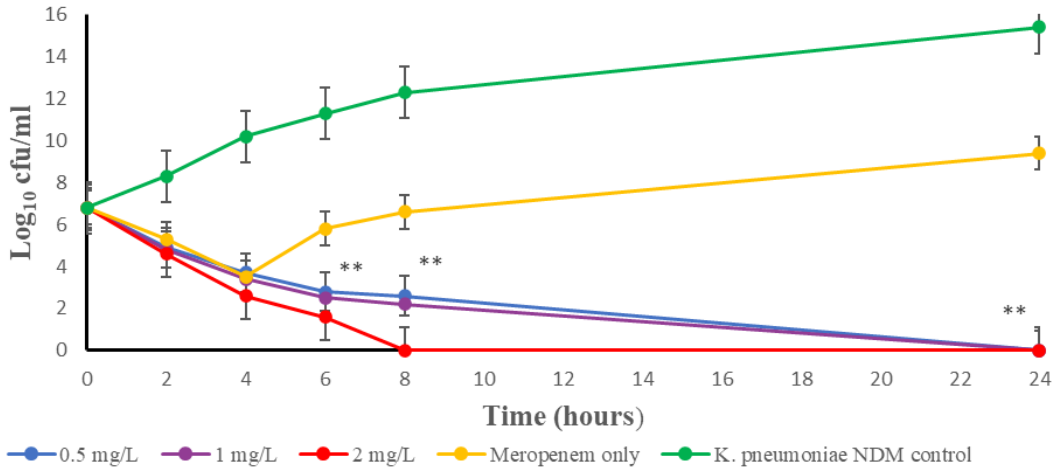


Figure 1: Cell viability studies conducted on HepG2 cells using varying concentrations of BP1. LDH was significantly reduced (***$p<0.0001$) at all BP1 treatment relative to the control. A dose-dependent decrease in cell viability was observed with a significant decrease occurring at 100 ($p<0.5$) and 200 ($p<0.01$) µg/mL relative to control. The IC_{50} generated by these experiments over a 24-hour exposure period was 1,818 mg/L. The concentration of BP1 tested in downstream experiments was < 50 mg/L, indicating that BP1 is non-toxic.

Time-kill kinetic studies (Figure 2) indicate that using 32 mg/L of BP1 in combination with varying meropenem doses of either 0.5, 1, or 2 mg/L, resulted in complete bactericidal activity over 24 h. The concentration of 32 mg/L of BP1 was determined by studying the minimum bactericidal concentration (MBC) of BP1 in combination with meropenem, which correlated to 2 x the MIC. Our findings show a similar time-kill response as the H_2dpa derivatives [24] however, their study employed a 2-fold dilution decrease in meropenem and inhibitor concentrations. Furthermore, BP1 achieved complete killing, whereas the H_2dpa derivatives reduced the bacterial load to 10 cfu/mL.



A fixed BP1 concentration of 32 mg/L was utilized

0.5, 1, 2 mg/L = Meropenem concentrations

** denotes $p < 0.001$

All assays were conducted in triplicate

Figure 2: Time-kill kinetic study of BP1 with Meropenem at varying concentrations. *K. pneumoniae* NDM control represents the bacterial growth curve unhindered by the addition of BP1 and meropenem. The meropenem only test group highlights the ineffectiveness of meropenem when administered without BP1. Sterilizing activity was achieved with BP1 at all concentrations of meropenem used by 24 h.

Enzyme inhibition studies were undertaken to establish the characteristics of pertinent enzyme parameters. Inhibition data were fitted to sigmoidal curves (Figure S11), the IC_{50} was determined using the ATT Bioquest tool [45] and the Ki was determined using the Cheng-Prusoff equation. Based on the data generated in Table 2, it is evident that BP1 displayed more potent interactions towards inhibiting VIM-2 (Ki= 24.8 µM) as compared to NDM-1 (Ki = 97.4 µM).

Table 2: Enzyme Inhibition exhibited by BP1

MBL	IC_{50} (µM)	Ki (µM)
NDM-1	253.3 ± 0.02	97.4
VIM-2	45.8 ± 0.004	24.8

All assays were conducted in triplicate

IC_{50} represents the mean ± SD

Ki was determined using the Cheng-Prusoff equation

Fluorescence quenching is a favourable approach to assess the interaction of a protein (MBL) [46] with a quenching molecule (BP1). NDM-1 and VIM-2 display intrinsic fluorescence due to the presence of fluorophore molecules in their protein structure, namely tryptophan. [47] In this study it is incorporated to measure the binding affinity of BP1 and MBLs; NDM-1 and VIM-2.

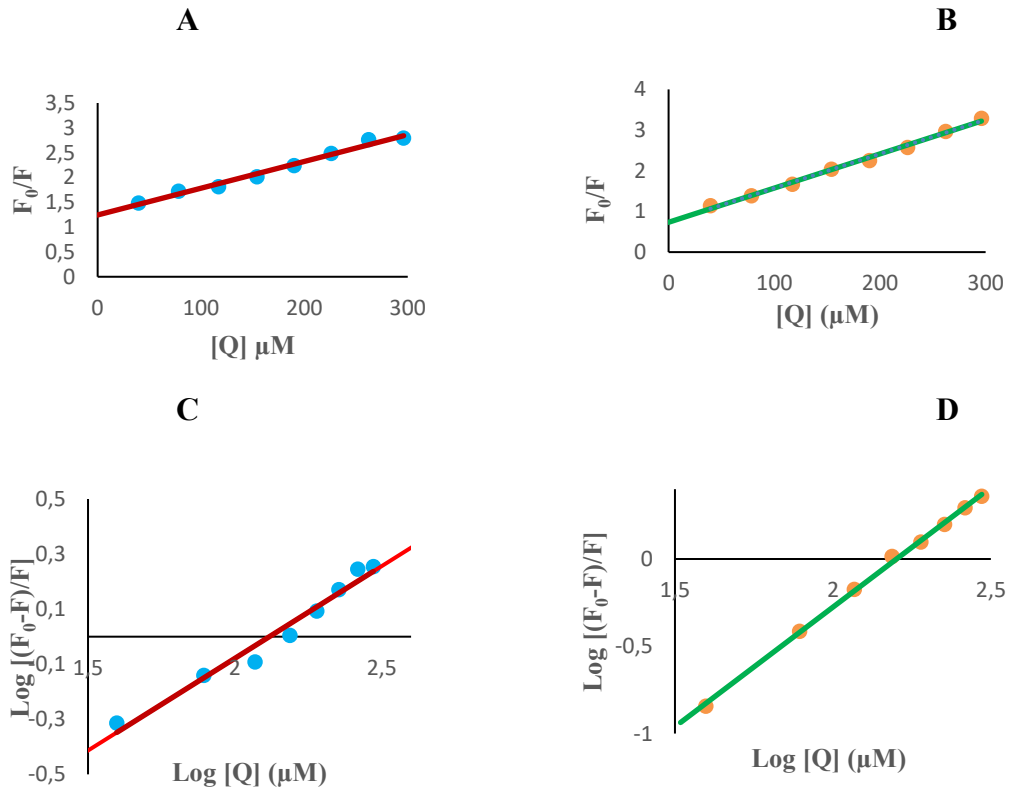


Figure 3: Stern-Volmer plots of BP 1 interacting with VIM-2 and NDM-1

BP1 was able to quench the tryptophan residues of 1nM VIM-2 (A) and 1nM NDM-1 (B). Modified Stern-Volmer plots of BP1 interacting with 1 nM VIM-2 (C) and NDM-1 (D) were then generated to determine quenching parameters. All assays were conducted in triplicate

Graphical observations (Figure 3; A & B) indicate that BP1 successfully interacts with the tryptophan residues in VIM-2, that results in the decrease in fluorescence intensity and hence the increase in quenching. This is primarily noted by the increasing concentration of BP1 that is directly proportional to the degree of quenching. This phenomenon is based on many factors however, the location of the tryptophan (trp) residue plays an important role, if the residue is present near the surface of the protein, then the emission intensity will decrease in the presence of a quencher[48], evident from the BP1 – VIM-2 interaction. With regards to NDM-1, Trp93, is 5.7 Å from the Zn (II) binding site (relatively close)[49] and therefore promotes a higher quenching rate (Figure 3;C & D) . However, if the tryptophan residues are located deeper in the protein structure, quenching will not have much effect on the emitted intensity. [48] This is evident as BP1 exhibits more affinity towards NDM-1 than VIM-2. The decrease in fluorescence results in a blue shift to lower wavelengths and is due to the increased hydrophobicity around the tryptophan sites. [50] This further substantiates the promise BP1 holds for further enzymatic evaluation.

Table 3: Summary of enzyme-inhibitor parameters obtained from the kinetics of inhibition and fluorescence quenching experiments

Enzyme - Inhibitor Parameters	NDM-1	VIM-2
Ksv [Q] (µM-1)	0.0083 ± 0.0001	0.0059 ±0.0005
Ka (µM)	2.813 ± 0.23	2.1147 ± 0.628
Kd (M)	0.357 ± 0.030	0.473 ± 0.174
n	1.275	0.964
R^2	0.998	0.997

Observations from Table 3 indicate the result of stronger binding affinity for BP1 to NDM-1 compared to VIM-2. This was noted by a higher Ka of 2.8 (NDM-1) in comparison to 2.1 (VIM-

2). Additionally, NDM-1 occupied more binding sites than VIM-2, which is dependent on the protein structure and the total number of binding sites available.

MBLs have been well known to possess active sites similar to the binding sites for mammalian enzymes, typically used for essential cellular functions. The human glyoxalase II (Glo2) enzyme shares a closely related protein fold and key zinc binding residues of MBLs.[51] The inhibitory activity of BP1 against the Glo2 (Figure 4) indicates that BP1 does not bind to zinc in this enzyme and is, by extension, less likely in other mammalian metallo proteins. This suggested that BP1 remained specific to binding to zinc within the MBLs at 50-500 µM, while zinc chelators; EDTA, and TPEN reduced the activity of the glyoxylase II by 39 and 48 %, respectively (Table S1).

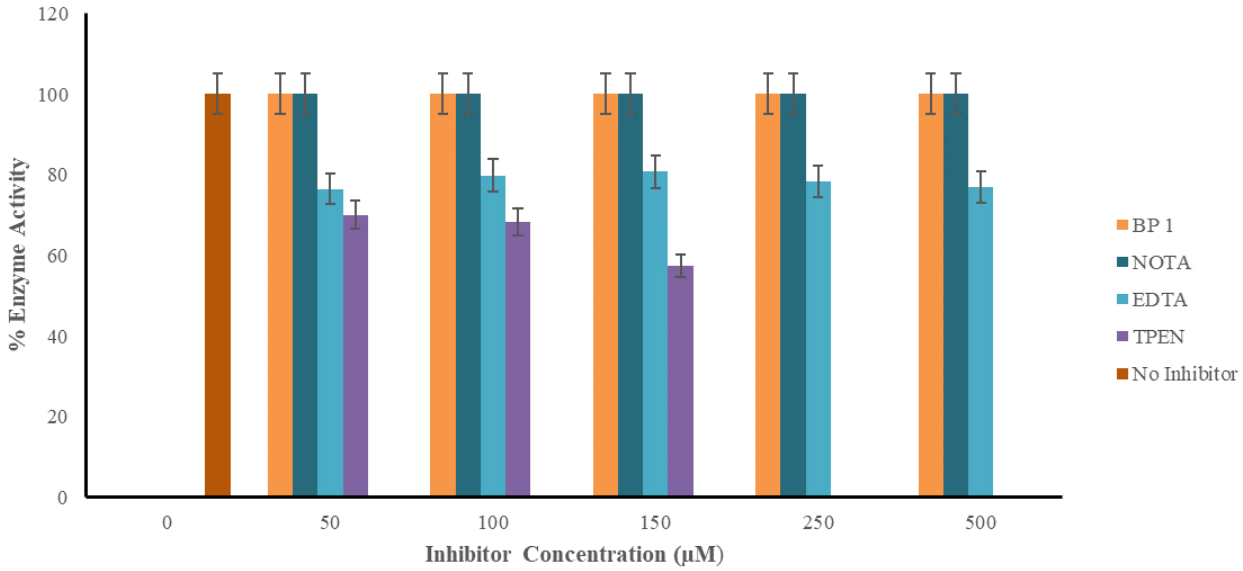


Figure 4: Activity of human recombinant glyoxylase II in the presence of four inhibitors. All assays were conducted in triplicate. Normal glyoxylase II activity is denoted by the legend representing no inhibitor added. BP1 did not decrease the activity of glyoxylase II in comparison to metal chelating agents EDTA and TPEN. NOTA a component of BP1, also did not reduce the activity of glyoxylase II

Next, we performed molecular docking of BP1 with NDM-1 and VIM-2, followed by a molecular dynamic simulation. The docked poses in NDM-1 and VIM-2 show that the chelator region of BP1 interacts with the Zn^{2+} ion via the carboxylic moiety (Figure 5). The NDM-1—BP1 had a docking score of -6.5 kcal/mol, while VIM-2—BP1 had -6.1 kcal/mol. It is possible that BP1 could ultimately remove the zinc ions from the active site, immobilizing the enzyme completely. This is supported by the absence of a MIC value when NOTA pre-complexed to zinc was evaluated as a potential MBL inhibitor (Table 1), confirming that BP1's zinc chelation is required for inhibition. Subsequent studies will use PACs-MD[52] to determine if NOTA chelation of the zinc ion is energetically feasible.

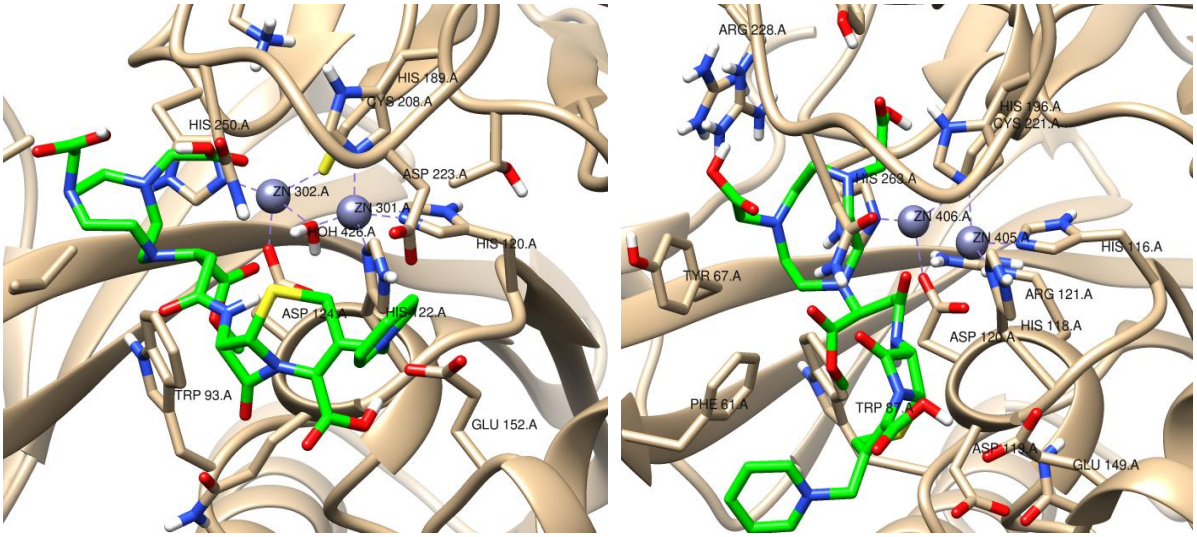


Figure 5: 3D structures of NDM1—BP1 and VIM2—BP1 complexes, respectively. BP1 compounds are shown in green. In NDM1, the Zn301 (grey sphere) is coordinated to three histidines, and a water molecule (HOH426) coordinates the two zinc ions, while Zn302 is coordinated to histidine, aspartate, and cysteine residues. In VIM2, the Zn405 is coordinated to three histidines, and Zn06 is coordinated to one histidine, one aspartate, and one cysteine residue.

To further elucidate the potential of BP1, *in vivo* studies were conducted to investigate if the *in vitro* effect can be successfully replicated in an animal model. Prior to the *in vivo* efficacy study, we conducted a single dose pharmacokinetic experiment to determine the dose of BP1 required to reach safe therapeutic concentrations in plasma and, thus, an appropriate dosing schedule. We found that BP1 and meropenem shared a similar PK profile reaching peak concentrations (C_{max}) at 15 minutes post drug administration before reaching trough concentrations at two hours (Figure S12). At a dose of 10 mg/kg.b.w, we achieved a plasma concentration of 1.93 µg/L, which was well below the cytotoxicity concentrations seen in the cell viability assays, advocating for the safe increase of the treatment dose to 100 mg/kg.b.w, which allowed us to reach therapeutic concentrations in plasma without the possibility of toxicity (Figure 6).

A murine thigh infection model was investigated to determine if combination therapy could reduce the bacterial burden. Prior to infection, animals were rendered neutropenic via the administration of cyclophosphamide, as discussed by Asempa *et al.*[53] This allows for the evaluation of the efficacy of the proposed therapy removing the confounding effect of the innate immune response (Appendix one), also suggesting that a larger dose was required to achieve a similar change in bacterial burden, and the inhibition of the immune system allowed for the establishment of an infection.[53] The mice were successfully infected with *Klebsiella pneumoniae* NDM, as inflammation of the localized area was visible (Figure S10); this correlated with the cfu/thigh data expressed (Table S2-S4). The mice were randomly grouped into three treatment regimens of S (saline only), M (meropenem only), and BP1 combination therapy (BP1 + meropenem). Since renal DHP-1 hydrolyzes meropenem at a rapid rate[54], dosing intervals with meropenem and BP1 were set two hours apart, to account for their short half-life (as determined by single-dose pharmacokinetic studies) and promote the *in vivo* efficacy trial (Figure 6). The mice received four

doses of both drugs (100 mg/kg each) over eight hours, with no observations of toxicity (total drug dose 800 mg/kg).

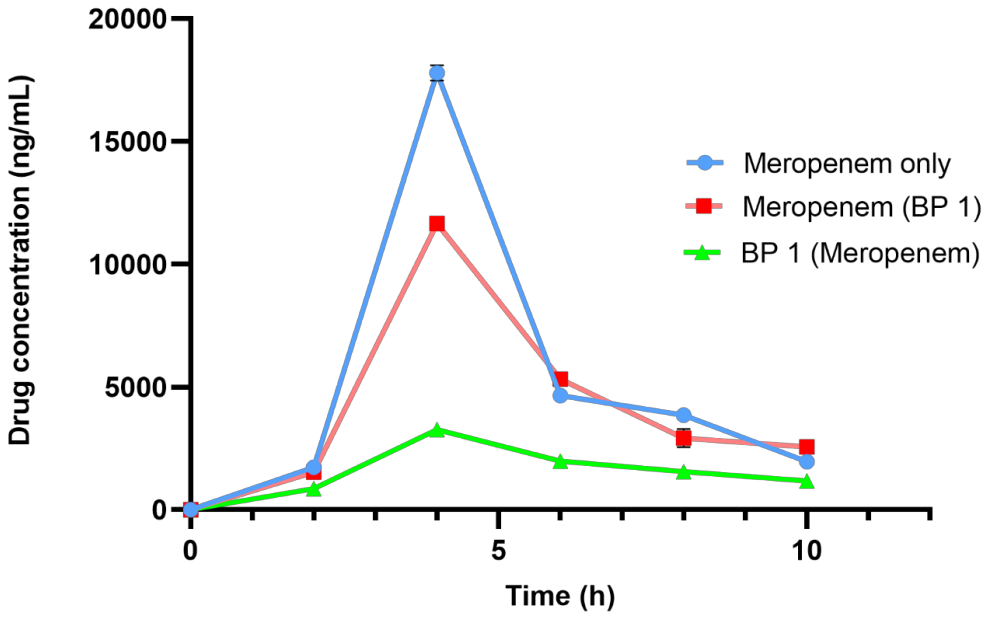
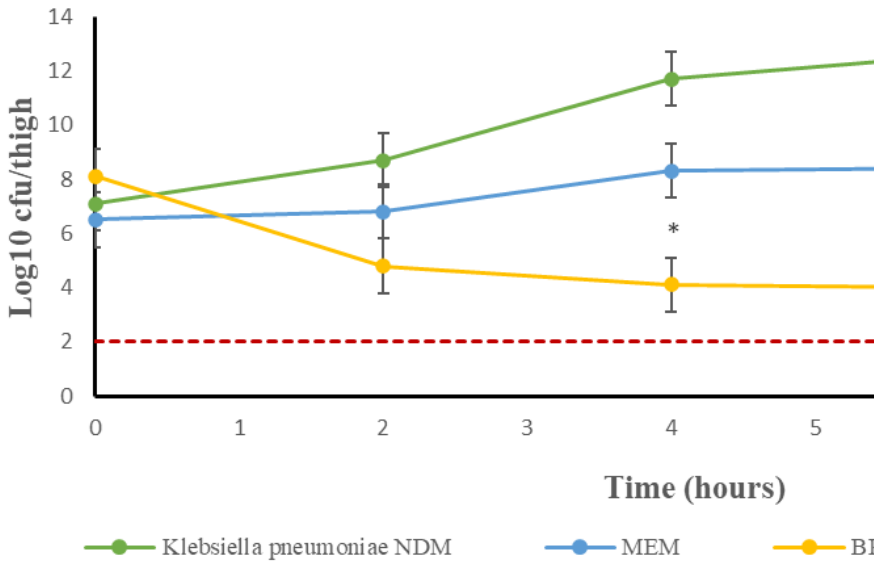


Figure 6: Plasma BP1 and meropenem concentrations over the eight-hour treatment period. BALB/c mice were infected i.m with *K. pneumoniae* NDM. Treatment was initiated 2 hours post-infection with either S, M, or BP1. Four treatment doses were administered via IP. Data are presented as a mean ± SD (n=6). Meropenem only - animals treated with meropenem only, Meropenem (BP 1) - meropenem concentration when animals were treated with the combination of BP 1 (Meropenem) - BP 1 concentration when animals were treated with the combination.

The outcome was successful (Figure S13), as shown by the significant reduction of > 3 $\log_{10}$ units in the cfu/thigh count (Figure 7). These results are highly significant (Table S2-S4), since the deviation amongst the data is < 10 % while maintaining the effectiveness of combination therapy ($p < 0.0018$), as highlighted in Table 4. Upon closer inspection of Figure 7, one can note that the mice belonging to the combination therapy regimen were infected with a higher initial

bacterial density than the S and M only treatment group. This could account for the reduction in the bacterial density not reaching a $\log_{10}$ cfu/mL count below 3.9. From our data we can extrapolate that the bactericidal effect would continue up until 24hr post-dosing however in terms of animal welfare, this is not ethically appropriate since the control animals will be at risk of severe inflammation and/or death. It is also impractical to repeatedly dose an experimental animal at 2hr intervals throughout a 24hr period. Nevertheless, the *in vivo* activity of BP1 is concordant with the *in vivo* efficacy of ZN148, where a decrease in the bacterial load is observed to a count of approximately 3 $\log_{10}$ units.[22]



MEM = Meropenem, LoD = Limit of Detection

n = 6 mice per time point per group, cfu/mL counts were done in triplicate

* Denotes $p<0.005$

Figure 7: The efficacy of BP1 combination therapy over monotherapy in a murine thigh infection model. The co-administration of BP1 and meropenem resulted in a significant decrease of *K.*

pneumoniae NDM cfu/mL, in comparison to S and M treatment. This clearly indicates that BP1 + meropenem is a favourable treatment strategy.

Table 4: Evaluation of statistical parameters measuring the bactericidal activity of BP1

Parameter	*Time-kill assay			*In vivo* efficacy study
	BP 1 + 0.5 mg/L MEM	BP 1 + 1 mg/L MEM	BP 1 + 2 mg/L MEM	BP 1 + MEM (100 mg/kg)
R^2	0.6367	0.6451	0.6617	0.6509
Mean squares	80.32	84.06	99.18	40.36
F ratio	13.14	13.63	14.67	11.19
P value	0.0005	0.0004	0.0003	0.0018

*BP 1 was administered as a fixed dose of 32 mg/L
MEM = Meropenem
All assays were done in triplicate

4. Conclusion

In summary, we have developed the first β-lactam-β-lactamase inhibitor that can restore the MIC of meropenem in MBL expressing carbapenem-resistant *Enterobacterales.* A preliminary evaluation found BP1 safe to administer at the utilized doses, with no signs of toxicity at reaching therapeutic concentrations. The co-administration of BP1 with meropenem; is highly efficacious in reducing the bacterial load to susceptible therapeutic concentrations, in which the bactericidal activity is evident, both *in vitro* and *in vivo*. Furthermore, the presence of human serum did not have a significant effect on the efficacy of this combination. Enzyme inhibition assays also demonstrated the potency of BP1, and computational studies support the hypothesis

that BP1 inhibits the MBL by binding to the Zn^{2+} ions. Therefore, our findings nominate BP1 as a promising MBL inhibitor with potential for further clinical development.

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Transparency declarations

BKP, HGK, PIA, TN, and TG have a patent PCT number PCT/IB2022/056748 on the technology. All other authors: none to declare.

Supplementary data

Further experimental details and spectra are available as supplementary data.

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Chapter five: Manuscript four

Neutralizing carbapenem resistance by co-administering meropenem with novel β-lactam-metallo-β-lactamase inhibitors

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Abstract

The rise of Carbapenem-Resistant *Enterobacterales* over the years has led to the global increase in morbidity and mortality rates that are primarily facilitated by the acquisition of serine and metallo β-lactamases genes. As a result, virulent *Enterobacterales* strains that have emerged are responsible for hard-to-treat infectious diseases. Whilst many serine β-lactamase inhibitors are in therapeutic use, an urgent global need for clinical metallo β-lactamase inhibitors have become dire. The utilization of metal chelators presents an important targeting strategy against metallo β-lactamases. This study evaluated the drug performance profile of three novel β-lactam derived

cyclic chelators (BP6, 10 and 14) co-administered with meropenem. According to the drug susceptibility results, the BP compounds potentiates the synergistic activity of meropenem to an MIC range of 0.03-1 mg/L and compounds are bactericidal over 24h. In addition, these metal chelating compounds are safe to administer at the selected concentrations. Further evaluation of BP10 showed synergism in restoring the meropenem MIC against 21 *Enterobacterale* isolates. Enzyme inhibition kinetics proved BP10 to be potent with a Ki of 30.9 μM and 26.2 μM against NDM-1 and VIM-2, respectively. Fluorescence quenching and molecular docking experiments indicated binding of BP10 to the MBLs, and it did not bind the glyoxylase II enzyme up to 500 μM. In a murine infection model, BP10 co-administered with meropenem was efficacious, observed by the > 3 log10 reduction of *K. pneumoniae* NDM cfu/thigh. Given the promising pre-clinical results, BP10 is a suitable candidate for further research and clinical development as an MBLI.

1. Introduction

The emergence of the global COVID-19 pandemic has exacerbated the antibiotic crisis. Many COVID-19 patients fell victim to secondary *Enterobacterale* infections, in which the symptoms could not be differentiated from COVID-19, resulting in the demise of an already crippled healthcare system [1]. The number of circulating Carbapenem-Resistant *Enterobacterales* (CRE) strains has thus tremendously increased in recent years [2, 3]. Since β-lactams are the most widely used class of antibiotics [4, 5], this poses a massive challenge to clinicians, with carbapenems being considered the "last line of defense in the treatment of infectious disease"[6]. CREs producing β-lactamases are classified as either serine β-Lactamases (SBLs) or metallo β-lactamases (MBLs) [7, 8]. Currently, the most difficult to treat infections emanate from CREs producing metallo β-lactamase enzymes [4, 9]. These enzymes hydrolyze the β-lactam ring of the

carbapenem antibiotic via the utilization of zinc ions at the active site, which creates a nucleophilic molecule, thereby rendering the β-lactam antibiotic ineffective [10, 11].

The most prevalent metallo β-lactamases globally are variants of New-Delhi metallo β-lactamase (NDM), Verona Integron-encoded metallo β-lactamase (VIM), and Imipenemase (IMP) [12-15]. There has been successful progress in developing many FDA-approved serine β-lactamase inhibitors (SBLIs) for commercial use [16-20]. However, progress towards an efficacious metallo β-lactamase inhibitor (MBLI) has been stagnant; although research is ongoing [21], there has not yet been an MBLI approved for commercial use [22]. Potential MBLIs to enter clinical trials should be novel, possess a good antimicrobial susceptibility profile, be safe and non-toxic to cells, and show activity over a considerable amount of time [23, 24].

Metal chelators have demonstrated activity against metallo-β-lactamases by sequestration of Zn^{2+} ions in the enzyme's active site, thus rendering the lactamases inactive against the β-lactam. The CRE's are re-sensitized through this process to the β-lactam antibiotic drug [25]. The use of metal chelators as a mechanism of combating metallo β-lactamases dates to the early twenty-first century when Docquier *et al* [26] found that the MBL VIM-2 is inhibited by metal chelators; EDTA, *o*-phenanthroline and dipicolinic acid (DPA), due to the arrangement of loosely bound Zn^{2+} ions present in its active site. Then in 2014, King *et al* [27] discovered a fungal-derived natural product, Aspergillomarasmine A (AMA), that functioned as a metal chelator and effectively inhibited NDM-1 and VIM-2 MBLs and thus restored the activity of meropenem. Although promising, recent advances in synthesizing derivatives of AMA could not surpass the biological potency of AMA [28]. There have been several other reports that indicate metal chelators could be rapid and potent MBLIs of the NDM, VIM, and IMP variants. These include some noteworthy contributions such as ZN148 [29], H_2dpa derivatives [30], ANT2681 [31], 1,2,4-

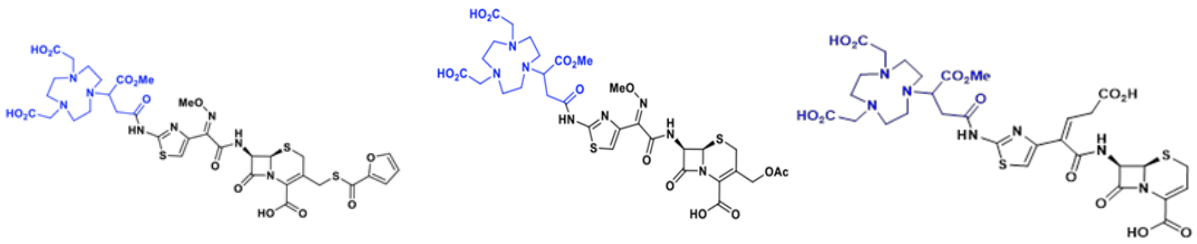
triazole-3-thione compounds with a 4-ethyl alkyl/aryl sulfide substituent [32], 2-quinazolinone derivatives [33] and *N*-sulfamoylpyrrole-2-carboxylates [34].

In some of these cases, the chelators have been tacked with dipeptide vectors that mimic the bacteria cells wall sequence. However, these have also been shown to possess eukaryotic cell toxicity [35]. The other challenge with potent chelators that would be expected is the grave off-target effects within the biological environment, thus limiting the therapeutic use of a simple zinc binder [1]. Our group recently investigated the antibacterial effects of a cyclic zinc chelator conjugated to a β-lactam antibiotic scaffold (Chapter 4 *manuscript under review*). Based on its excellent results, herein we expand the scope of the most potent derivatives to BP 6, BP 10, and BP 14, which can also be used as potential MBLI's to restore the efficacy of carbapenems against metallo-β-lactamases.

2. Methods

2.1.Synthesis of MBLIs

The metal chelators/inhibitors were synthesized and characterized by the Catalysis and Peptide Research Unit of the University of Kwa-Zulu Natal, Westville, Durban, South Africa. Our previous studies indicated that the chelators alone exhibit very good β-lactamase inhibition; however, poor pharmacokinetics were observed [36, 37] (Chapter three *Accepted*); hence we envisaged the need to explore conjugation of the chelator to enhance its properties. This was achieved by attaching a chelator to known β-lactam antibiotic cores **(PCT Filing-PCT/IB2022/056748)**.

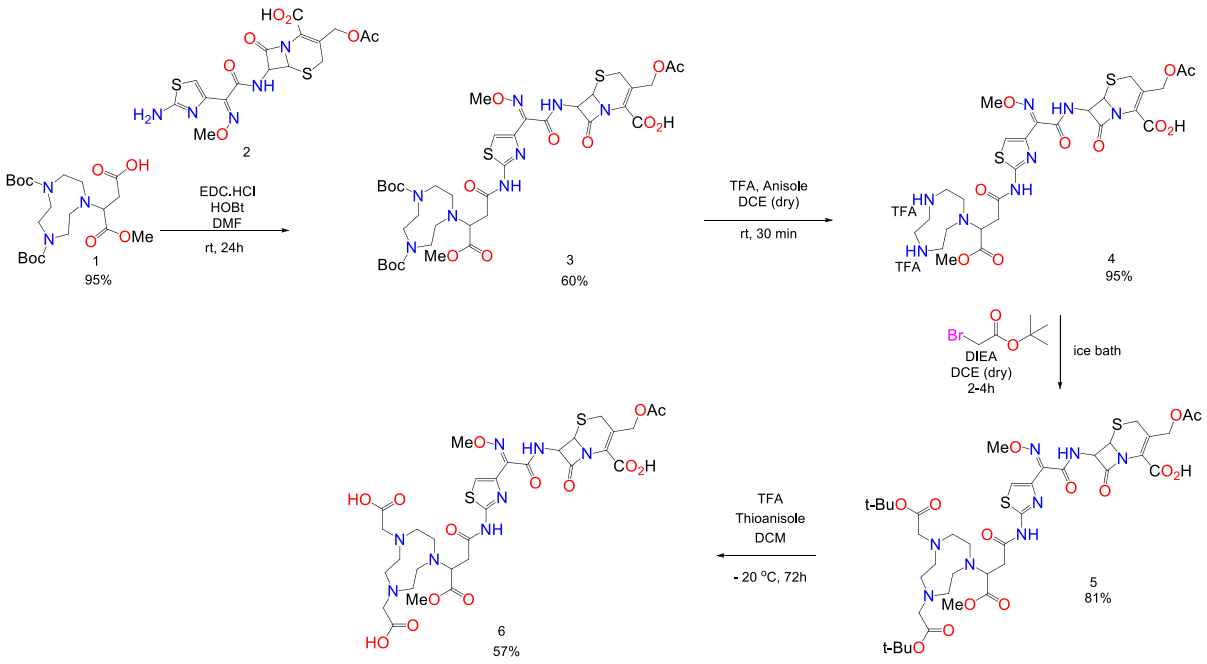


BP 6 **BP 10** **BP 14**

Figure 1. Molecular structures of the potential MBLI's BP 6, BP10 and BP14

2.1.1. Synthesis of inhibitors

As per Scheme 1, 4-Methoxy-1,4,7-triazacyclononane-butanoic acid analogue (**1**) was coupled with a commercially available cephalosporin (**2**) using the peptide coupling agent EDC to furnish (**3**) in 60% yield in its racemic form. The Boc protecting groups were then removed with TFA to yield 95% of compound (**4**). Thereafter the amines on the cyclononane were alkylated **(5),** and the t-butyl protecting groups were subsequently removed using trifluoroacetic acid (TFA) to produce the de-protected final product (**6** aka BP10) in 57% yield as an off-white solid. All reactions were monitored and optimized using liquid chromatography-mass spectrometry (LC-MS), and products were fully characterized using standard methods.



Scheme 1: Synthetic route to BP10

The synthetic route for BP6 and BP14 followed an analogous route using the respective cephalosporin for the coupling step.

2.2. Bacterial Source

CRE strains producing MBLs were acquired from Patrice Nordmann at the Institut National de la Santé et de la Recherche Médicale (U914), Paris, France [38]. The bacterial strains used included *Escherichia coli* NDM-1, *E. coli* NDM-4, *E. coli* IMP-1, *E. coli* IMP-8, *Enterobacter cloacae* NDM-1, and *E. cloacae* VIM-1. *Klebsiella pneumoniae* NDM was acquired from David P. Nicolau at the Center for Anti-Infective Research and Development, Hartford Hospital, USA [39]. An *E. coli* ATCC 25922 was employed as a carbapenem-susceptible control. All bacterial stock solutions were preserved in Trypticase soy broth and 10% glycerol containing 4mm glass beads at -80°C. Meropenem was obtained from Sigma-Aldrich (Schnelldorf, Germany), and the BP compounds were synthesized and characterized by the Catalysis and Peptide Research Unit, University of KwaZulu Natal, South Africa. Meropenem was prepared in distilled water (m/v),

and the BP compounds were prepared in 50% (m/v) DMSO. The final DMSO concentration was < 1.0 %. Antimicrobial stock solutions were stored at -80^{o}C.

2.3. Drug Susceptibility Testing

2.3.1. Broth Microdilution Assay

The Minimum Inhibitory Concentration (MIC) of meropenem, BP 6, BP 10, and BP 14 were determined utilizing the broth microdilution assay, as described by the Clinical and Laboratory Standards Institute (CLSI) antimicrobial susceptibility guidelines [40]. The mono-therapeutic effect of each antibiotic and MBLI was evaluated across a panel of MBL producing *Enterobacterales.*

2.3.2. Checkerboard Assay

The drug susceptibility profile of meropenem in combination with BP 6, BP 10, and BP 14 were studied using the checkerboard assay. This assay was used to ascertain the effect of two drugs on antimicrobial resistance targeting CRE strains. The assay was performed according to the protocol described previously [41] and was in accordance with CLSI antimicrobial susceptibility guidelines [40]. Briefly, twofold dilutions of meropenem with each chelator were made in Cation-adjusted Mueller Hinton Broth (CAMHB) in a 96 well microtiter plate. A 0.5 McFarland-standardized bacterial inoculum was added to each well to obtain a final volume of 100 µl per well. Thereafter plates were incubated at 37°C for 18h under aerobic conditions. The checkerboard assays were performed in triplicate. The MIC was determined as the lowest concentration at which no visible growth was present. The fractional inhibitory concentration index (FICI) was calculated for each combination according to the equation FICI = FIC_a (MIC of drug A in combination/MIC of drug

A alone) + FIC_b (MIC of drug B in combination/MIC of drug B alone) [42]. The FICI was interpreted as follows: synergy, FICI $\leq$0.5; additive 0.5-1; indifference, >0.5 FICI < 4; and antagonism, FICI $\geq$ 4 [43].

2.3.3. Effects of human serum

To study the effects of human serum on the MIC values, the above antimicrobial susceptibility testing protocol was adopted. However, the broth was prepared differently. MHB was prepared according to the manufacturer's instructions (Oxoid Ltd, ThermoFisher Scientific, United Kingdom). Thereafter equal volumes of broth and 100% human serum were utilized to generate a medium that contained 50% human serum

2.4.Time-kill Study

Time-kill studies were performed according to previously published methods [44], including those described by CLSI document M26-A [45]. Briefly, an overnight culture of *K. pneumoniae* NDM was diluted to a 0.5 McFarland standard that correlated to approximately 10^6 cfu/ml. The prepared bacterial suspensions were added to 96 plate wells containing a fixed dose of 32 mg/L of each BP MBLI and meropenem in concentrations of 0.5, 1 or 2 mg/L. Plates were incubated at 35ºC and 100 rpm shaking. A bacterial control without adding any drugs was included, and a meropenem-only control employing similar conditions. Viability counts were performed at 0, 2, 4, 6, 8, and 24 h by sampling 0.1 ml, diluting as appropriate, and spreading onto Mueller Hinton agar (MHA). These plates were incubated at 35ºC for at least 18 h. Colonies were enumerated as cfu/ml.

2.5.Cytotoxicity assay

2.5.1. Cell culture

HepG2 cells were cultured in 25 ml cell culture flasks using Eagle's minimum essentials medium (EMEM) supplemented with 10% fetal bovine serum, 1% pen-strep-fungizone and 1% L-glutamine, maintained in a humidified incubator (37 °C, 5% CO_2) until approximately 80% confluent.

2.5.2. Methyl Thiazol Tetrazolium Assay

The MTT assay was one of the methods used to determine *in vitro* cell viability of BP on HepG2 cells. HepG2 cells (15,000 cells/well) were seeded into a 96-well microtiter plate and allowed to adhere overnight (37 °C, 5% CO_2). Thereafter, the cells were incubated (37 °C, 5% CO_2) with a range of BP concentrations (0, 1, 8, 10, 50, 100 and 200 µg/ml) in triplicate for 6 h. After the 6 h incubation, the cells were washed with 0.1 M phosphate-buffered saline (PBS) and incubated with MTT salt solution (5 mg/ml in 0.1 M PBS) and 100 µl CCM for 4 h (37 °C, 5% CO_2). The MTT salt solution was removed, and DMSO (100 µl/well) was added and incubated for 1 h. The optical density was measured using a spectrophotometer (Bio-Tek µQuant) at 570/690 nm. Results are expressed as % cell viability versus BP concentration (µg/ml).

2.5.3. Lactate Dehydrogenase Assay

The LDH assay was used to assess membrane damage of HepG2 cells. Supernatant collected from control and BP treated cells were centrifuged (400 x g, 24°C, 10 minutes) and dispensed (100 µl/well) in triplicate into a 96-well microtiter plate. LDH reagent (100 µl, 11644793001, Sigma Aldrich) was added to each well. The plate was incubated for 30 minutes at room temperature in the dark. Absorbance was read using a spectrophotometer (Bio-Tek µQuant,) at 500 nM. Results are represented as relative fold change compared to untreated control.

2.6.Enzyme Assays

2.6.1. Inhibition of kinetics

A dose-dependent enzyme inhibition assay was performed using a Biotek PowerWave XS2(Biotek Instruments, Inc.USA) plate reader. NDM-1 and VIM-2 enzymes were purchased from RayBiotech (RayBiotech Life Inc.USA). Enzymes in the quantity of 1 nM (NDM-1) or 0.5 nM (VIM-2) were used in this study with a fixed nitrocefin concentration of either 120 μM (NDM-1) or 50 μM (VIM-2) and varying BP 10 concentrations ranging from 5-200 μM (NDM-1) and 2.5-300 μM (VIM-2) in 50 mM HEPES buffer supplemented with 100 μg/ml BSA and 10 μM $ZnCl_2$. Inhibition was measured at 482 nm at 25°

2.6.2. Fluorescence quenching

Since intrinsic tryptophan residues in NDM and VIM exhibit fluorescence, the ability of BP 10 to quench this was evaluated spectrofluorimetrically using a Biotek Synergy Mx (Biotek Instruments, Inc. USA) plate reader. Tryptophan residues were excited at 280 nm, with emission recorded at 355 nm. A 4 mM stock of BP 10 was added to 1 nM NDM-1 or VIM-2 in 1 μl increments in the presence of 50 mM HEPES buffer. Each BP 10 increment was incubated for 10 minutes with NDM-1 or VIM-2 at 25°C, before measuring the fluorescence intensity. EDTA (9 mM) was used as a control. The assay was conducted in triplicate. An increase in BP10 concentration resulted in a decrease in fluorescence and indicated inhibitor quenching of the tryptophan fluorophores in NDM-1 and VIM-2.

2.6.3. Non-specific binding of the inhibitor to zinc in non-metallo β-Lactamases

To determine the binding specificity of the inhibitors to other zinc-containing enzymes, glyoxylase (BioVision Inc, USA) was utilized to measure the level of specificity exhibited by BP10. The methodology has been previously described [29]. EDTA (purchased from Merck KGaA, Germany) was included as a positive control.

2.7. Ethical statement

All animal experiments carried out in this study were approved by the Institutional Animal Research Ethics Committee at the University of KwaZulu-Natal, with approval reference AREC/00002618/2021 (for the *in vivo* efficacy study). All sample sizes used in this study were estimated using G*Power Version 3.1.9.4.

2.8. *In vivo* efficacy study

A murine thigh infection model described by Michail *et al.*[46] was performed with minor modifications. Male Bragg inbred albino c-strain (BALB/c) mice weighing 20-25 g (n=90) were used in this study. Each of the three groups constituted n=30 mice, with n=6 mice receiving treatment every two hours. Prior to infection, mice were treated intraperitoneally (IP) with 150 mg/kg cyclophosphamide on day one and 100 mg/kg on day four of the trial. This was done to induce neutropenia in the mice, neutropenia was confirmed by a neutrophil count of $<100/mm^3$. On day five of the trial, 0.1 ml inoculum containing 10^6-10^8 cfu/ml of *K. pneumoniae* NDM, were intramuscularly injected into the right thigh of the mice, to initiate infection. Meropenem monotherapy, BP10 + Meropenem combination therapy, or normal saline was administered every two hours in an eight-hour treatment period. Mice were euthanized by isoflurane overdose, at 2h, 4h, 6h and 8h post-dosing. The right thigh muscle was then aseptically removed and homogenized in 5 ml of PBS. Homogenates were spread onto Mueller-Hinton agar and MacConkey agar plates, followed by incubation at 35^{o}C for 24h and enumeration of the cfu/ml.

2.9. LC-MS/MS Quantification

A Shimadzu Nexera Series (Shimadzu Corporation, Kyoto, Japan) liquid chromatography system was coupled with Shimadzu LCMS-8050 tandem mass spectrometer (Shimadzu, Kyoto, Japan). The chromatographic separation was achieved using a Shim-Pack Velox SP-C18 column (100mm × 2.1mm, 2.7 µm particle size) with a gradient mobile phase comprised of Millipore water (0.1% v/v trifluoroacetic acid) (A) and Acetonitrile (0.1% v/v trifluoroacetic acid) (B). The gradient method started from 5 to 95% B in 8 minutes, then held at 95% B for up to 12 minutes; thereafter, it was brought back to 5 % B at 12.1 minutes. The column was equilibration time was 2.9 minutes with a flow rate of 0.4 mL min^{-1} and the column oven temperature at 40 ºC. The injection volume was 25 µL, and the total run time of the method was 15 minutes.

Quantitative and qualitative studies were conducted using MRM mode *via* an ESI interface, with the following source parameters: nitrogen nebulizer gas flow 3 L/min; heat gas 10 L/min and interface temperature of 300°C. The precursor and product ions optimized were *m/z* 813.2→330.0 for BP10, *m/z* 384.50→68.25 for Meropenem and *m/z* 350.50→304.40 for Ampicillin (IS). Results were analyzed using LabSolutions Insight LCMS. All data are expressed as a mean ± SD.

2.10. Computational Methods

These methods have been detailed in the Appendices section

2.11. Statistical Analyses

GraphPad Prism version 8.0.2 (GraphPad Inc., San Diego, CA, USA) was utilized to analyze the data generated from the time-kill assay. The rate at which combination therapy resulted in bactericidal activity was determined per time point using a linear regression model. Comparative analysis between each combination of BP co-administered with meropenem was assessed against meropenem monotherapy utilizing the student's t-test. The effects of combination therapy with all BP compounds tested were measured against meropenem monotherapy and no therapy (*K.*

pneumoniae NDM control) using the two-way analysis of variance (ANOVA). In the *in vivo* efficacy study, the three treatment groups, S (saline only), M (meropenem monotherapy) and BP 10 and meropenem combination therapy (BP10 and meropenem), were compared. Statistical significance was represented by a decrease in the p-value ($p< 0.05$) and an increase in the F ratio.

3. Results and Discussion

The utilization of metal chelators in combination therapy to target CREs proves to be a promising treatment strategy observed in reports of TPA-based zinc chelators[47] as well as DPA [48, 49]. This was also indicated by our previous results, that is BP1 (Chapter four). This led us to investigate other derivatives of BP1 (cyclic zinc chelators attached to a β-lactam moiety) in order study other aspects for optimization of the derivatives/hits with regards to synthetic route viability, solubility affinity, selectivity, efficacy/potency, metabolic stability, and oral bioavailability. The results from the drug susceptibility assay (Table 1) indicate that virulent strains of NDM-producing bacteria were observed to be highly resistant to meropenem monotherapy but susceptible to n combination therapy (Table 1). In combination therapy, the BP compounds (6, 10, and 14) were highly potent in restoring the efficacy of seven MBL variants to meropenem concentrations ≤ 1 mg/L. When comparing the biological activity of the BP inhibitors reported herein to other metallo-β-lactamase inhibitors, the MICs generated by BP are highly efficacious. These observations further indicate BP is superior to the concentrations reported by Ishii *et al*., for the malic acid derivative ME1071 [50], as well as Everett *et al*., for ANT431 [51]. When comparing our results to structurally similar MBLIs; ZN148 [29] a tris-picolylamine ligand, and the $h_2$dpa derivatives [30] with pentadentate ligands, the reported activities concur. However, the BP compounds exhibited better efficacies with lower MBLI concentrations.

Therefore, the findings from Table 1 suggest BP 6, 10, and 14 are effective MBLIs against *Enterobacterales* expressing*;* $bla_{\text{NDM-1}}$, $bla_{\text{NDM-4}}$, $bla_{\text{VIM-1}}$, $bla_{\text{IMP-1,}}$ and $bla_{\text{IMP-8}}$ genes. According to the FICI (Table 2), meropenem administered with the BP compounds displayed a synergistic relationship against inhibition of these MBL-expressing bacteria. Table 2 further highlights that the FICI obtained with the BP compounds are much lower than the recommended FICI ($\leq$0.5) for synergism, thus substantiating the high level of synergy achieved and low MICs obtained. Serum had no considerable effect on the MICs of BP 6,10, and 14 for the seven bacteria studied.

Table 1: MICs obtained for meropenem and BP chelators alone, as well as from combination therapy, against CREs harboring MBLs

			MIC (mg/L)		
MBL Producing Bacteria	**MEM alone**	**BP 6/10/14 alone**	**BP 6 + MEM**	**BP 10 + MEM**	**BP 14 + MEM**
E. coli NDM-1	> 128	> 256	8 + 0.125	16 + 0.25	8 + 0.125
E. coli NDM-4	128	> 256	8 + 0.25	16 + 0.5	8 + 0.06
E. coli IMP-1	8	> 256	8 + 0.5	16 + 0.03	8 + 0.03
E. coli IMP-8	4	> 256	8 + 0.25	32+ 0.06	8 + 0.125
E. cloacae NDM-1	32	> 256	8 + 0.25	16+ 0.125	8+ 0.25
E. cloacae VIM-1	16	> 256	8 + 0.25	16 + 0.25	8 + 0.5
K. pneumoniae NDM	> 128	> 256	8 + 0.5	16 + 0.25	8 + 0.25
E. coli ATCC 25922	S	N/A	S	S	S

MEM = meropenem
S = Susceptible to meropenem at a concentration of 0.06 mg/L: no bacterial growth
N/A = Not applicable
All assays were conducted in triplicate

Table 2: FIC index obtained from combination therapy with MBLI and meropenem against CREs expressing MBLs

MBL Producing Bacteria	**BP 6 + MEM**	**BP 10 + MEM**	**BP 14 + MEM**

E. coli NDM-1	0.03	0.13	0.03
E. coli NDM-4	0.03	0.07	0.03
E. coli IMP-1	0.09	0.04	0.04
E. coli IMP-8	0.09	0.09	0.06
E. cloacae NDM-1	0.04	0.07	0.04
E. cloacae VIM-1	0.05	0.13	0.06
K. pneumoniae NDM	0.04	0.07	0.03

Synergy, FICI $\leq$0.5; additive 0.5-1; indifference, >0.5 FICI < 4; and antagonism, FICI $\geq$ 4

Table 3: Half the maximal inhibitory concentrations of MBLIs on HepG2 cells

MBLI	IC_{50} (µg/ml)
BP 6	51.83
BP 10	61.98
BP 14	42.34

The BP compounds were then investigated for cytotoxicity using a human liver (HepG2) cell line According to the cytotoxicity data (Table 3), BP10 possessed the lowest cytotoxicity results with an IC_{50} of 61.98 µg/ml, whilst BP 14 displayed the highest of 42.34 µg/ml. For this study, MBLI concentrations of $\leq$ 32 mg/L were required for subsequent experiments, allowing us to proceed. In addition, the cell viability was not affected by the concentrations of BP 6 (Figure 2) and 10 (Figure 3). BP6 increased cell viability compared to the control, with 200ug/mL being significant (Figure 2). The MTT assay measures cellular metabolic output, and compound BP6 increases metabolic output. It is known that cancer cells utilize anaerobic glycolysis for ATP generation and minimizes mitochondrial output, as this will activate apoptosis. The LDH assay further confirmed the non-toxic properties of all compounds. Compound BP6 significantly decreased LDH membrane leakage at all concentrations tested (Figure 2). BP10 significantly decreased LDH membrane leakage from 8-200 ug/mL (Figure 3), while BP14 significantly decreased LDH membrane leakage at 8 ug/mL (Figure 4). An added advantage of compound BP6 (increases metabolic output and

decreases LDH leakage) will aid eukaryotic cells in increasing ATP production and help fight bacterial insult. Thus, the overall opinion of the cytotoxicity data suggests that these inhibitors are non-toxic at the selected doses and, therefore, safe to administer *in vivo*.

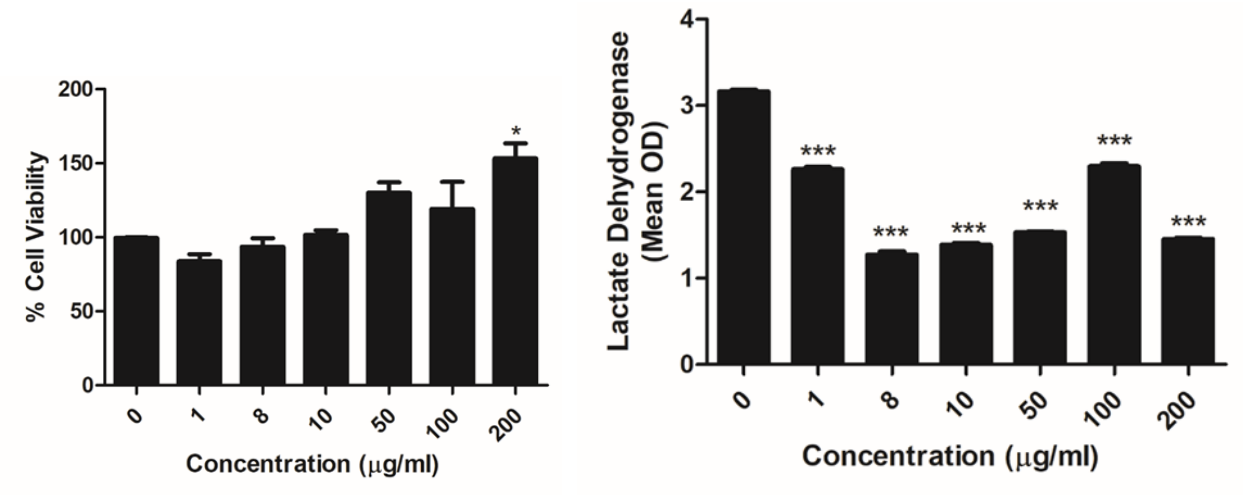


Figure 2: Cell viability studies conducted on HepG2 cells using varying concentrations of BP 6. BP6 induced a dose-dependent increase in the cell viability of HepG2 cells, however, cell viability was only significantly altered at 200 μg/ml. *p<0.05 relative to control. LDH was significantly reduced at all concentrations, indicating that BP-6 does not induce necrosis after exposure. ***p<0.0001 relative to control.

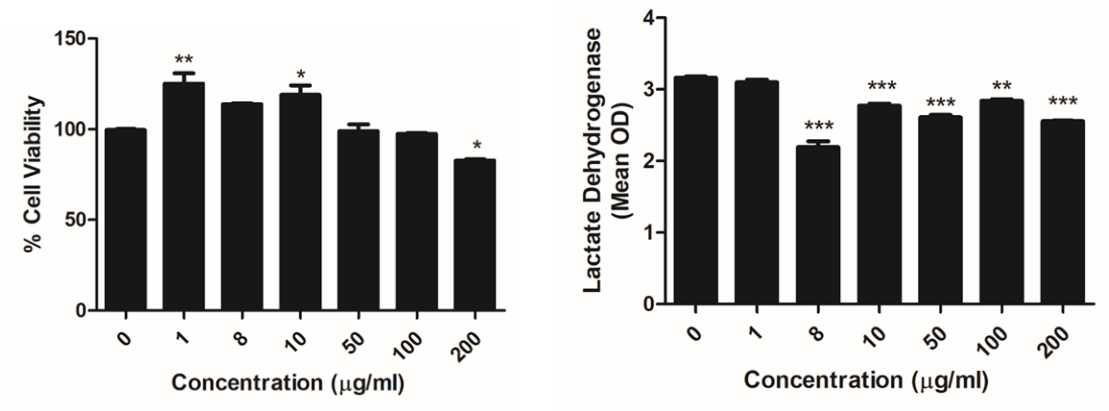


Figure 3: Cell viability studies conducted on HepG2 cells using varying concentrations of BP 10. Cell viability was not significantly altered at 50-100 μg/ml; however significantly reduced at 200 μg/ml. *p<0.5 and **p<0.01 relative to control. LDH levels remained unaffected at 1 μg/ml and were significantly reduced at 8-200 μg/ml, indicating BP10 does not induce necrosis in HepG2 cells after exposure. **p<0.01 and **p<0.001 relative to control.

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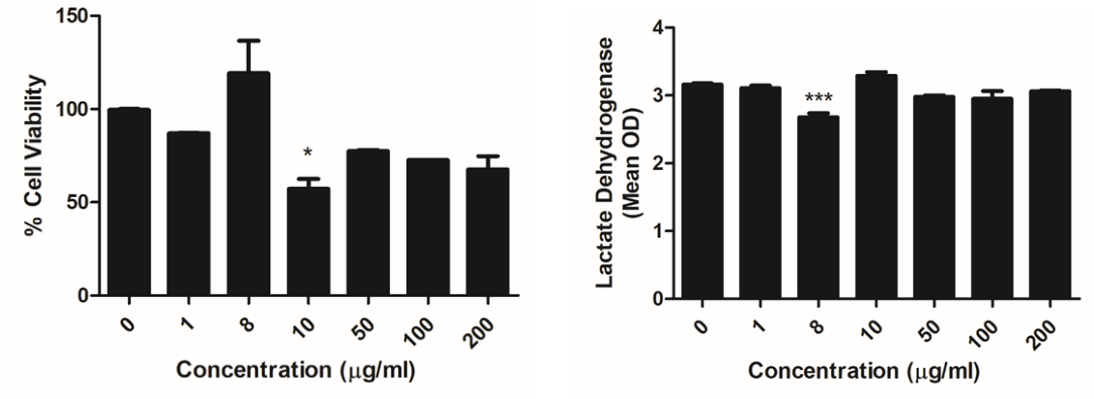


Figure 4: Cell viability studies conducted on HepG2 cells using varying concentrations of BP 14. BP14 reduced cell viability at 1 μg/ml and 10-200 μg/ml; however, proliferation occurred at 8 μg/ml. *p<0.5 and **p<0.01 relative to control. LDH levels were significantly reduced at 8 μg/ml and remained unaffected at 1 μg/ml and 10-200 μg/ml, indicating BP14 does not induce necrosis in HepG2 cells after exposure. **p<0.001 relative to control.

Figures 5-7 represent the effect exhibited by the BP compounds + meropenem over 24 hours against the virulent *K. pneumoniae* NDM-expressing bacteria. BP - 6, 10, and 14 displayed excellent bactericidal activity against *K. pneumoniae* NDM with meropenem concentrations of 0.5, 1, and 2 mg/L. A > 3 $\log_{10}$ decrease in the bacterial load was observed for each time point relative to the bacterial control. Meropenem monotherapy produced a > 2 $\log_{10}$ increase of bacteria in comparison to the BP + meropenem treated groups. However, a sharp decrease was observed at 4 hours post-inoculation for the meropenem control group. This effect was short-lived as the bacteria continued to grow exponentially for the subsequent time points. This was expected as bacterial resistance to meropenem was observed and noted in Table 1. Similar bactericidal trends of a 3 $\log_{10}$ bacterial reduction were found with BP1 and the $h_2$dpa derivatives [30], however, $h_2$dpa derivatives utilized a concentration two-fold lower compared to BP and achieved a bacterial reduction to 10 cfu/ml, whilst BP achieved complete killing. When comparing our results to ZN148, the BP compounds employed half the meropenem and ZN148 concentrations for the time-kill studies and were, therefore, superior [29]. Reports by Sosibo *et al*., [44] are also concordant

with this study since similar bactericidal trends of $\geq$ 3 $\log_{10}$ reduction in the growth of MBL-producing bacteria using meropenem co-administered with pyridyl type chelators, were observed experimentally. These findings are characteristic of β-lactams and indicate that meropenem's efficacy has been restored due to the co-administration of BP. No observations of bacterial re-growth were observed as complete killing was achieved, as illustrated by Figures 5-7.

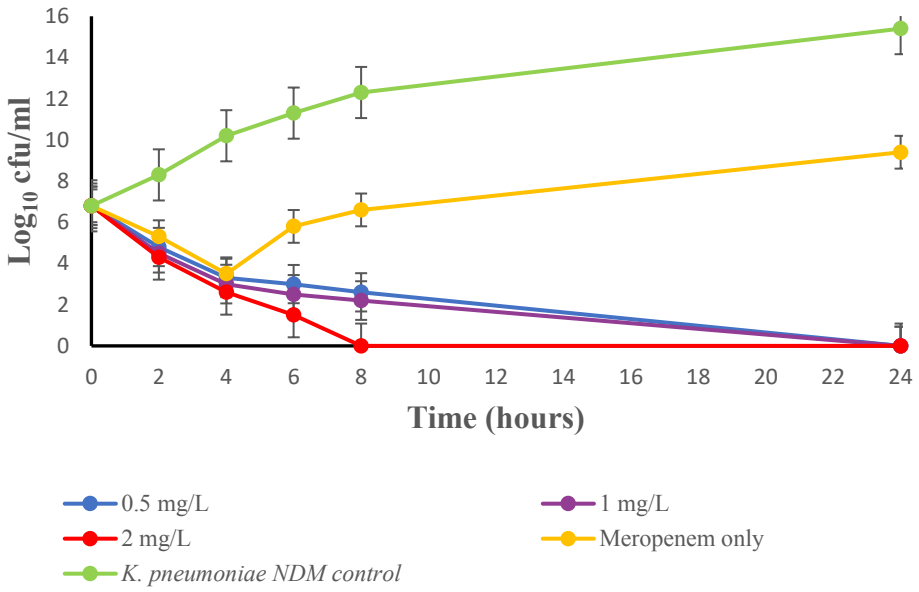


Figure 5: Time-kill kinetic study of *K. pneumoniae* NDM using BP 6 MBLI + meropenem.

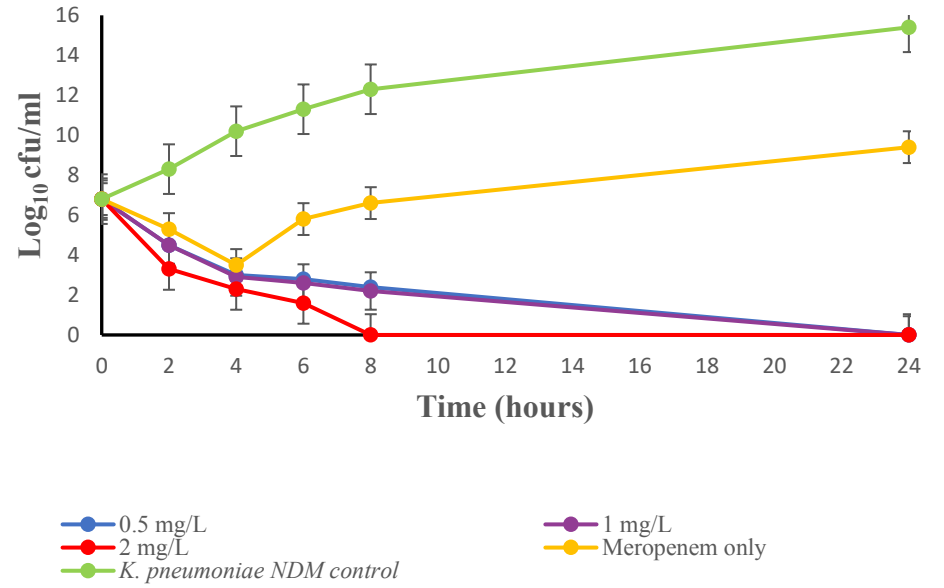


Figure 6: Time-kill kinetic study of *K. pneumoniae* NDM using BP 10 MBLI + meropenem.

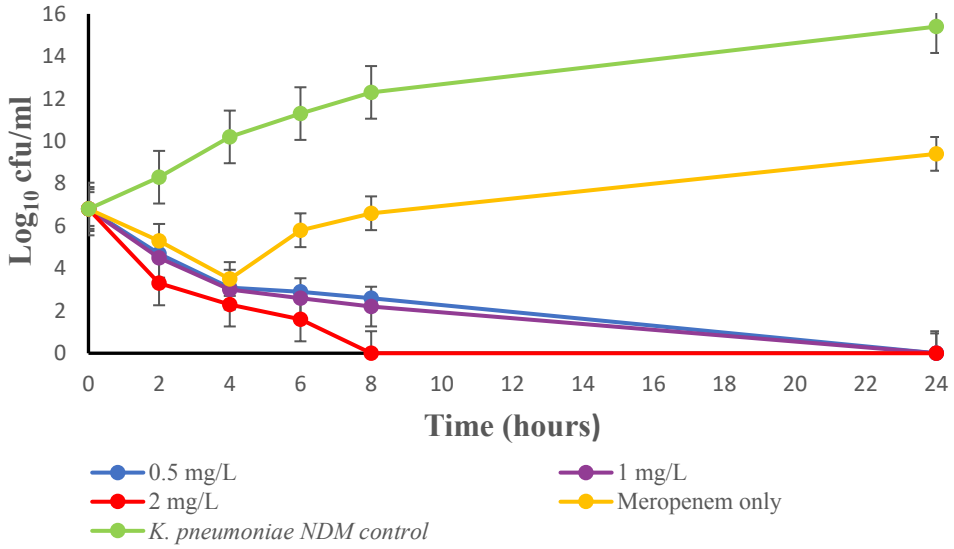


Figure 7: Time-kill kinetic study of *K. pneumoniae* NDM using BP 14 MBLI + meropenem.

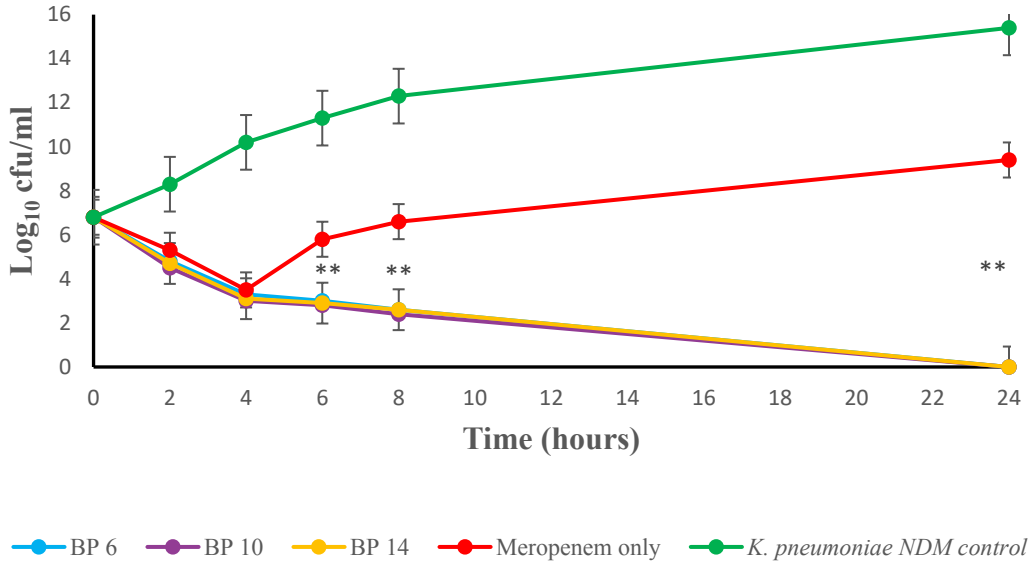


Figure 8: Comparison of time-kill data for BP – 6, 10 and 14.

All three BP compounds behaved similarly. Statistical significance is represented by ** in which $p<0.005$

The results from our study indicate that combination therapy between BP and meropenem produces a bactericidal effect by a measure of ≥ 4 $\log_{10}$ cfu/ml, over a 24-hour period and at meropenem concentrations as low as 0.5 mg/L for all three BP chelators investigated (Figure 8). Another observation to note from Figure 8 and Table 4 is that all the BP compounds administered behaved similarly, P = 0.9986.

Table 4: Comparative analyses of combination therapy vs. monotherapy

Compound	P value	R squared
BP 6 + Meropenem	0.0441	0.3464
BP 10 + Meropenem	0.0347	0.3738
BP 14 + Meropenem	0.04	0.3577

Analyses were conducted on the lowest dose administered, 32 mg/L BP + 0.5 mg/L meropenem

The difference in the $\log_{10}$ cfu/ml count across the treatment and non-treatment groups investigated, has generated a huge statistical difference, P = 0.0013 (Table 5), thereby indicating

the success of combination therapy. Many recently reported studies do not boast such favorable results, where killing is initiated immediately and rapidly over a prolonged timeframe. Therefore, the overall finding is that BP - 6, 10, and 14 are potent, efficacious, and safe therapeutic alternatives that potentially become promising MBLIs.

Table 5: Statistical analyses of the variation exhibited by combination therapy vs. monotherapy and no therapy

Parameter	BP + Meropenem*
Mean square	98.4
F ratio	17.14
% of total variation	77.19
P value	0.0013

*Represents the mean $\log_{10}$ cfu/ml for all BP compounds per time point.
There was great similarity (P= 0.9986) amongst the performance of the BP compounds
Analyses were conducted on the lowest dose administered, 32 mg/L BP + 0.5 mg/L meropenem

This conclusion led us to investigate the BP compounds further. Based on the excellent results achieved by BP10, and since it was more synthetically viable in comparison to BP6 and BP14 (overall yield and solubility issues), we decided to assess it more closely. We initiated this assessment by evaluating the antimicrobial susceptibility profile of BP10 in combination with meropenem against 21 bacterial isolates from different geographies (Table 6). BP10 successfully restored the meropenem MIC to therapeutically acceptable levels, defined herein as < 2 mg/L coupled with a BP10 MIC of < 64 mg/L (Figure S16), therefore conforming to the standards outlined by CLSI [52] and ISO 20776-2 [53] regulatory bodies. The data presented in Table 6, offers a more thorough and intense approach to assessing the MIC of BP10 and reiterates the efficacy of BP10 in combination with meropenem, evidenced by a highly synergistic drug effect of a FICI $\leq$ 0.14. The MICs depicted in Table 6 were also subjected to multiple tests measuring the effect of different brands of Mueller-Hinton broth cation adjusted or not, namely; Sigma and

Millipore, Oxoid, as well as Neogen. This was done to determine if there was any variability in the MICs due to the effect of zinc in the broth [54]. The end results showed no discrepancies in the MIC across the different brands of broth used, thus validating the data produced.

Table 6: The MICs of BP10 and meropenem from a panel of 21 carbapenem-resistant bacteria

Bacterial reference	Bacterial strain	MBL produced	MIC (mg/L)			FICI
			MEM alone	BP 10 alone	BP 10 + MEM	
ATCC 25922	*E. coli*	N/A	0.06	N/A	0 + 0.06	N/A
AUS 271	*E. coli*	NDM-1	> 128	> 256	16 + 0.25	0.13
FEK	*E. coli*	NDM-4	>32	> 256	16 + 0.5	0.07
JAP	*E. coli*	IMP-1	8	> 256	16 + 0.03	0.04
TWA	*E. coli*	IMP-8	8	> 256	32 + 0.06	0.09
BM 14	*E. coli*	VIM-1	32	> 256	8 + 0.25	0.04
TC CARF	*E. coli*	VIM-2	8	> 256	4 + 0.5	0.08
DIH	*E. coli*	VIM-19	>32	> 256	16 + 0.125	0.07
IR 386	*E. cloacae*	NDM-1	32	> 256	16 + 0.125	0.07
BM 5	*E. cloacae*	IMP-1	> 32	> 256	32 + 0.5	0.14
TWA	*E. cloacae*	IMP-8	0.125	> 256	16 + 0.06	0.5
KAR	*E. cloacae*	VIM-1	4	> 256	16+ 0.25	0.13
USA 449	*K. pneumoniae*	NDM	> 32	> 256	16 + 0.25	0.07
6852	*K. pneumoniae*	IMP-1	> 32	> 256	64 + 1	0.3
TWA	*K. pneumoniae*	IMP-8	8	> 256	16 + 0.25	0.09
ENNES	*K. pneumoniae*	VIM-1	> 32	> 256	8 + 0.5	0.05
DIH	*K. pneumoniae*	VIM-19	32	> 256	16 + 0.125	0.07
	S. marcescens	IMP-11	> 32	> 256	64 + 1	0.3
BM 20	*S. marcescens*	VIM-2	> 32	> 256	8 + 0.5	0.05
PSTU	*P. stuartii*	NDM-1	32	> 256	8 + 0.25	0.04
IR38	*P. rettgeri*	NDM-1	32	> 256	32 + 0.5	0.14

MEM = Meropenem

N/A = Not applicable

Synergy, FICI $\leq$ 0.5; additive 0.5-1; indifference, >0.5 FICI < 4; and antagonism, FICI $\geq$ 4

All assays were conducted in triplicate

Compound **(2)** i.e., the cephalosporin component of BP10, displayed no activity on its own
NOTA pre-complexed to zinc displayed no activity towards the MBL harboring bacteria
BP10 + Meropenem did not show activity towards *S. marcescens* KPC-2 and *E. coli* OXA-28 indicating BP10 has specific activity towards MBLs

BP10 was then subjected to enzyme analyses to determine essential enzyme parameters for assessing the level of potency exhibited. The IC_{50} was calculated using the ATT Bioquest tool [55] from the generated sigmoidal curves (Figure S17), and the Ki was calculated using the Cheng-Prusoff equation. Table 7 indicates that BP10 is fairly potent against VIM-2 , with an IC_{50} very similar to that of BP1 (24.8 μM, previously evaluated by our group), however BP10 is three times more potent with inhibiting NDM-1 than BP1 (97.4 μM).

Table 7: Enzyme Inhibition exhibited by BP10

MBL	**IC_{50} (μM)**	**Ki (μM)**
NDM-1	57.7 ± 4.5	30.9
VIM-2	48.5 ± 2.9	26.2

All assays were conducted in triplicate

IC_{50} represents the mean ± SD

Tryptophan (trp) is a fluorescent amino acid within the protein structure of NDM (trp-93) and VIM (trp-87) MBLs. Therefore fluorescent quenching was employed to measure the binding affinity of the quencher (BP10) to the fluorophore (MBLs) [56]. Observations from Figure 9 depict a reduction in fluorescence intensity that correlates to an increase in quenching. This indicates that BP10 at increasing concentrations influences the intensity of fluorescence. More importantly, it conveys that on a molecular level, BP10 is bound to the MBLs.

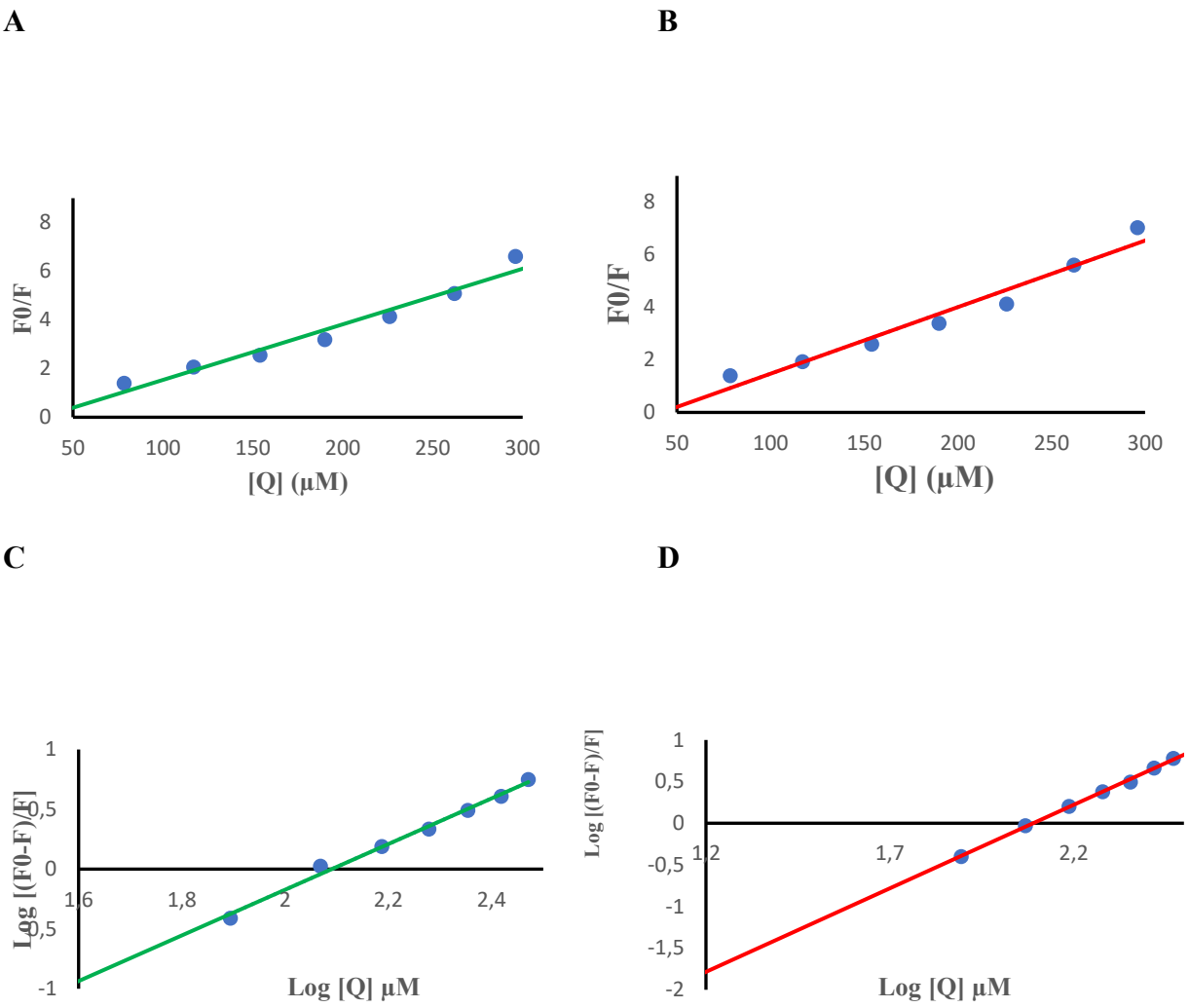


Figure 9: Stern-Volmer plots of BP 10 interacting with VIM-2 and NDM-1. BP10 was able to quench the tryptophan residues of 1nM VIM-2 (A) and 1nM NDM-1 (B). Modified Stern-Volmer plots of BP10 interacting with 1 nM VIM-2 (C) and NDM-1 (D) were then generated to determine quenching parameters. All assays were conducted in triplicate

Although BP10 displays strong binding affinity to NDM-1 and VIM-2 MBLs, data from Table 8 suggests that BP10 interacts more strongly with NDM-1 as compared to VIM-2. This is implied from a higher Ka with NDM-1. These findings are consistent with the binding affinity generated by BP1.

Table 8: Summary of enzyme-inhibitor parameters obtained from the kinetics of inhibition and fluorescence quenching experiments

Enzyme - Inhibitor Parameters	NDM-1	VIM-2
Ksv [Q] (µM-1)	0.0253 ± 0.0001	0.0228 ±0.0016
Ka (µM)	4.19 ± 0.06	3.99 ± 0.09
Kd (M)	0.239 ± 0.007	0.251 ± 0.01
n	2.01± 0.02	1.91 ± 0.05
R^2	0.99	0.98

Metal chelators are known to suffer from off-target specificity and as a result have been criticized as potential MBLIs *in vivo* [57]. This experiment was designed to investigate the level of specificity exhibited by BP10. Recombinant human glyoxylase II (Glo2), an essential zinc-containing enzyme structurally similar to MBLs [58], was monitored for interaction in the presence and absence of BP10. Based on the reults from Figure 10, concentrations up to 500µM of BP10, did not reduce the activity of Glo2, indicating that it does not bind to the zinc ions within the active site of Glo2. BP10 exhibits specific activity towards the NDM-1 and VIM-2 MBLs tested herein. Whilst metal chelating agent EDTA, significantly reduced the activity of Glo2 from 50 -500 µM. These findings corresponds to reports from chelators, BP1 and ZN148 [29].

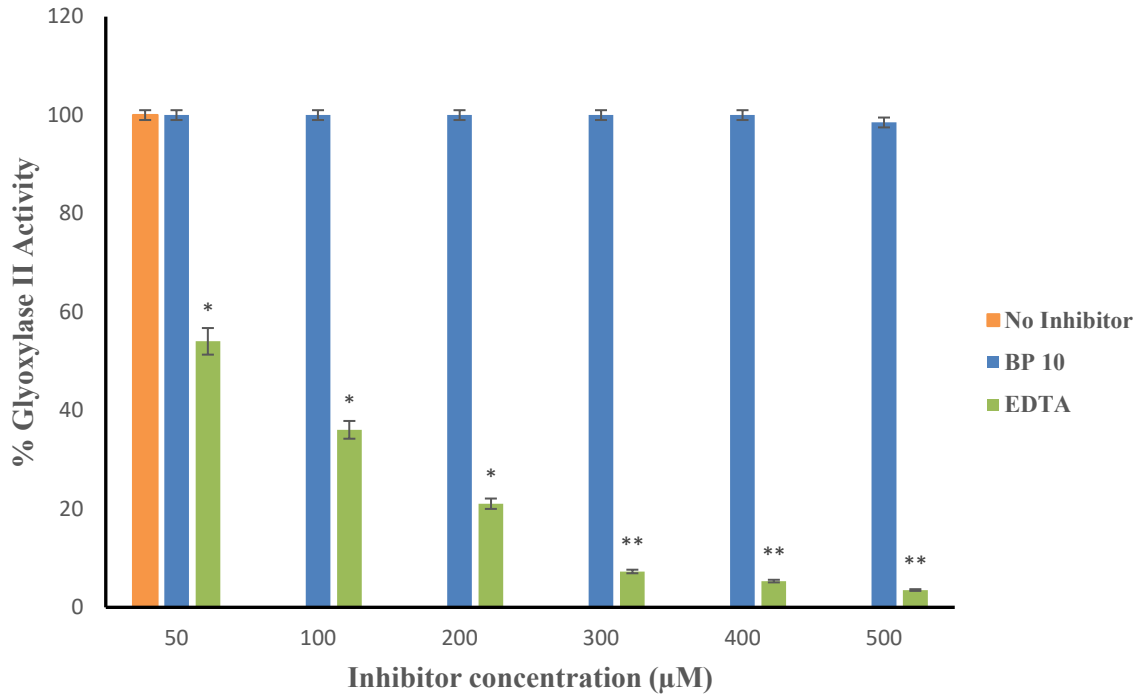


Figure 10: Glyoxylase II activity in the presence of BP10 and EDTA. Normal glyoxylase II activity is denoted by the legend representing no inhibitor added. BP10 did not decrease the activity of glyoxylase II compared to metal chelating agent EDTA. Statistical significance is denoted by $p < 0.005$ (*) and $p < 0.001$ (**) Experiment conducted in triplicate

Next, molecular docking of BP10 with NDM-1 and VIM-2 was performed (Figure 11). The docked complexes were evaluated by elucidating the interactions formed and the stability of the conformations over 100ns MD simulations. The ligand interaction diagram (Figure S18) shows that the carboxylic arms of the chelator remained coordinated to the Zn^{2+} throughout the MD simulation. This observation implies that the investigated systems have the potential to abstract the Zn^{2+} ions from the enzyme active site, as we hypothesized. The binding affinities of different BP10 configurations were determined using MMGBSA post-MD simulations. The binding free energies with their corresponding docking scores (Table S11) revealed NDM-1—BP10 had a docking score of -6.20 kcal/mol, while VIM-2—BP10 had -6.40kcal/mol. The BP10_SS

configuration has the best binding free energies compared to BP10_SR. 11). It is possible that BP10 ultimately removes the zinc ions from the active site, immobilizing the enzyme completely. This is supported by the absence of a MIC value when NOTA pre-complexed to zinc was evaluated as a potential MBL inhibitor (Table 6), confirming that BP10's zinc chelation is required for inhibition. Subsequent studies will use PACs-MD[59] to determine if NOTA chelation of the zinc ion is energetically feasible.

a) **b)**

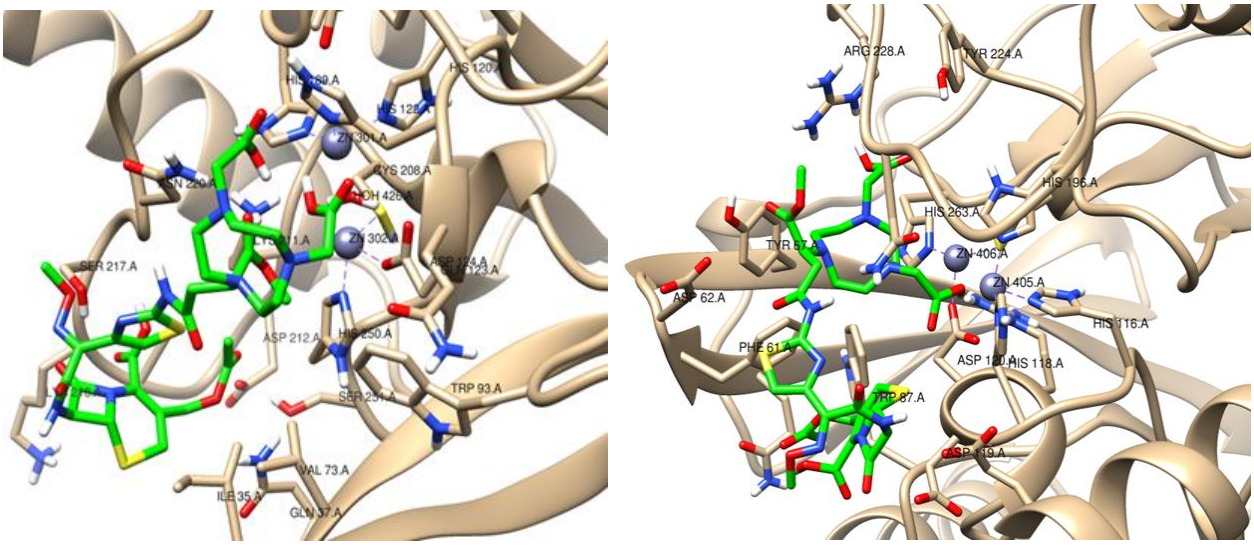


Figure 11: 3D structures of NDM1—BP10 and VIM2—BP10 complexes, respectively. BP10 compounds are shown in green. In NDM1, the Zn301 (grey sphere) is coordinated to three histidines, and a water molecule (HOH426) coordinates the two zinc ions, while Zn302 is coordinated to histidine, aspartate, and cysteine residues. In VIM2, the Zn405 is coordinated to three histidines, and Zn06 is coordinated to one histidine, one aspartate, and one cysteine residue.

Delving deeper into the examination of BP10, *in vivo* efficacy studies were undertaken to assess the potency of BP10 in a murine infection model. This five day animal trial was initiated by immune-suppressing the mice with cyclophosphamide, to allow the progression of infection and allow BP10 + meropenem treatment to be studied over eight hours on day five of the trial. In addition, it excludes the effects of the innate immune response as a potential variable [60]. Since

many carbapenem-resistant infections are acquired as secondary infections in immue-suppressed hosts [61]. The mice were successfully infected with *Klebsiella pneumoniae* NDM, evidenced by visible inflammation of the localized area; this correlated with the cfu/thigh data expressed (Table S7-S9). Three treatment regimens of S (saline only), M (meropenem only), and BP10 combination therapy (BP10 + meropenem) were used to randomly categorize mice.

Based on previous studies conducted by our research group, we know that BP10 should be administered every two hours with meropenem to account for the short half-life of BP and meropenem (PK studies, Figure S12) as well as the rapid renal DHP-1 hydrolysis rate of meropenem [51]. Therefore, a total of four doses (100 mg/kg of each drug) over eight hours were administered with no visible observations of toxicity (total drug dose 800 mg/kg). Treatment was restricted to eight hours to ensure the trial was logistically viable. Moreover, injecting an animal of that size repeatedly is unethical and does not concur with the ARRIVE guidelines used to guide the trial's design.

The results from Figure 12 indicate that although BP10 is more potent *in vitro*, it shares a similar *in vivo* efficacy to BP1. This is expected since they are structurally related compounds with the same action mechanism.

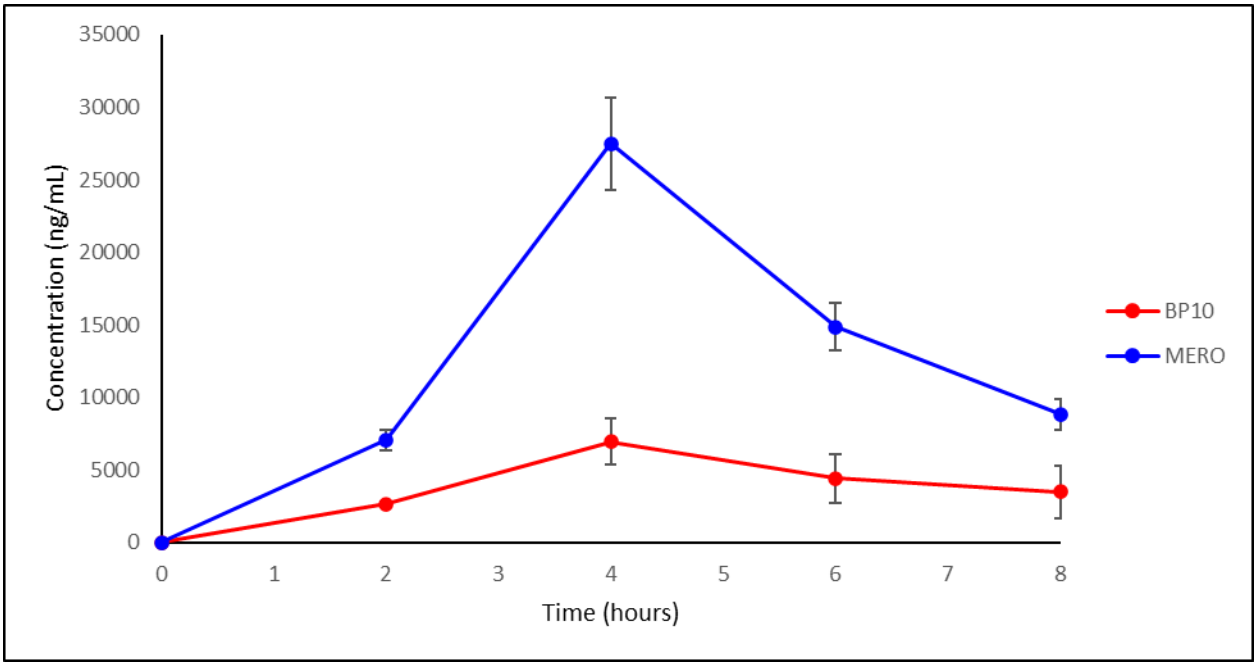
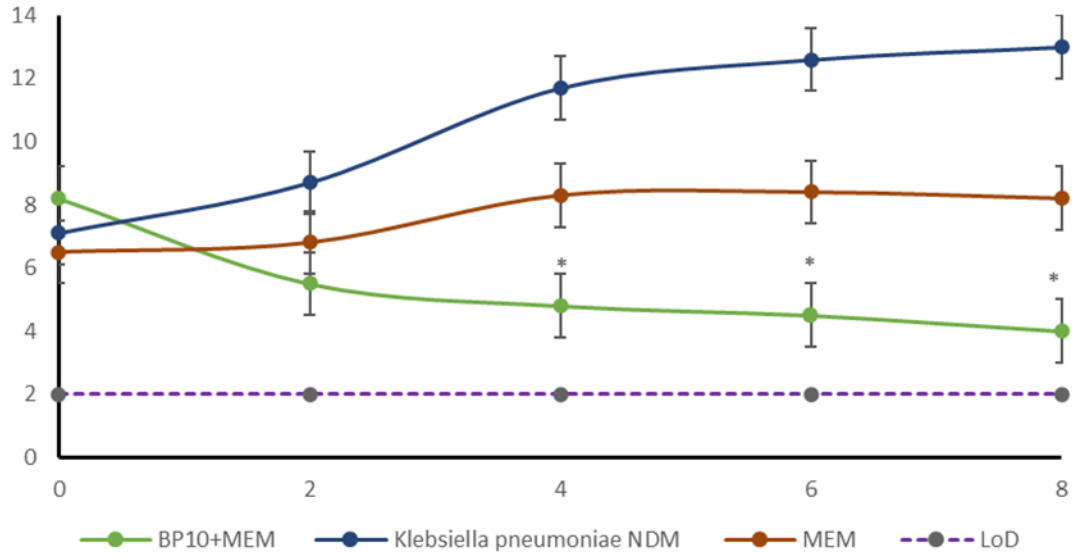


Figure 12: Plasma BP10 and meropenem concentrations over the eight-hour treatment period. BALB/c mice were infected i.m with *K. pneumoniae* NDM. Treatment was initiated 2 hours post-infection with either S, M, or BP10. Four treatment doses were administered via IP. Data are presented as a mean ± SD (n=6).

Many MBLI candidates produce good *in vitro* efficacies but fail to reach efficacy *in vivo*. These include NOTA [36] poor bioavailability and TPEN [37] cytotoxicity [62], both previously researched by our group. Figure 13 depicts the outcome of a successful murine infection model, *K. pneumoniae* NDM colonies were reduced by > 3 $\log_{10}$ units, the deviation between the doses of BP10 + meropenem administered was < 8% (Table S7-S9), indicating statistical significance ($p<0.005$). Based on the extrapolation of the treatment curve of Figure 13, a further decline in the cfu/thigh count would have been observed with continued treatment. However, considering the animal's welfare, we could not risk the fate of severe inflammation/animal demise. Based on the results (Figure 13), the *in vivo* activity of BP10 is concordant with the *in vivo* efficacy of BP1 and ZN148, where a decrease in the bacterial load is observed to a count of approximately 3 $\log_{10}$ units [29].



MEM = Meropenem, LoD = Limit of Detection

n = 6 mice per time point per group, cfu/mL counts were done in triplicate

* Denotes $p<0.005$

Figure 13: The efficacy of BP10 combination therapy over monotherapy in a murine thigh infection model. The co-administration of BP10 and meropenem resulted in a significant decrease of *K. pneumoniae* NDM cfu/mL, in comparison to S and M treatment. This indicates that BP10 + meropenem is a favourable treatment strategy.

4. Conclusion

The metal chelators: BP 6, BP 10, and BP 14, described in this study function as promising MBLIs and restore the efficacy of meropenem to susceptible antimicrobial concentrations against various MBL expressing bacteria. Very few MBLIs have the potential to restore meropenem to such low concentrations without causing an adverse effect to the eukaryotic cells. Furthermore, the co-administration of meropenem with the BP compounds has proven to have a synergistic effect across a panel of MBL-harboring bacteria. Analyses from the time-kill experiments also indicated that the metal chelators displayed a bactericidal effect on *K. pneumoniae* NDM. All BP compounds tested, in combination with meropenem, demonstrated excellent bactericidal activity. BP restored

meropenem's potency, facilitating the 24-hour killing of *K. pneumoniae* NDM at all concentrations tested. In addition, bacterial re-growth was absent as complete killing was achieved. Given the successful results obtained from the *in vitro* tests conducted, BP10 was pursued for further susceptibility testing in conjunction with enzyme studies and *in vivo* experiments. The *in vitro* potency of BP10 continued to generate excellent outcomes, complemented by a successful murine infection study. The overall findings of this study indicate that BP10 is a therapeutic solution aimed at targeting MBL-mediated carbapenem resistance and should be prioritised for further pre-clinical assessment.

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Declaration of interests

HGK, PIA, TN, and TG have a patent PCT number PCT/IB2022/056748 on the technology. All other authors: none to declare.

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Chapter six: Summary and Recommendations

In recent years, carbapenem-resistance has escalated to alarming rates due to the number of β-lactamase-producing bacterial strains in circulation. Leading to a global appeal by the World Health Organization for therapeutic alternatives to become available. In response to this urgent crisis, drug candidates have been developed that re-sensitize the pathogen to β-lactam/ carbapenem antibiotics. However, most of these drugs only target bacteria harbouring serine β-lactamases, and there has not yet been a metallo-β-lactamase inhibitor that can be safely administered in the clinic. Thus, the dire need for metallo-β-lactamase inhibitors to become commercially available is of paramount importance in the fight against antibiotic resistance. To tackle the resistance conferred by metallo-β-lactamases, for this study, our research group synthesized six novel metallo-β-lactamase inhibitors, namely, NOTA, NO3PY, BP1, BP6, BP10, and BP14 and evaluated its potential on a cellular, molecular, and biochemical level.

In vitro evaluation of these inhibitory compounds has demonstrated significant potential in restoring the MIC of meropenem to susceptible breakpoints outlined by CLSI and EUCAST guidelines. All the inhibitors mentioned above were non-toxic at the selected doses and displayed plasma stability over 24 hours. When assessing the *in vivo* potential of the initial inhibitor, NO3PY, it was found to be below the limit of detection for the pharmacokinetic study and hence could not be further pursued. On the contrary, the next inhibitor, NOTA was well detected by the *in vivo* pharmacokinetics, however, the drug could not be quantified in the plasma due to poor bioavailability. This finding piqued our interest and was therefore further explored. Subsequent synthetic experiments derivatized NOTA by coupling it to known cyclic β-lactams to improve the *in vivo* pharmacokinetics of the drug, which we now refer to as the novel BP series of inhibitors.

Based on the synthetic viability, solubility and yields of the BP series of inhibitors as well as the requirement for the compound to be easily upscaled for animal studies, two candidates were selected and pursued further. These were BP1 and BP10; before animal studies, enzyme analyses were completed and favoured BP1 and BP10. The mode of action for the inhibitors was evidenced by observations of potent inhibitory activity and site-specific binding interactions, coupled with computational experiments that depict the carboxylic moiety of BP1 and 10 removing the Zn^{2+} ions from the MBLs active site, thereby neutralizing the MBLs.

A murine infection model of *K. pneumoniae* NDM in the thigh was undertaken to assess the efficacy of BP1 and 10, each in combination with meropenem. This study aimed to ascertain if the *in vitro* efficacy could be replicated in an animal model. The outcome of combination therapy was successful and conveyed > 3 $\log_{10}$ reduction in the cfu/thigh count against meropenem monotherapy. The BP compounds were quantified in plasma because of increased bioavailability, which indicated that derivatizing NOTA was an excellent strategy. The overall findings of this research are that BP1 and BP10 are the first β-lactam metallo-β-lactamase derived inhibitor that offers potent activity in restoring meropenem's efficacy to susceptible MICs against carbapenem-resistant *Enterobacterales*. BP1 and 10 have proven to be lead MBLI compounds and demonstrate outstanding potential for further research and development. As part of the drug discovery process, future studies are required to assess the full potential of BP- 1 and 10 before advancing into clinical development. Future recommendation for these studies could include:

1. Microbiology and pharmacology packages with data against contemporary MDR strains from different geographies.

This will enable comparisons to be made using a larger bacterial population, providing more insight into resistance patterns and the predominant genes in circulation. The information obtained from these packages will also establish the drug concentrations needed *versus* the selected concentrations frequently utilized.

1.1. Acquisition and evaluation of a larger panel of MDR bacteria from different geographies

With the excellent bactericidal effect elicited by the BP series of inhibitors, testing on a larger bacterial panel of MDR isolates will provide more information on the inhibitors' antimicrobial spectrum of activity

1.2. Predicting the efflux ratio of the drug

This experiment will determine if the compounds are subject to efflux out of the cell due to the presence of efflux pumps. If it is predicted to efflux, the ratio at which it occurs is essential to determine if these drugs are a viable option or not, to further develop as potential MBLIs.

1.3. *In vivo* safety assessment with maximum tolerated dose (MTD) and Dose-Range-Finding studies.

The maximum tolerated dose and escalation of a single dose will provide more information on the characteristics and safety of the compounds at the determined dosage and promote regulatory studies using this accepted dose response. This needs to be achieved for more than eight hours for the BP compounds, using a larger study animal that can handle multiple dosing without adverse effects.

1.4. PK profile and compound tissue distribution.

A thorough investigation of the PK parameters (absorption, distribution, disposition, metabolism, and excretion) is still needed to determine the compounds' biodistribution. Drug elimination rate and accumulation of the drug and/or metabolites need to be determined, especially in relation to potential sites of action.

2. Chemistry management and controls (CMC) program strategy

In this category, research, including thermal hazard assessment of intermediates and products, generates enough candidate molecules to support the formal toxicology of the research compound according to good laboratory practice (GLP-tox).

3. Drug delivery insight

The goal here is to ensure the drug reaches the targeted area, doesn’t affect healthy tissue yet is still effective. The best route of administration i.e., oral, topical, membrane, intravenous, and or inhalation needs to be assessed *via* various formulation optimization methods.

Appendices

Appendix one: Chapter three

Biological Activity

The MIC for meropenem + NO3PY (mg/L): 0.06 + 4

Results from the *in vitro* time-kill assays conducted on NO3PY

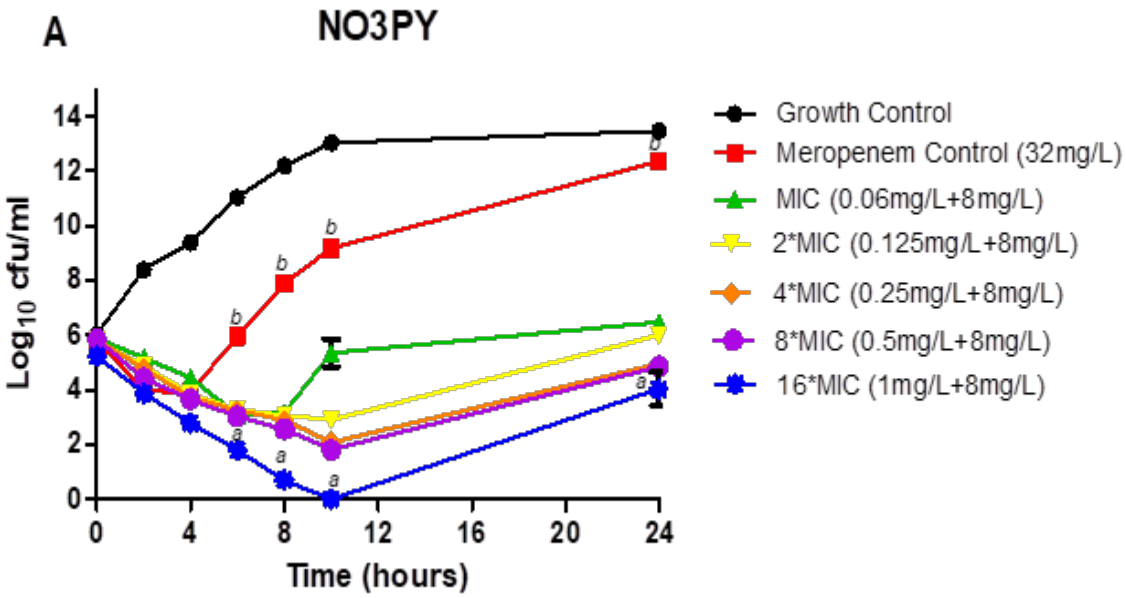

Figure S1: *E. coli* NDM-1 treated with multiple meropenem concentrations + 8 mg/L of NO3PY. Inhibitor, NO3PY indicates a significant difference ($P < 0.05$) between the meropenem control and 8*MIC (1 mg/L+8 mg/L) Symbol *a* indicates a significant increase in the rate of kill as compared to *b*. Data is represented as a mean ± SD

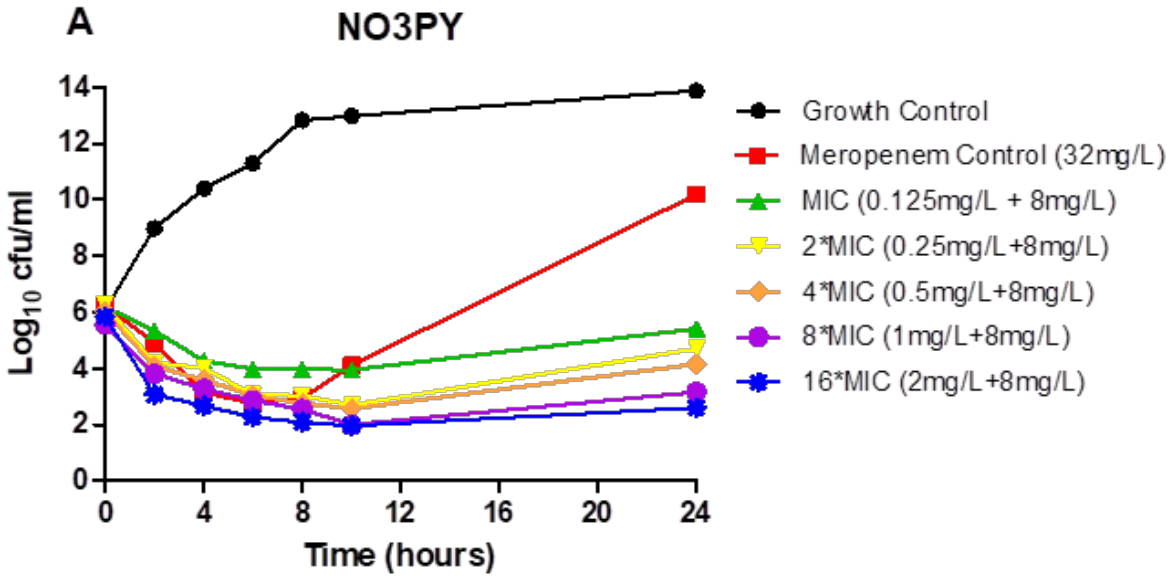

Figure S2: *K. pneumoniae* NDM-1 treated with multiple meropenem concentrations + 8 mg/L NO3PY. Data is represented as a mean ± SD.

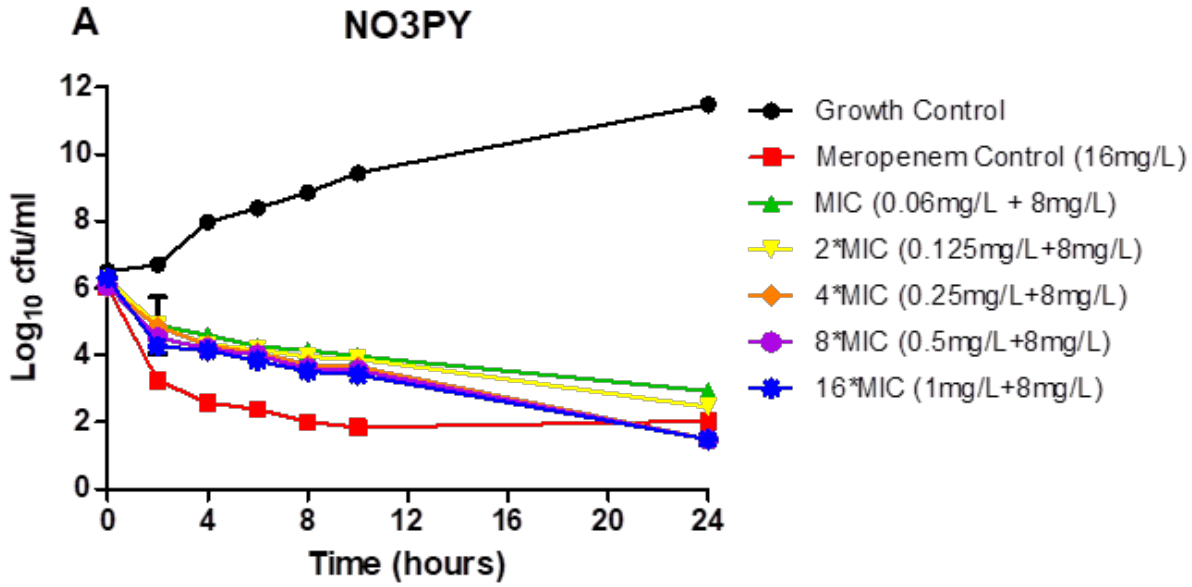

Figure S3: *E. coli* IMP-1 treated with multiple meropenem concentrations + 8 mg/L NO3PY Data is represented as a mean ± SD.

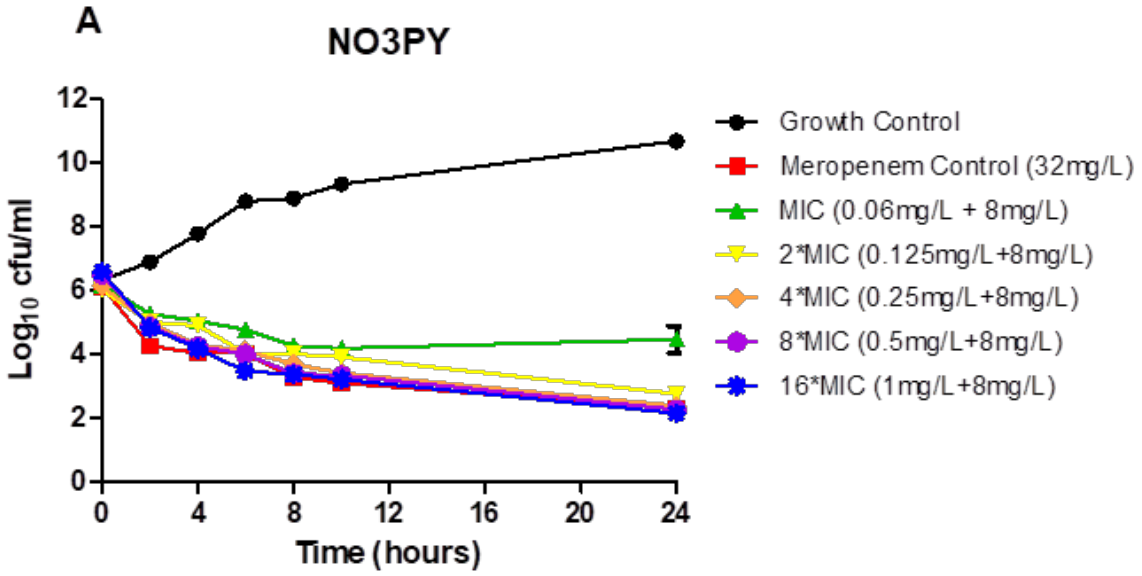

Figure S4: *E. cloacae* NDM-1 treated with multiple meropenem concentrations + 8 mg/L NO3PY. Data is represented as a mean ± SD.

Appendix two: Chapter four and five collectively

Biological Activity

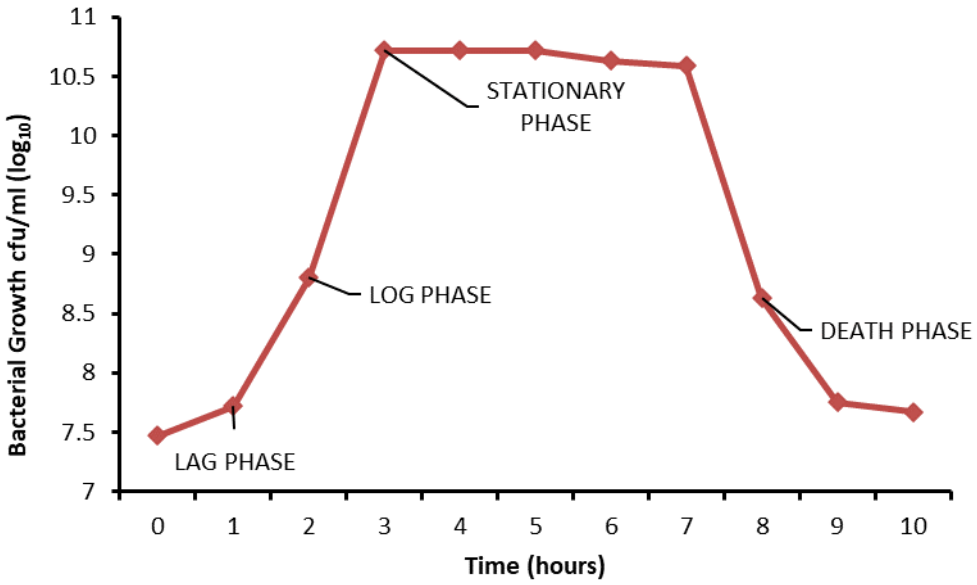

Figure S5: *K. pneumoniae* NDM bacterial growth curve, starting with a 0.5 McFarland standardized inoculum (7 cfu/ml $\log_{10}$ units). All bacterial growth phases were observed. Deduction made for the *in vivo* efficacy study the *K. pneumoniae* NDM takes approximately 2 hours to enter the log phase of growth (bacterial replication)

Calculation of the DMSO content for the preparation of BP 1, 6, 10 and 14 in the MIC assay:

5 mg of BP dissolves in 50 % DMSO to make a 1 mL stock

256 µl of this BP stock is diluted in 744 µl water to make a 1 mL working solution of 128 mg/L (12.8% DMSO)

700 µl of the working solution is diluted in 700 µl water to yield 1.4 mL of the desired concentration, 64 mg/L (6.4 % DMSO)

10 µl of the above desired concentration is added to each well of the micro titre plate with a final well volume of a 100 µl (0.64% DMSO)

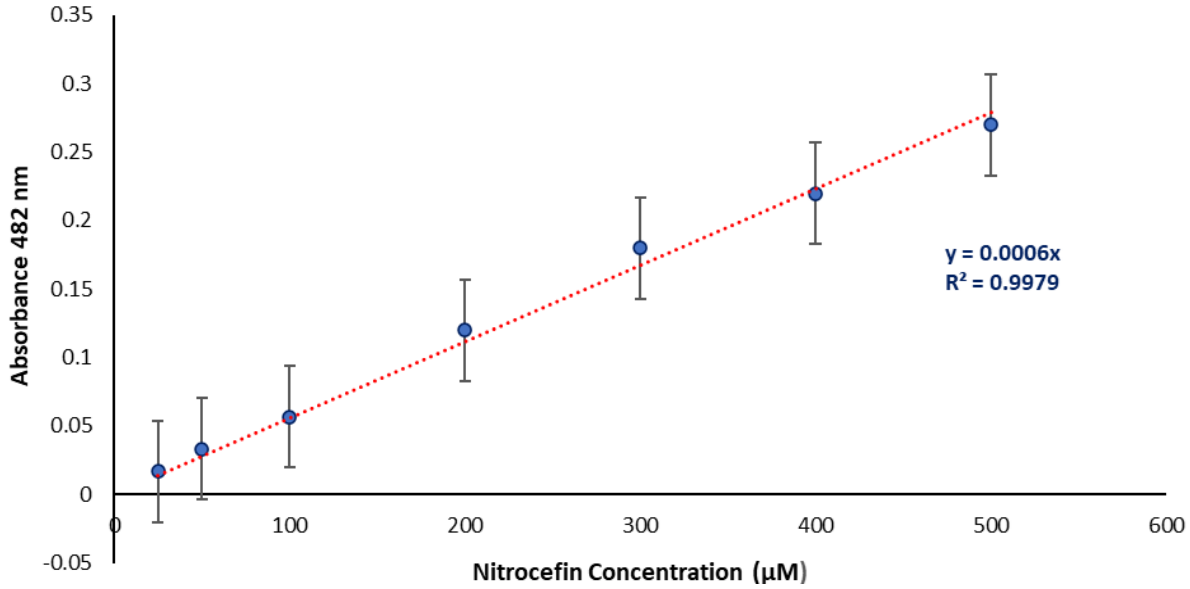

Fig S6: Nitrocefin calibration curve using a concentration range of 25-500 µM conducted in triplicate at three independent times

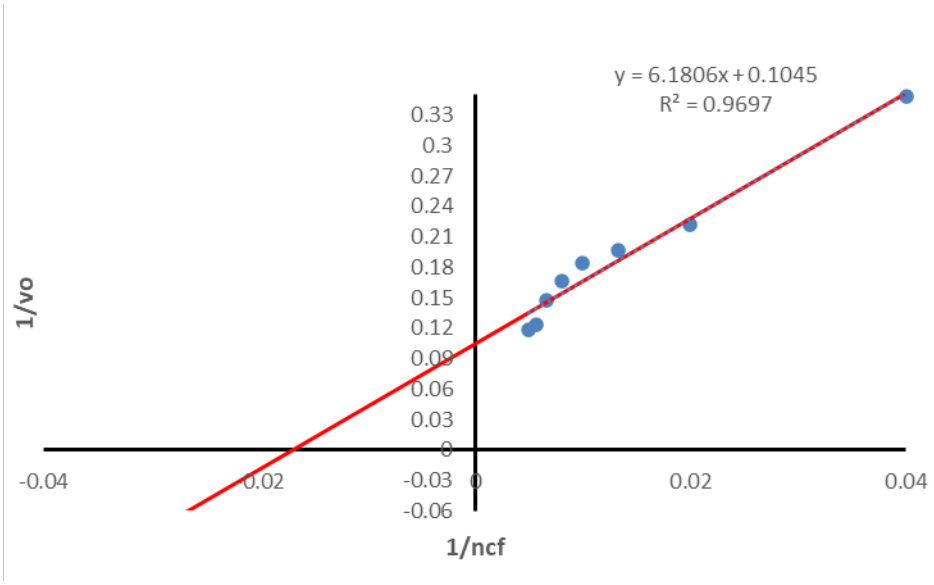

Figure S7: Kinetic assay of VIM-2, represented on a Lineweaver-burk plot

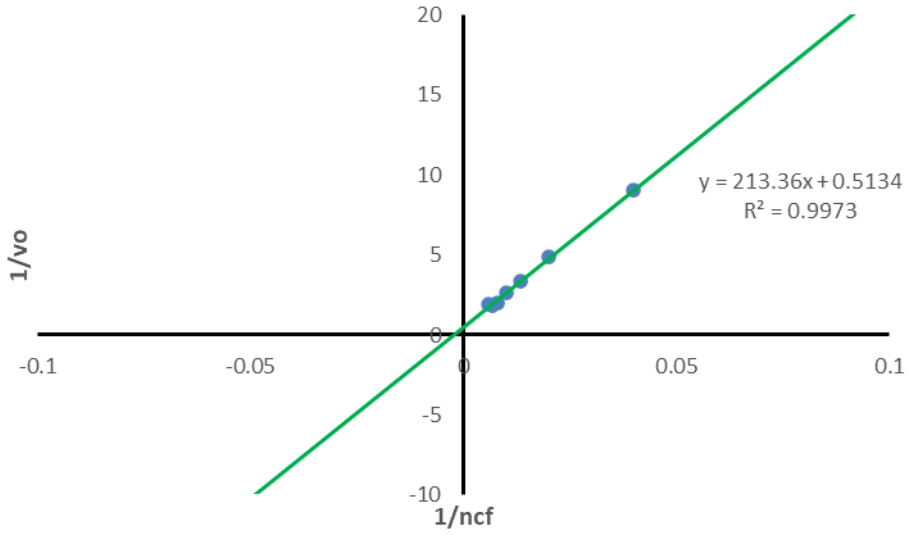

Figure S8: Kinetic assay of NDM-1, represented on a Lineweaver-burk plot.

The Cheng-Prusoff equation was used to calculate the inhibition constant (K*i*) to ascertain the level of specific inhibitor binding

$$\mathbf{K}i = \frac{\mathbf{IC50}}{\mathbf{1}+\frac{[\mathbf{S}]}{\mathbf{Km}}} \qquad \text{(eq.1)}$$

Since the intensity of quenching is directly proportional to the Stern-Volmer quenching constant (Ksv), Figure 3; A & B (Chapter four) and Figure 9 A & B (Chapter five) were generated using equation (2). Ksv was determined from the slope of F_0/F vs [Q].

$$\boldsymbol{F0/F = 1 + KSV[Q]} \qquad \text{(eq. 2)}$$

Modified Stern-Volmer plots were used to determine the binding constant (Ka) and the number of binding sites (n) from the slope and intercept of Figure 3; C & D (Chapter four) and Figure 9 C & D (Chapter five) using equation 3:

$$\mathbf{Log(F0 - F)/F = logKa + n\ log[Q]} \qquad \text{(eq.3)}$$

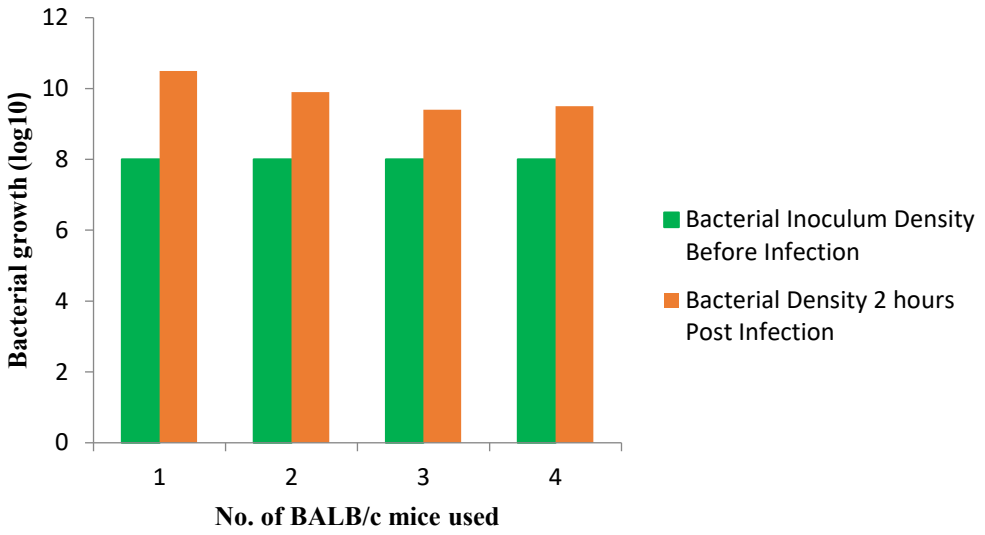

Figure S9: Growth of *K. pneumoniae* NDM in mice over 2 hours

Calculation of the immune suppressant (IS), cyclophosphamide doses per treatment group

150 mg/kg.b.w

If n=30 mice, make up drug for n=35 mice to ensure the volume is not lost in the needle

n=35 mice with an average weight of 23g

therefore, 35 x 23 = 805g

= 805g/1000g

= 0.805 kg

IS dose =0.805 x 150

= 120.75 mg in n=35 x 0.2 mL (volume) saline

= 120.75 mg in 7 mL saline

100 mg/kg.b.w

If n=30 mice, make up drug for n=35 mice to ensure the volume is not lost in the needle

n=35 mice with an average weight of 23g

therefore, 35 x 23 = 805g

= 805g/1000g

= 0.805 kg

IS dose =0.805 x 100

= 80.5 mg in n=35 x 0.2 mL (volume) saline

= 80.5 mg in 7 mL saline

Multiple dosing of animals for *in vivo* efficacy study per treatment regimen; S, M or BP-1/10:

Group 1 of mice received 0 dose

Group 2 of mice received 1 dose, n=24

Group 3 of mice received 2 doses, n=18

Group 4 of mice received 3 doses, n=12

Group 5 of mice received 4 doses, n=6

Calculation of BP-1/10 and meropenem doses in the *in vivo* efficacy study

A total of 60 doses are required for each treatment regimen, therefore make up extra for n=65

Using an average weight of 23g and dose of 100 mg/kg.b.w for meropenem and for BP-1/10:

n=65 x 23g

= 1495g/1000g

= 1.495 kg

= 1.495 kg x 100

=149.5 mg of M or BP-1/10

Dissolved in saline for meropenem and saline + DMSO (< 10 %) for BP

0.2 ml x 65 doses

= 13 mL

= 149.5 mg in 13 mL saline

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

06 May 2021

Mrs Nakita Reddy (209502875)
School of Health Sciences
Westville Campus

Dear Mrs Reddy,

Protocol reference number: AREC/019/019D
Project title: An in vivo efficacy study of novel metallo beta-lactamase inhibitors, in a murine infection model.

Full Approval – Renewal Application

With regards to your renewal application received on 06 May 2021. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 05 May 2022.

Please note: the study renewal in 2022 must be uploaded to the RIG online system as a new application.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Prof T Naicker
cc BRU Manager: Dr Jaca

Animal Research Ethics Committee
Telephone: 031 2608850
Email: animalethics@ukzn.ac.za
University Road
Chiltern Hills
Westville
3629
South Africa

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INYUVESI YAKWAZULU-NATALI

10 August 2021

Mrs Nakita Reddy (209502875)
School of Life Sciences
Westville Campus

Dear Mrs Reddy,

Protocol reference number: AREC/00002618/2021
Project title: An in vivo efficacy study of novel metallo beta-lactamase inhibitors co-administered with meropenem, in a murine infection model.

Full Approval – Research Application

With regard to your revised application received on 17 June 2021, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 09 August 2022.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee

/kr
cc Supervisor: Prof Tricia Naicker
cc BRU Manager: Dr Jaca

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Neutrophil and white blood cell count

This was done on mice for each time point to ensure that they were adequately immune suppressed

UPL

Universal Pathology Laboratory

12 The Avenue East, Prospecton,4133

PO Box 26411, Isipingo Beach, 4133

Tel: (031) 817 5570

Dr Khatija Ahmed Inc

Practice No: 0916803

Rgno /Barcode	: 177467 / 243650	**Registered On**	: 20/12/2021 9:15:30 AM
IDNo	: NA	**Sample Coll on**	: 20/12/2021 9:15:35 AM
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Patient Name	: T0 MOUSE 4	**Sample Type**	: EDTA-WB
Age / Sex	: 0 Years / Male	**Report Status**	: Final
DOB	: 20/12/2021	**Policy no**	:
Doctor/Client	: Dr.Catalysis and peptide research unit	**Tel no**	: 0000000000
Address	:		
Consult Doctor	: --SELECT--		6th Floor E Block, Room E1-06
Investigations	: FULL BLOOD COUNT		

HAEMATOLOGY

Investigation		Result	Flag	Units	Reference
FULL BLOOD COUNT					
Haemoglobin	:	**11.8**	L	g/dl	13.5 - 19.5
RBC Count	:	**7.29**	H	10^6/ul	5.00 - 7.00
PCV (Haematocrit)	:	**35.6**	L	%	41 - 70
MCV	:	48.9		fL	
MCH	:	**16.2**	L	pg/dl	24 - 34
MCHC	:	33.1		g/dl	30 - 35
RDW	:	**18.7**	H	%	11 - 16
Platelet Count	:	**454**	H	10^3/ul	150 - 450
WBC Count	:	**0.3**	L	10^3/ul	10 - 26
DIFFERENTIAL COUNT%					
Absolute Neutrophil Count	:	**0.0**	L	10^9/l	2.0 - 7.0
Neutrophilis	:	**4.5**	L	%	40 - 80
Absolute Lymphocyte Count	:	**0.3**	L	10^9/l	1.0 - 3.0
Lymphocytes	:	**86.6**	H	%	20 - 40
Absolute Eosinophil Count	:	**0.0**	L	10^9/l	0.02 - 0.5
Eosinophilis	:	**0.6**	L	%	1 - 6
Absolute Monocyte Count	:	**0.0**	L	10^9/l	0.2 - 1.0
Monocytes	:	6.4		%	2 - 10
Absolute Basophil Count	:	**0.0**	L	10^9/l	0.02 - 0.1

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PO Box 26411, Isipingo Beach, 4133
Tel: (031) 817 5570
Dr Khatija Ahmed Inc
Practice No: 0916803

Rgno /Barcode	: 177471 / 243654	**Registered On**	: 20/12/2021 9:17:39 AM
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Patient Name	: T2 MOUSE 2	**Sample Type**	: EDTA-WB
Age / Sex	: 0 Years / Male	**Report Status**	: Final
DOB	: 20/12/2021	**Policy no**	:
Doctor/Client	: Dr.Catalysis and peptide research unit	**Tel no**	: 0000000000
Address	:		
Consult Doctor	: --SELECT--		6th Floor E Block, Room E1-06
Investigations	: FULL BLOOD COUNT		

HAEMATOLOGY

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PCV (Haematocrit)	:	**30.2**	L	%	41 - 70
MCV	:	46.4		fL	
MCH	:	**15.4**	L	pg/dl	24 - 34
MCHC	:	33.1		g/dl	30 - 35
RDW	:	**20.3**	H	%	11 - 16
Platelet Count	:	**494**	H	10^3/ul	150 - 450
WBC Count	:	**0.4**	L	10^3/ul	10 - 26
DIFFERENTIAL COUNT%					
Absolute Neutrophil Count	:	**0.0**	L	10^9/l	2.0 - 7.0
Neutrophilis	:	**4.8**	L	%	40 - 80
Absolute Lymphocyte Count	:	**0.3**	L	10^9/l	1.0 - 3.0
Lymphocytes	:	**77.6**	H	%	20 - 40
Absolute Eosinophil Count	:	**0.0**	L	10^9/l	0.02 - 0.5
Eosinophilis	:	**0.0**	L	%	1 - 6
Absolute Monocyte Count	:	**0.1**	L	10^9/l	0.2 - 1.0
Monocytes	:	**16.4**	H	%	2 - 10
Absolute Basophil Count	:	**0.0**	L	10^9/l	0.02 - 0.1

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Practice No: 0916803

Rgno /Barcode	: 177461 / 243652	**Registered On**	: 20/12/2021 9:10:45 AM
IDNo	: NA	**Sample Coll on**	: 20/12/2021 9:10:50 AM
Passport no	:	**Reported On**	: 20/12/2021 10:59:45
Patient Name	: T4 MOUSE 1	**Sample Type**	: EDTA-WB
Age / Sex	: 0 Years / Male	**Report Status**	: Final
DOB	: 20/12/2021	**Policy no**	:
Doctor/Client	: Dr.Catalysis and peptide research unit	**Tel no**	: 0000000000
Address	:		
Consult Doctor	: --SELECT--		6th Floor E Block, Room E1-06
Investigations	: FULL BLOOD COUNT		

HAEMATOLOGY

Investigation		Result	Flag	Units	Reference
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Haemoglobin	:	**13.2**	L	g/dl	13.5 - 19.5
RBC Count	:	**8.30**	H	10^6/ul	5.00 - 7.00
PCV (Haematocrit)	:	**38.4**	L	%	41 - 70
MCV	:	46.3		fL	
MCH	:	**15.9**	L	pg/dl	24 - 34
MCHC	:	34.4		g/dl	30 - 35
RDW	:	**17.2**	H	%	11 - 16
Platelet Count	:	**485**	H	10^3/ul	150 - 450
WBC Count	:	**0.9**	L	10^3/ul	10 - 26
DIFFERENTIAL COUNT%					
Absolute Neutrophil Count	:	**0.1**	L	10^9/l	2.0 - 7.0
Neutrophilis	:	**12.2**	L	%	40 - 80
Absolute Lymphocyte Count	:	**0.7**	L	10^9/l	1.0 - 3.0
Lymphocytes	:	**76.3**	H	%	20 - 40
Absolute Eosinophil Count	:	**0.0**	L	10^9/l	0.02 - 0.5
Eosinophilis	:	2.3		%	1 - 6
Absolute Monocyte Count	:	**0.1**	L	10^9/l	0.2 - 1.0
Monocytes	:	7.9		%	2 - 10
Absolute Basophil Count	:	**0.0**	L	10^9/l	0.02 - 0.1

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Rgno /Barcode	: 177476 / 243768	**Registered On**	: 20/12/2021 9:22:01 AM
IDNo	: NA	**Sample Coll on**	: 20/12/2021 9:22:06 AM
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Patient Name	: T6 MOUSE 4	**Sample Type**	: EDTA-WB
Age / Sex	: 0 Years / Male	**Report Status**	: Final
DOB	: 20/12/2021	**Policy no**	:
Doctor/Client	: Dr.Catalysis and peptide research unit	**Tel no**	: 0000000000
Address	:		
Consult Doctor	: --SELECT--		6th Floor E Block, Room E1-06
Investigations	: FULL BLOOD COUNT		

HAEMATOLOGY

Investigation		Result	Flag	Units	Reference
FULL BLOOD COUNT					
Haemoglobin	:	**10.4**	L	g/dl	13.5 - 19.5
RBC Count	:	6.61		10^6/ul	5.00 - 7.00
PCV (Haematocrit)	:	**31.9**	L	%	41 - 70
MCV	:	48.2		fL	
MCH	:	**15.7**	L	pg/dl	24 - 34
MCHC	:	32.6		g/dl	30 - 35
RDW	:	**23.2**	H	%	11 - 16
Platelet Count	:	317		10^3/ul	150 - 450
WBC Count	:	**0.3**	L	10^3/ul	10 - 26
DIFFERENTIAL COUNT%					
Absolute Neutrophil Count	:	**0.0**	L	10^9/l	2.0 - 7.0
Neutrophilis	:	**3.0**	L	%	40 - 80
Absolute Lymphocyte Count	:	**0.2**	L	10^9/l	1.0 - 3.0
Lymphocytes	:	**76.7**	H	%	20 - 40
Absolute Eosinophil Count	:	**0.0**	L	10^9/l	0.02 - 0.5
Eosinophilis	:	**0.5**	L	%	1 - 6
Absolute Monocyte Count	:	**0.1**	L	10^9/l	0.2 - 1.0
Monocytes	:	**19.8**	H	%	2 - 10
Absolute Basophil Count	:	**0.0**	L	10^9/l	0.02 - 0.1

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Dr Khatija Ahmed Inc
Practice No: 0916803

Rgno /Barcode	: 177478 / 243765	**Registered On**	: 20/12/2021 9:23:46 AM
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Patient Name	: T8 MOUSE 2	**Sample Type**	: EDTA-WB
Age / Sex	: 0 Years / Male	**Report Status**	: Final
DOB	: 20/12/2021	**Policy no**	:
Doctor/Client	: Dr.Catalysis and peptide research unit	**Tel no**	: 0000000000
Address	:		
Consult Doctor	: --SELECT--		6th Floor E Block, Room E1-06
Investigations	: FULL BLOOD COUNT		

HAEMATOLOGY

Investigation		Result	Flag	Units	Reference
FULL BLOOD COUNT					
Haemoglobin	:	**12.6**	L	g/dl	13.5 - 19.5
RBC Count	:	**7.69**	H	10^6/ul	5.00 - 7.00
PCV (Haematocrit)	:	**38.1**	L	%	41 - 70
MCV	:	49.5		fL	
MCH	:	**16.4**	L	pg/dl	24 - 34
MCHC	:	33.1		g/dl	30 - 35
RDW	:	**17.8**	H	%	11 - 16
Platelet Count	:	283		10^3/ul	150 - 450
WBC Count	:	**2.0**	L	10^3/ul	10 - 26
DIFFERENTIAL COUNT%					
Absolute Neutrophil Count	:	**0.0**	L	10^9/l	2.0 - 7.0
Neutrophilis	:	**1.0**	L	%	40 - 80
Absolute Lymphocyte Count	:	**0.6**	L	10^9/l	1.0 - 3.0
Lymphocytes	:	30.4		%	20 - 40
Absolute Eosinophil Count	:	0.2		10^9/l	0.02 - 0.5
Eosinophilis	:	**8.6**	H	%	1 - 6
Absolute Monocyte Count	:	**1.2**	H	10^9/l	0.2 - 1.0
Monocytes	:	**59.4**	H	%	2 - 10
Absolute Basophil Count	:	**0.0**	L	10^9/l	0.02 - 0.1

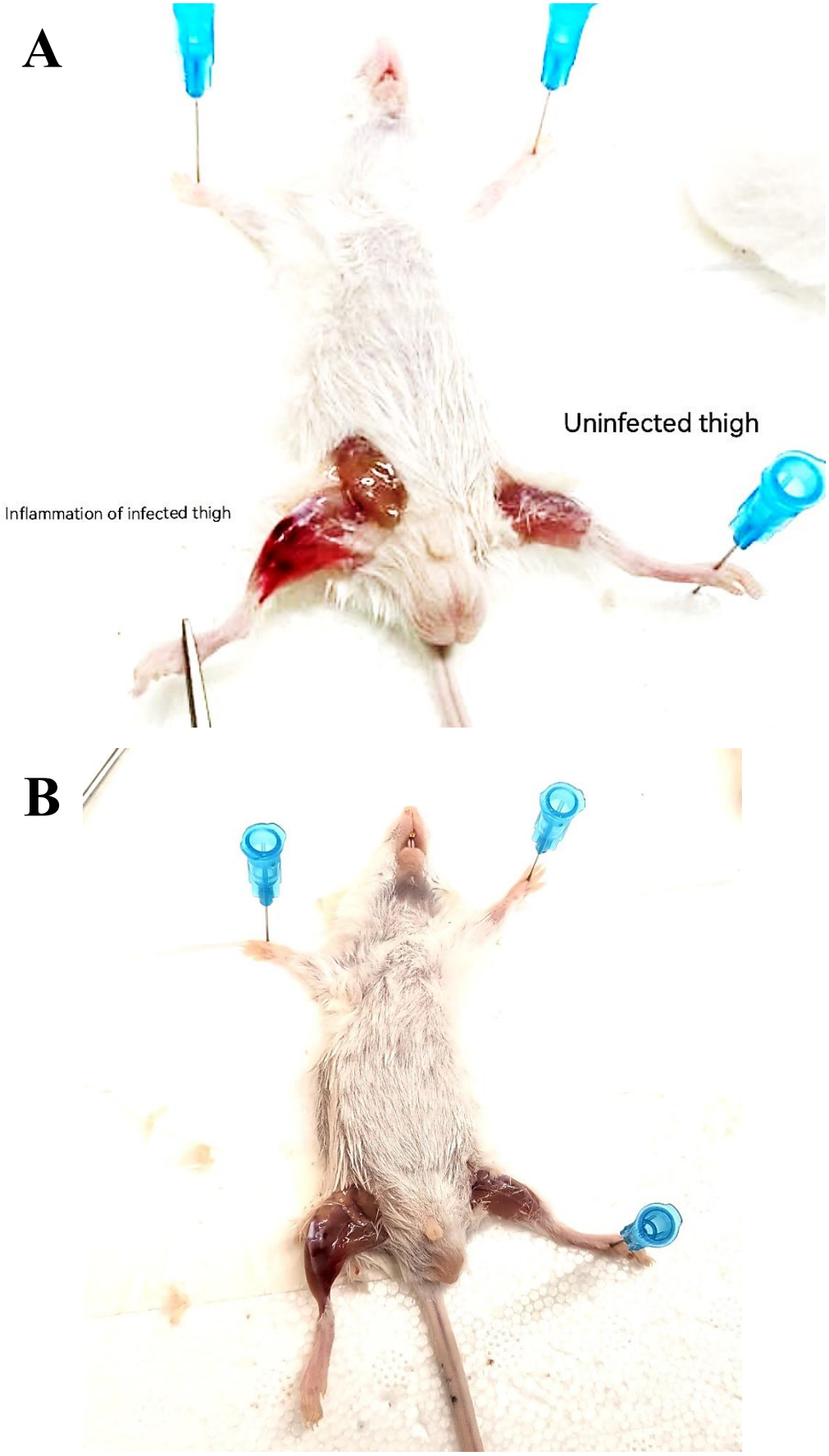

Figure S10: Inflammation of the right infected thigh. This image is of 2 different animals belonging to the meropenem monotherapy regimen. The uninfected thigh is shown for comparison and to

indicate infection was localized to the right thigh. **A** Shows red inflammation of the tissue. **B** shows swelling and a softer consistency in the feel of the tissue, another indication of inflammation

Computational Methods

System preparation

The wild-type NDM-1 and VIM-2 single x-ray crystal structures from bacterial strains were retrieved from the protein data bank (PDB ID 5LSC & 4RL0) [1, 2]. The crystal water molecules were removed, and only chain A was considered for each enzyme. Both enzymes have two Zn^{2+} metal ions in the active site trivalently coordinated by the histidine's (HIS), aspartate(ASP), and cysteine (CYS) amino acid residues. The atomic positions of the crystal-bound ligands in the active site of both enzymes were used to generate the grid box and later removed. The NDM-1 enzyme has a catalytic water molecule participating in the coordination of the two zinc metal ions. The BP-1/10 compound was modeled using the GaussView program and further optimized with the DFT B3LYP/6-31+G(d) level of theory.

Molecular docking of BP-1/10 into NDM-1 and VIM-2

Molecular interaction analysis of BP-1/10 compound with both NDM-1 and VIM-2 was performed by molecular docking using AutoDock Vina[3] implemented in UCSF Chimera 1.15 [4]. The grid box generated using the active site ligand position was used to dock the BP-1/10 compound, and the docking exhaustiveness was set to 8 with an energy interval of 3. The docking results were ranked according to their docking scores combined with the RMSD values. The best conformation was selected based on visual inspection (the chemical intuition) and the RMSD cut-off of <2 Å.

MD simulation

Post molecular docking, the bond orders' assignment and hydrogenation for the complexes were performed using Protein Preparation Wizard in Schrodinger Maestro (Schrödinger

Release 2021-4: Maestro, Schrödinger, LLC, 2021). The ionization state of the BP-1/10 was determined using Epik at a suitable pH of 7.0 ± 2.0 [5]. The protonation state was determined using PROPKA embedded in Maestro [6]. The restrained energy minimization of the complexes was performed using the OPLS4 force field [7]. MD simulations were performed using Desmond [8] to assess the interactions and evaluate the binding free energy profiles of the complexes. The "System Setup" utility in Maestro was used to set up all the energy minimized systems by placing them in an orthorhombic box with a buffer distance of 10 Å. A TIP3P [9] solvation model with a 9 Å cut-off for van der Waals was used with time step, initial temperature, and pressure of the systems set to 2.0 fs, 300K, and 1.01325 bar, respectively, neutralized with a 0.15 M NaCl buffer. Furthermore, the sampling interval during the simulation was set to 50 ps, and the MD simulations were performed under the NPT ensemble for 1 μs.

Post-MD trajectory analyses

The MD trajectories were analyzed using the "simulation interaction diagram" tool in Maestro to assess the interactions between the BP-1/10 and the enzymes investigated in this study. The root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), and solvent accessible surface area (SASA) were assessed to provide the systems' stability, dynamic behavior (fluctuations), and solvent accessibility metrics, respectively, during the MD simulation run. The Desmond trajectory clustering tool in Maestro was used to attain representative structures for calculating the binding free energy. The extraction interval was 10 frames, and 2000 frames were used for clustering after MD simulations. The Prime MMGBSA tool in Maestro was used to conduct the binding free energy calculations. The VSGB solvation model [10] and OPLS4 force field [7] were set for binding free energy calculation.

References

1. Christopeit T, Yang K-W, Yang S-K, Leiros H-K: **The structure of the metallo-β-lactamase VIM-2 in complex with a triazolylthioacetamide inhibitor**. *Acta Crystallographica Section F: Structural Biology Communications* 2016, **72**(11):813-819.
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4. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE: **UCSF Chimera—a visualization system for exploratory research and analysis**. *Journal of computational chemistry* 2004, **25**(13):1605-1612.
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Appendix three: Chapter four

Biological Activity

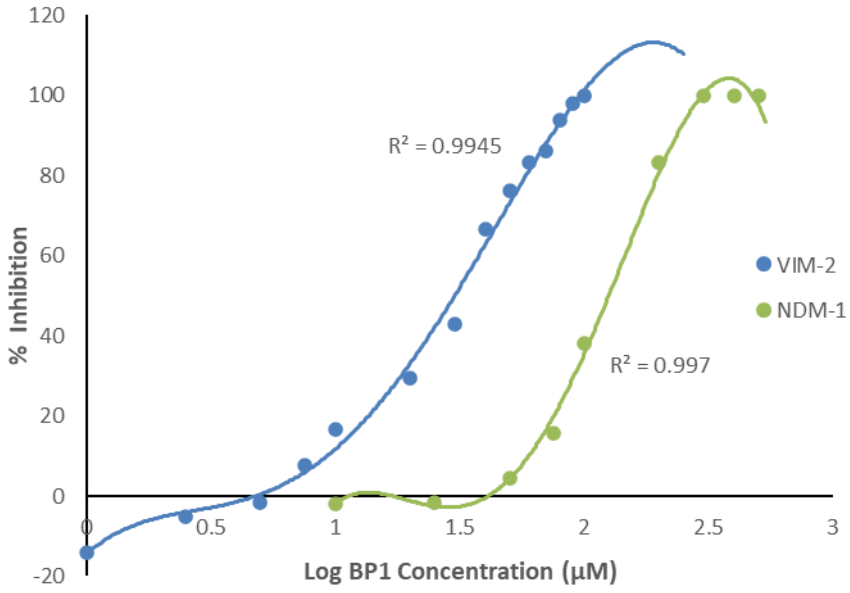


Figure S11: Half the maximal inhibitory concentration of BP1 against NDM-1 and VIM-2

Table S1: Inhibition exerted by BP 1, NOTA, EDTA and TPEN on glyoxylase II

Inhibitor	% Inhibition	
	100 µM	500 µM
BP1	0.17	4.4
NOTA	0.85	5.98
EDTA	38.8	41
TPEN	47.6	N/A

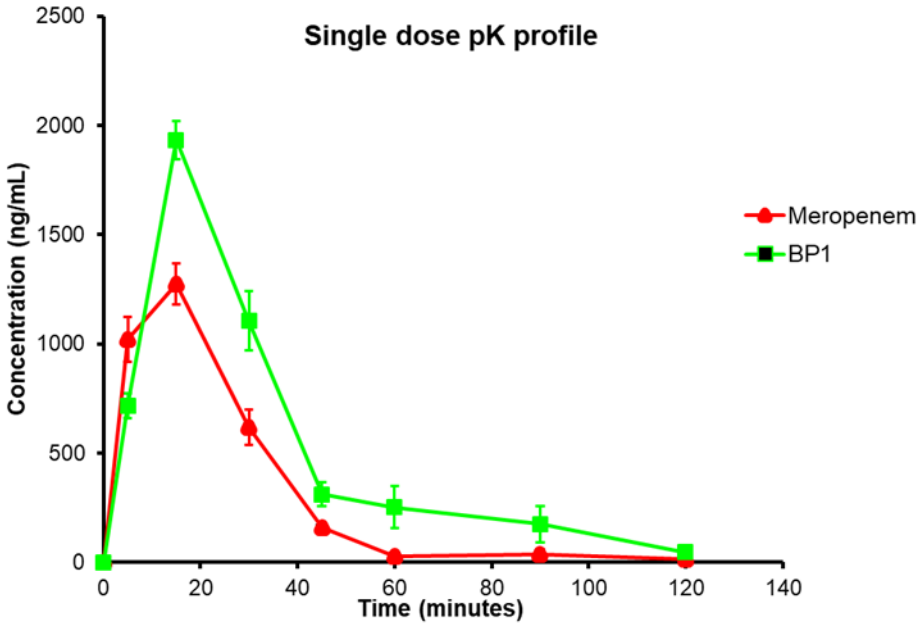


Figure S12: Single dose pharmacokinetic profile of BP1 and Meropenem (10 mg/kg.b.w each). Data is represented as a mean ± SD (n=3)

Balb/c mice were injected intraperitoneally with meropenem only as well as with BP 1 only. These results indicate that BP1 and meropenem shared a similar pharmacokinetic profile, dosing therefore, needs to occur every two hours, in addition it is safe to increase the dose, and administer BP1 and meropenem at 100 mg/kg.b.w. *Dose calculated in the same as (IS-Appendix one)

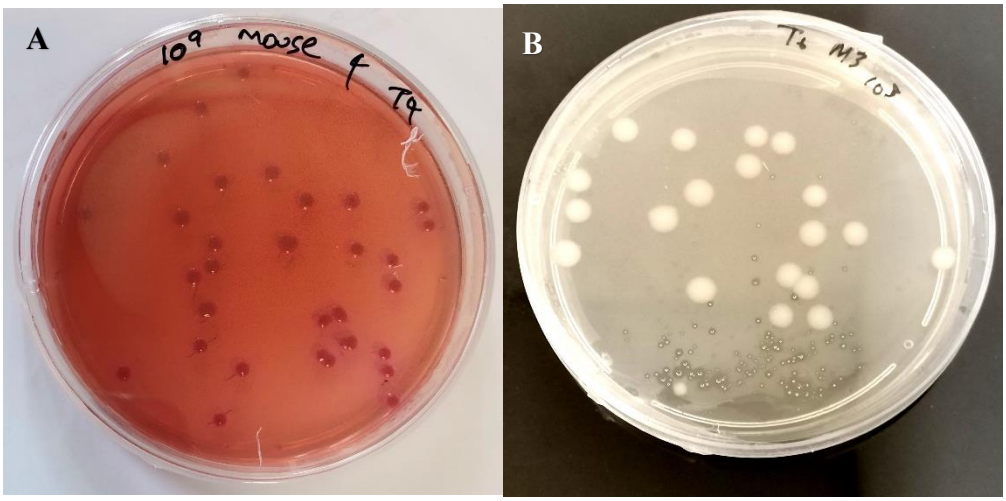


Figure S13: Images of the cfu/thigh count from the BP1 + meropenem treatment group. **A** show a higher dilution factor (10^9) four-hour post infection (two doses of combination drug) as compared to **B** (10^3) six hours post infection (three doses of combination drug), indicating combination therapy is working

Table S2: *Klebsiella pneumoniae* NDM infected mice receiving saline only

Treatment Time (h)	Best Log10 Cfu/thigh	Mean Log10 Cfu/thigh	Std Dev	RSD (%)
2	8.1	8.7	0.8	9.7
4	11.3	11.7	0.6	5.1
6	12.6	12.6	1	8
8	12.8	13	0.7	5.3

Table S3: *Klebsiella pneumoniae* NDM infected mice receiving meropenem only treatment

Treatment Time (h)	Best Log10 Cfu/thigh	Mean Log10 Cfu/thigh	Std Dev	RSD (%)
2	7.2	6.8	0.4	6.5
4	8	8.3	0.5	5.5
6	8.3	8.4	0.3	3.6
8	8.3	8.2	0.2	2.1

Table S4: *Klebsiella pneumoniae* NDM infected mice receiving BP1 and meropenem treatment

Treatment Time (h)	Best Log10 Cfu/thigh	Mean Log10 Cfu/thigh	Std Dev	RSD (%)
2	4.8	4.8	0.1	2
4	4.1	4.1	0.4	9.7
6	3.9	4	0.2	4
8	3.5	3.7	0.1	3.6

Table S5: Summary of plasma drug concentrations from the *in vivo* efficacy study

Time (Hour)	Meropenem Only (ng/mL)	Meropenem with BP1 (ng/mL)	BP1 with Meropenem (ng/mL)	SD	SD	SD
0	0	0	0	0	0	0
2	1724	1535	857	10.6	50.2	143.5
4	17790	11657	3265	313.4	32.9	168.1
6	4650	5321	1977	98.5	42	111.3
8	3855	2922	1557	4.3	375.4	140.2
10	1958	2563	1171	32.9	15.4	192.5

Computational Results

There were slight differences in the final orientations of BP1 for NDM1 versus VIM2 after the docking process. The carboxylic arm of the chelator coordinated Zn302 in NDM1 and Zn405 in VIM2.

A 100ns MD simulation was conducted to evaluate the complexes' stability and determine their binding free energy profiles. The MD trajectories reveal that the BP1 remained intact or coordinated to the Zn302 in NDM1 with the carboxylic moiety stabilized by hydrogen bond and cation-pi interactions, while in VIM2, Zn405 was coordinated by the carboxylic group of the linker, as depicted in **Figure S14** In the NDM-1 active site, the two Zn^{2+} ions coordinate the water molecule's oxygen, which may play a significant role in the reaction mechanism. It can be observed that a water molecule forms a hydrogen bond with the BP1 carbonyl oxygen. The BP1 diastereomers (SR and SS) were evaluated to provide the binding free energy landscape of the two structures.

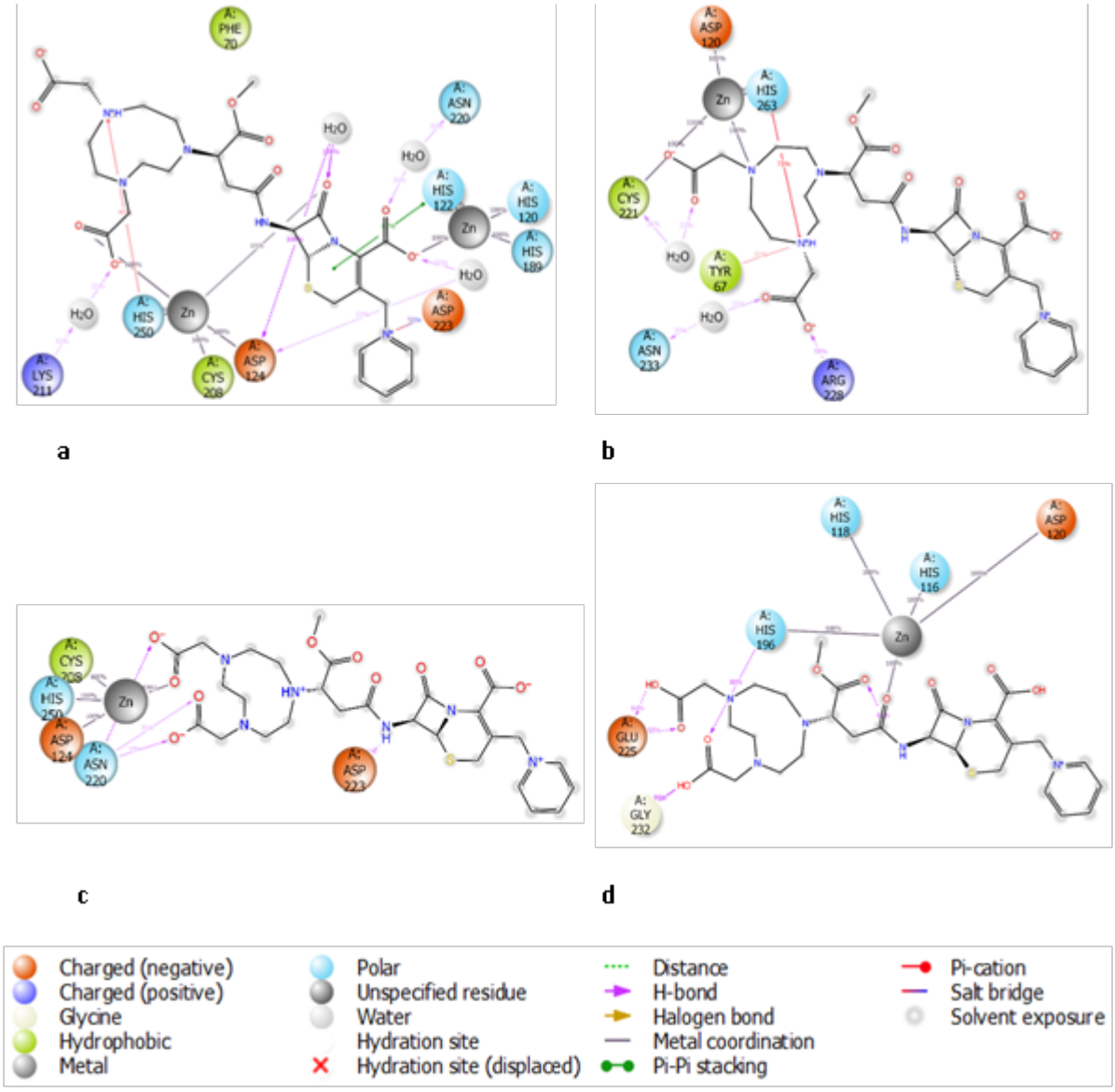


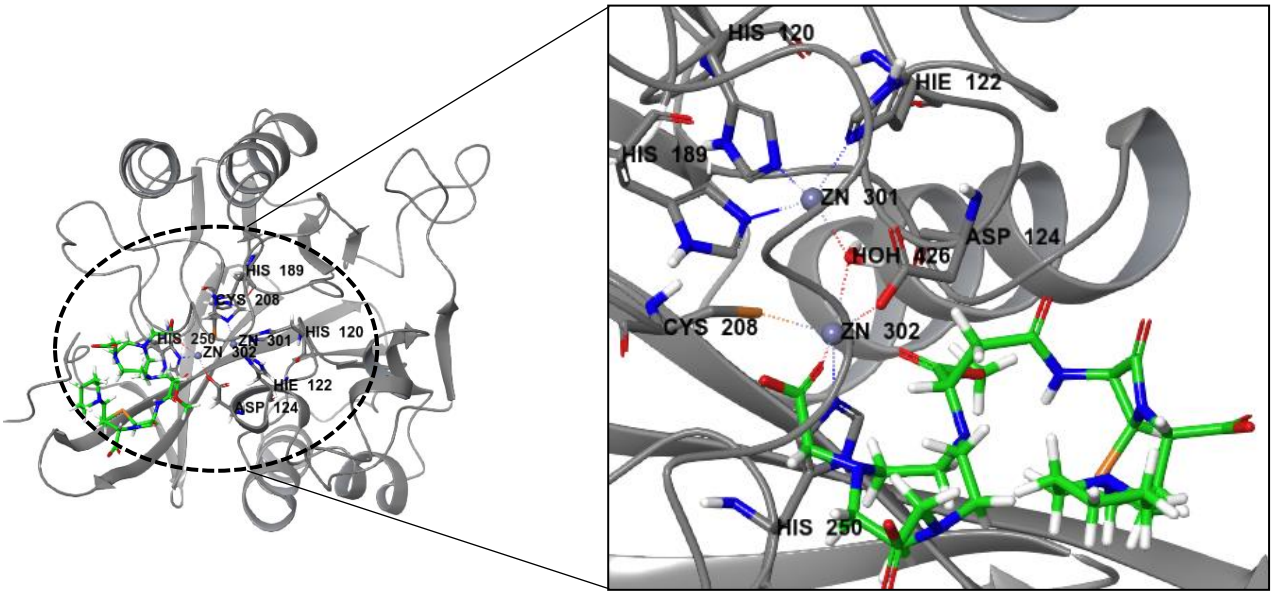
Figure S14: a) NDM1—BP1_SR, b) VIM2—BP1_SR, c) NDM—BP1_SS, and d) VIM2—BP1_SS plots showing the interactions in the active site. As observed, the strength of the coordinated species of the chelator is 100% to the zinc ion, indicating full coordination.

The prime MM-GBSA method implemented in Schrodinger was used to assess the binding free energies of the investigated systems. The binding affinity results of BP1 towards NDM1 and VIM2 are thermodynamically favorable except for the NDM1—BP1-SR complex, as reflected by the binding free energies in **Table S6** and have the potential to chelate the Zn^{2+} ions from the active sites. The energy discrepancy between the investigated systems is marginal. The NDM—BP1_SS diastereomer coordinated the Zn^{2+} (302) ion with two carboxylic groups of the chelator, indicating the possibility of chelating the zinc out of the

active site. Subsequent studies will use PACs-MD[11] to determine if the process of NOTA chelation of the zinc ion is energetically feasible.

Table S6: Docking scores and the binding free energies of the investigated systems. The energies are presented in KJ/mol.

Complexes	Docking score (KJ/mol)	DG_{bind} (KJ/mol)
NDM1_BP1_SR	-25.10	40.25
NDM1—BP1_SS	-25.94	-23.14
VIM2—BP1_SR	-27.20	-46.28
VIM2—BP1_SS	-25.52	-42.63



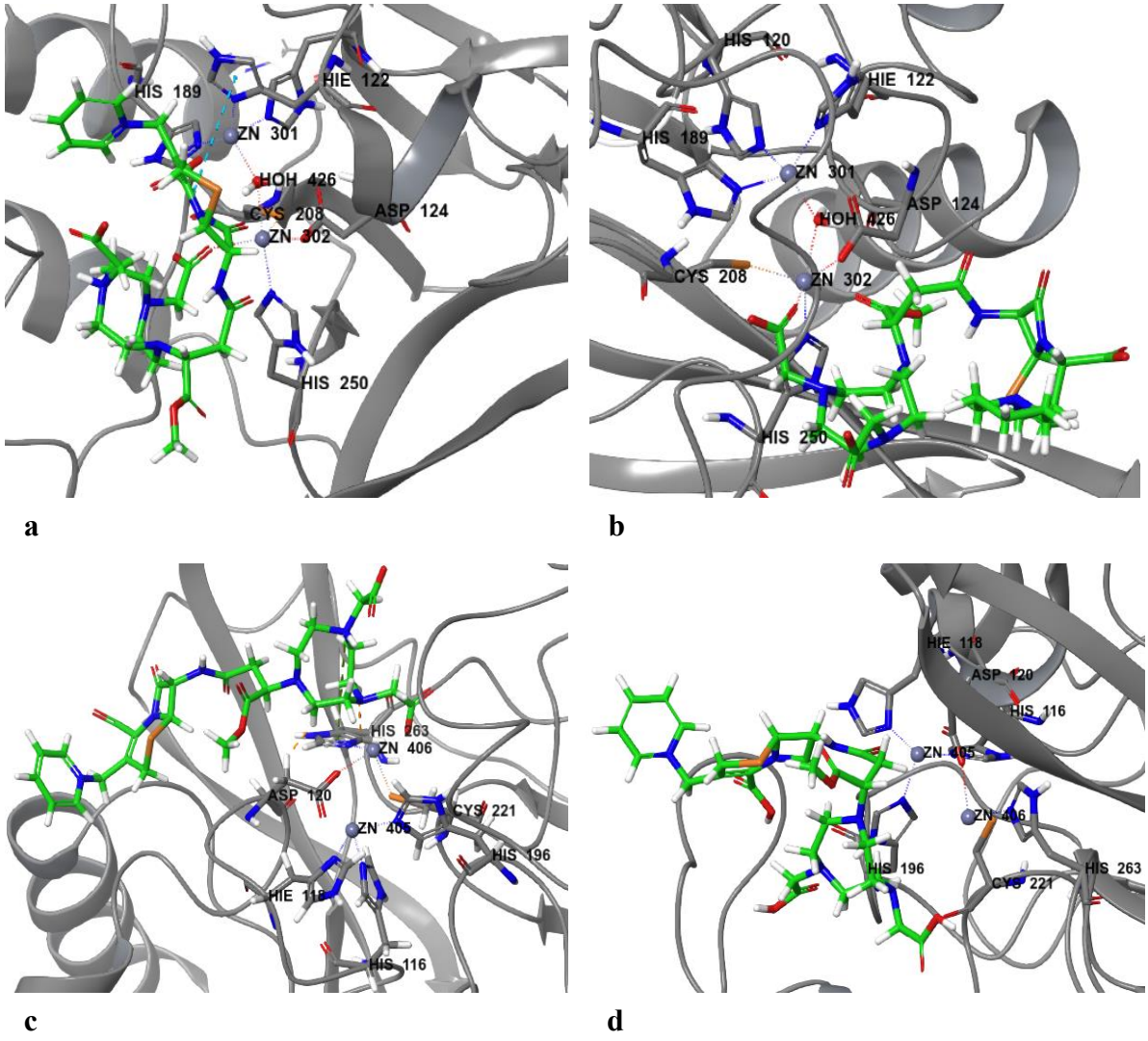


Figure S15: 3D representation of the complexes investigated. **a**) NDM1—BP1_SR, **b**) NDM1—BP1_SS, **c**) VIM2—BP1_SR, and **d**) VIM2—BP1_SS, where the 2D representations in Figure 2 were captured.

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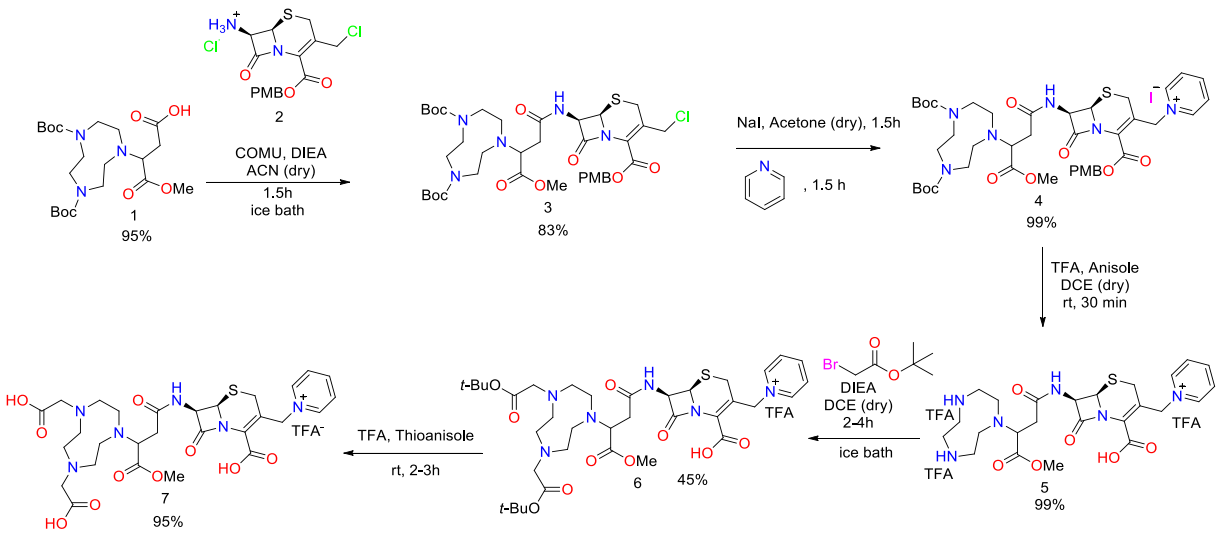
Synthesis of BP1

Antibiotics (lactams) were purchased from Sigma Aldrich (Germany), DLD Scientific (South Africa) and Hangzhou Dayangchem Co., Ltd (China). Reagents and solvents were purchased from Sigma Aldrich and Merck. All solvents were dried by means of standard procedures. Thin Layer Chromatography (TLC) was performed using Merck Kieselgel 60 F254 plates. All the synthetic steps were monitored using LC-MS (Shimadzu 2020 UFLC-MS, Japan). The LC-MS method used a gradient of 5% ACN: H_2O (0.1% formic acid) to 95% ACN: H2O (0.1% formic acid) over 9 minutes on an XBridgeTM C18 5um 4.6x150mm column, where the flow rate is 1mL/min. Purification of the intermediates was done by either gravity column chromatography (mesh particle size, 40-63 μm) and preparatory supercritical fluid chromatography performed on a Sepiatec Prep SFC basic/basic 30 (Germany). High resolution mass spectrometric data were obtained with a Bruker micrOTOF-Q II instrument that operated at ambient temperatures and at a sample concentration of 2 μg/ml. Infrared spectrometric data were recorded on a Perkin Elmer spectrum 100 instrument with a universal ATR attachment. NMR data were recorded using a Bruker AVANCE III 400 MHz at room temperature. Chemical shifts are expressed in ppm and coupling constants are reported in Hz. All melting points are uncorrected.

Note for the NMR spectra of the final compounds:

The NMR spectra of the final compounds appeared with much overlapping due to conformers. This was attributed to the presence of rotamers and/ or the ability of the chelator moieties to appear bent due to the 3D structure of the lactam (four membered ring adjacent to five/ six membered ring).[12] The floppy nature of the molecule is likely to result in the broadening of the signals leading to the poor resolution of the multiplets. This is further complicated by overlapping signals arising from the many protons in similar environments. The integration corresponded to the number of protons on the products however the spectra appeared ‘messy’ due to this overlap. After consulting with NMR experts (collaborators in Sweden), by subjecting the samples in their labs to temperature variation as well as complexing the chelators with either Zn or Cu, no significant changes in the spectra were observed.[13] Hence the NMR spectra of all starting material was recorded and confirmed. The NMR spectra of all chelators on its own also displayed much overlap. For these reasons, the final compounds were further treated and characterized as per peptides in organic synthesis in which the NMR spectra is not

recorded however supported by other means of characterization i.e. HRMS, LCMS and IR.[14] All compounds herein have a final purity of >95% as evidenced by the LCMS traces.



7-Amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (2)

Off-white solid powder, ^{1}H NMR (400 MHz, DMSO): δ 7.33 (d, J = 8.69 Hz, 2H), 6.90 (d, J = 8.65 Hz, 2H), 5.2 (m, 4H), 4.56 (d, J = 11.46 Hz, 1H), 4.47 (d, J = 11.48 Hz, 1H), 3.76 (d, J = 18.10 Hz, 2H) , 3.71 (s, 3H), 3.66 (d, J = 18.10 Hz, 2H); ^{13}C NMR (100 MHz, DMSO): δ 161.1, 160.8, 159.8, 130.8, 127.9, 127.2, 125.3, 114.2, 67.9, 58.3, 55.6, 54.6, 43.7, 27.0.

di-tert-butyl 7-(4-(((6R,7R)-3-(chloromethyl)-2-(((4-methoxybenzyl)oxy)carbonyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-7-yl)amino)-1-methoxy-1,4-dioxobutan-2-yl)-1,4,7-triazonane-1,4-dicarboxylate (3)

Compound **1** (1.0 equiv.)[15] was dissolved in dry ACN (1.0 mL/mmol), dry DIEA (3.0 equiv.) was then added, followed by COMU (1.1 equiv.), in an ice bath at 0 ºC. After 30 seconds to 1 minute, compound **2** (1.0 equiv.) was added, and the reaction was allowed to gently warm up to room temperature whilst in the ice bath. After 1.5 hours, the reaction was completed (monitored by LC-MS). Purification of **3** was performed by wet-loading the reaction mixture without concentration over a short neutral alumina column packed with EtOAc:Hexane (1:9) and immediately eluted with EtOAc:Hexane (1:1). TLC was done in EtOAc:Hexane, 50:50 Rf

~ 0.35). Relevant fractions were collected and concentrated *in vacuo* (water bath temperature 40°C and below) to afford 83 % yield. MS (ESI, H_2O:ACN): *m/z* = 810 ($(m+H)^+$). HRMS (ESI+) m/z (m+H) calculated for $C_{37}H_{52}ClN_5O_{11}S$: 810.3145. Found: 810.3095. MS (ESI, H_2O:ACN): *m/z* = 810 ($(m+H)^+$). I.R. (υ_{max}/cm^{-1}) 3297.14, 2917.23, 2850.63, 2163.37, 2027.70, 1781.68, 1729.59, 1682.79, 1514.94, 1462.88, 1392.11, 1365.21, 1244.86, 1159.14, 1030.41, 990.92, 822.56, 773.34, 718.76, 569.21, 518.69.

1-(((6R,7R)-7-(3-(4,7-bis(tert-butoxycarbonyl)-1,4,7-triazonan-1-yl)-4-methoxy-4-oxobutanamido)-2-(((4-methoxybenzyl)oxy)carbonyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methyl)pyridin-1-ium iodide (4)

Compound **3** (1.0 equiv.) was dissolved in dry acetone (4.0 mL/mmol to 4) and the flask was covered with aluminum foil to shield the reaction mixture from outside light. Sodium iodide (1.1 equiv.) was then added, in one shot at room temperature and the reaction stirred for 1.5 hours. Thereafter, pyridine (1.1 equiv.) was added, and the reaction was stirred for another 1.5 hours (progress of this step monitored by LC-MS). Quick inspection revealed that the solution had turned to a red/purple. The solvent was then removed *in vacuo* (water bath temperature 40°C and below), producing a red slurry, which was washed using the following washing procedure.

Washing procedure

The compound was sonicated (5 minutes) in Et_2O (~10 mL) and centrifuged between 5000-9000 rpm (over minimum 2 minutes) thereafter the washing decanted this was repeated twice. The solid was allowed to dry without absorbing moisture under a gentle stream of argon gas to afford pure **4** as a pale yellow fluffy powder (99 % yield). MS (ESI, H_2O:ACN): *m/z* = 853 ($(m+H)^+$). HRMS (ESI+) m/z (m+H) calculated for $C_{42}H_{57}IN_6O_{11}S$: 853.3806. Found: 853.3742. MS (ESI, H_2O:ACN): *m/z* = 853 ($(m+H)^+$). I.R. (υ_{max}/cm^{-1}) 3421, 2932, 2031, 1776, 1720, 1611, 1513, 1396, 1364, 1302, 1245, 1173, 1110, 1065, 1025, 894, 840, 771, 678, 637, 557.

1-(((6R,7R)-2-carboxy-7-(4-methoxy-4-oxo-3-(1,4,7-triazonan-1-yl)butanamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methyl)pyridin-1-ium (5)

To compound **4** (1.0 equiv.) was dissolved in dry DCE (8.0 ml/mmol) and anisole (8.0 equiv.) and TFA (6.0 ml/mmol) was added, the reaction was complete after 30-50 minutes (progress

of this step monitored by LC-MS). The TFA was gently removed using a stream of N_2 gas. Once all the TFA fumes had subsided the residue was washed using the washing procedure mentioned above to afford pure **5** as a pale-yellow fluffy powder that was taken to the next step without further purification (70 – 99% yield).

1-(((6R,7R)-7-(3-(4,7-bis(2-(tert-butoxy)-2-oxoethyl)-1,4,7-triazonan-1-yl)-4-methoxy-4-oxobutanamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methyl)pyridin-1-ium (6)

To compound **5** (1.0 equiv,) was dissolved in dry DCE (5.0 ml/mmol) and DIEA (20.0 equiv.). Tert-butylbromoacetate (2.05 equiv.) was added and the reaction was carried out in an ice bath. Progress of the reaction was monitored using LC-MS and the reaction was complete after 2-4 hours. The reaction mixture was then concentrated *in vacuo*, and the residue was washed using the washing procedure mentioned above and dried under a stream of argon gas to afford crude **6** as a pale-yellow fluffy powder. Further purification was carried out using prep-SFC with the following parameters: sample concentration = 10–50 mg/mL (ACN) and filtered, injection volume = 100-200 µL, column = Ethylpyridine (250x10 mm, 5 Å) at 40 °C, mobile phase program (Run time 10-11 min) = 10 –30% for 5 min, then 50% for 2 min wash , followed by 10% for 3-4 min equilibration time, MeOH:ACN (1:1) with 0.3% triethylamine(TEA) as the modifier, tech grade-wet CO2 the balance of the flow, in 10 min, flow = 10 mL/min, BPR setting = 150 bar, monitoring and collection at 220 nm. Fraction(s) were confirmed by LC-MS and concentrated (water bath temperature below 40 ℃) to obtain compound **6** with 40-45 % yield as a clear oil. Fractions should not be left in the modifier for longer than 24 hours since the methanol can hydrolyse the β-lactam. MS (ESI, H_2O:ACN): *m/z* = 761 $((m)^+)$. HRMS (ESI+) m/z (m) calculated for $C_{36}H_{53}IN_6O_{10}S^+$: 761.3538. Found: 761.3693. I.R. (υ_{max}/cm^{-1}) 3407, 2934, 2661, 1758, 1680, 1611, 1485, 1392, 1201, 1155, 1132, 1063, 1020. 930, 799, 775, 720, 682, 577, 501.

1-(((6R,7R)-7-(3-(4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl)-4-methoxy-4-oxobutanamido)-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl)methyl)pyridin-1-ium (7)

To compound **6** (1.0 eq.) was added thioanisole (4.0 mL/mmol to **7**) and then TFA (44 mL/mmol to **7**). The reaction was allowed to stir for 2-4 hour at room temperature (progress of this step monitored by LC-MS), after which the volatiles were removed by passing a gentle stream of N_2 over the reaction until a pale-yellow residue remained. The residue was washed using the washing procedure mentioned above with Et_2O three times as before and dried under a gentle stream of argon gas producing pure compound **7**. Compound **7** was produced with 85-95 % yield as white/ off-white solid. Melting point 177-178 ºC. MS (ESI, H_2O:ACN): *m/z* = 649 ($(m)^+$). HRMS (ESI+) m/z (m) calculated for $C_{28}H_{37}IN_6O_{10}S^+$: 649.2286. Found: 649.2415. I.R. (υ_{max}/cm^{-1}) 3399, 2956, 1979, 1667, 1534, 1486, 1432, 1178, 1130, 1015, 833, 798, 764, 720, 678, 598, 517.

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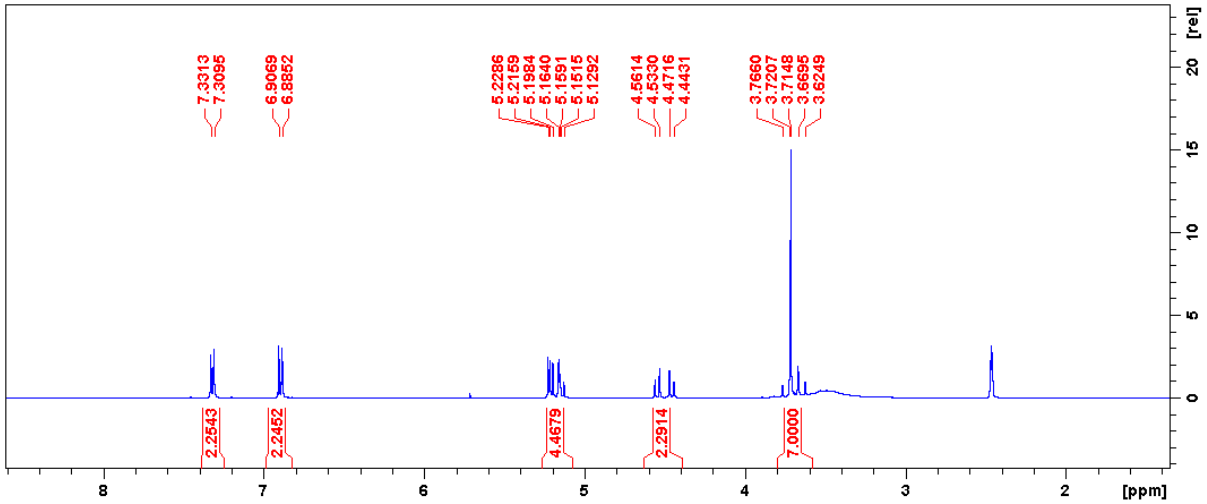
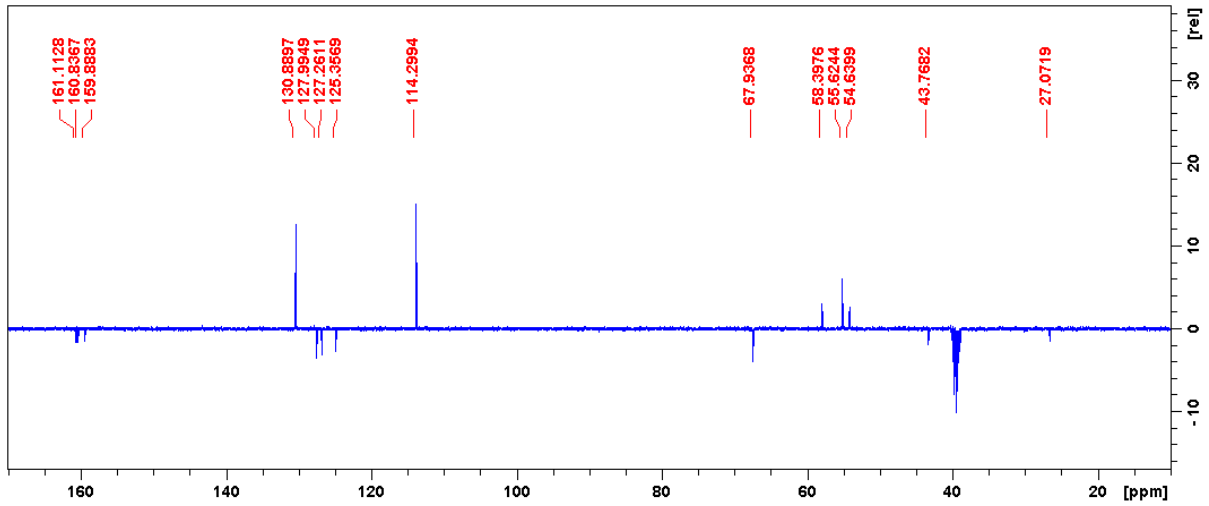
Appendix four: Spectra

^{1}H NMR of **2**



^{13}C NMR of **2**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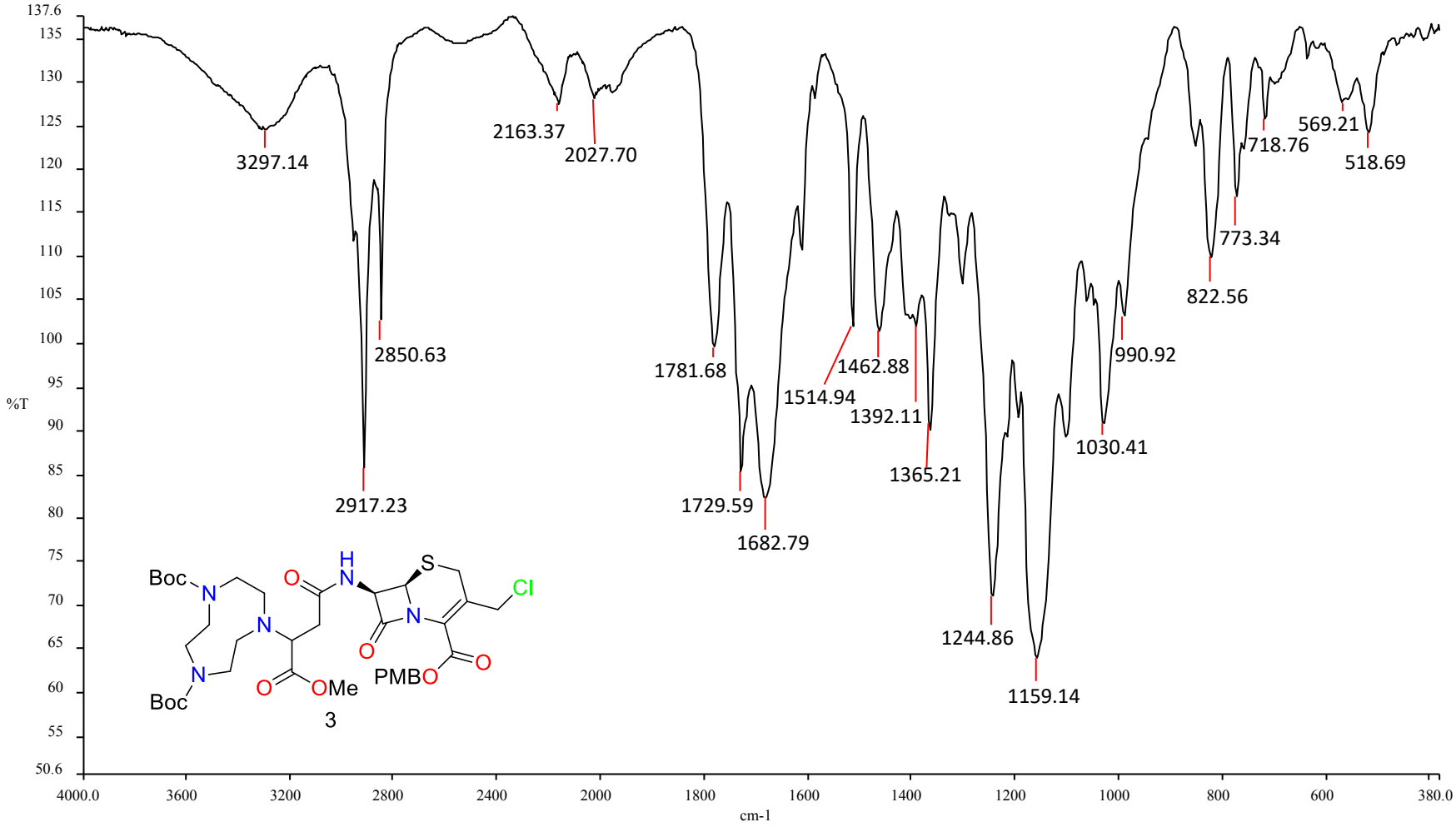
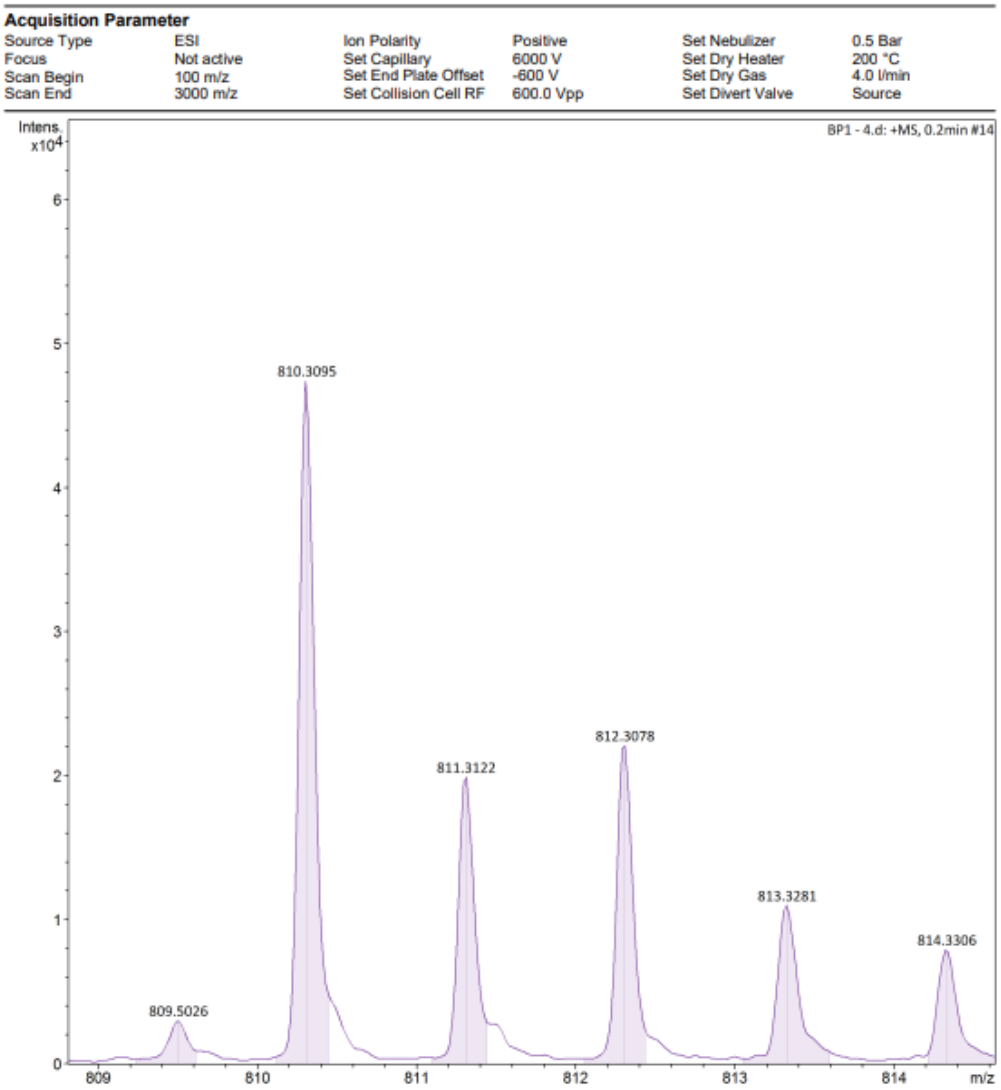
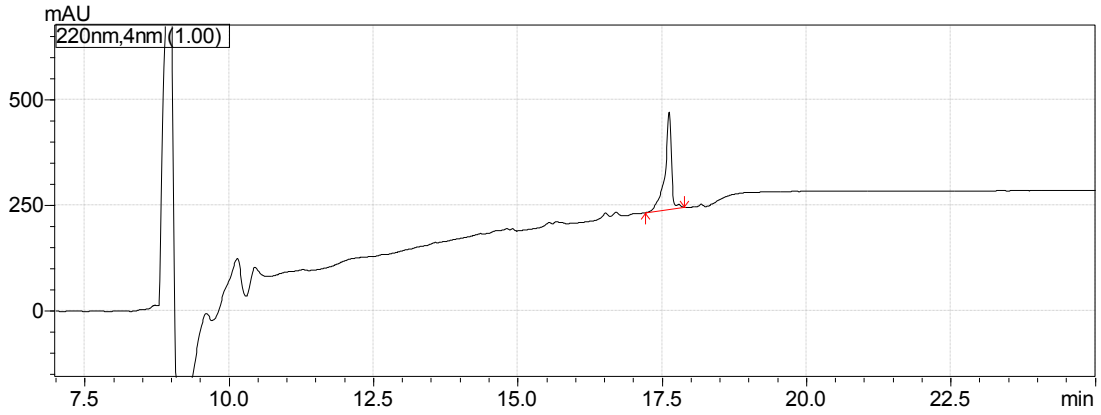
IR spectrum of **3**



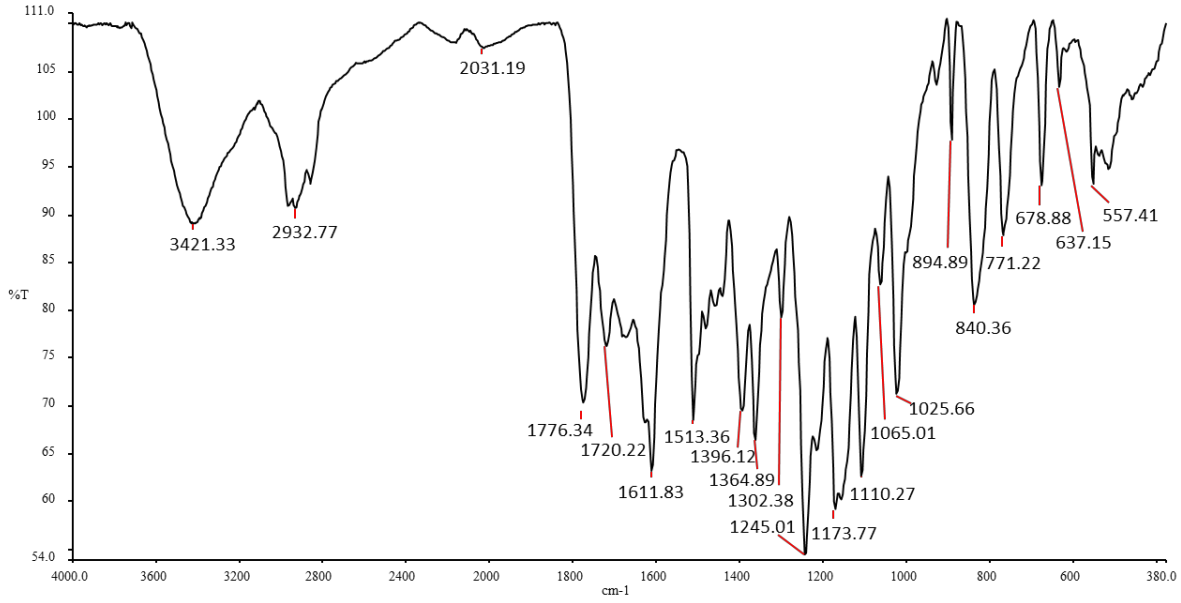
HRM spectrum of **3**

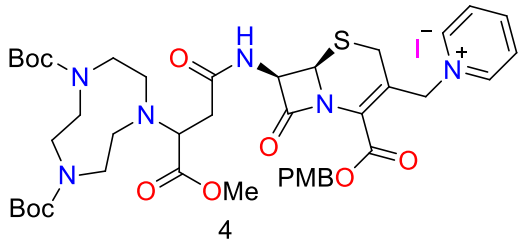


Chromatogram of **3**


mAU
220nm,4nm (1.00)
500
250
0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min

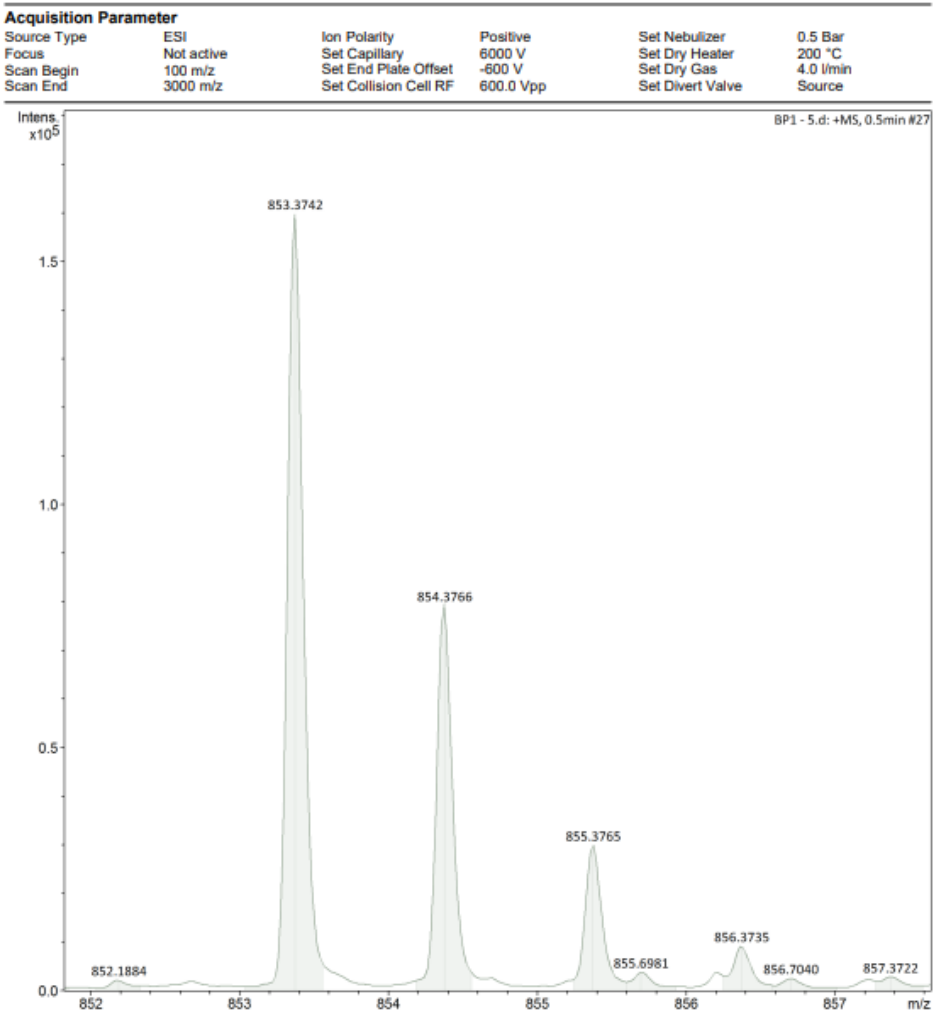
IR spectrum of **4**



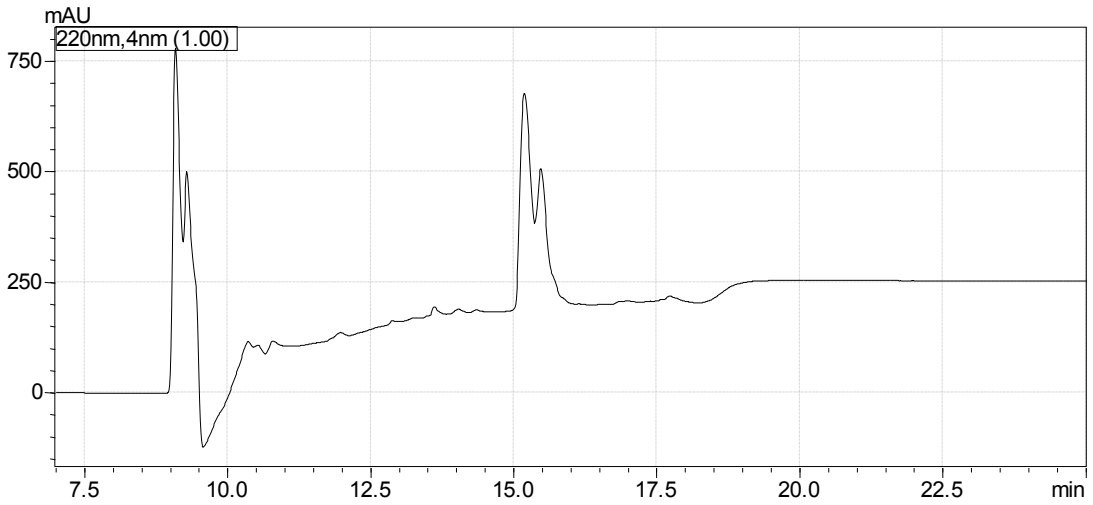


4

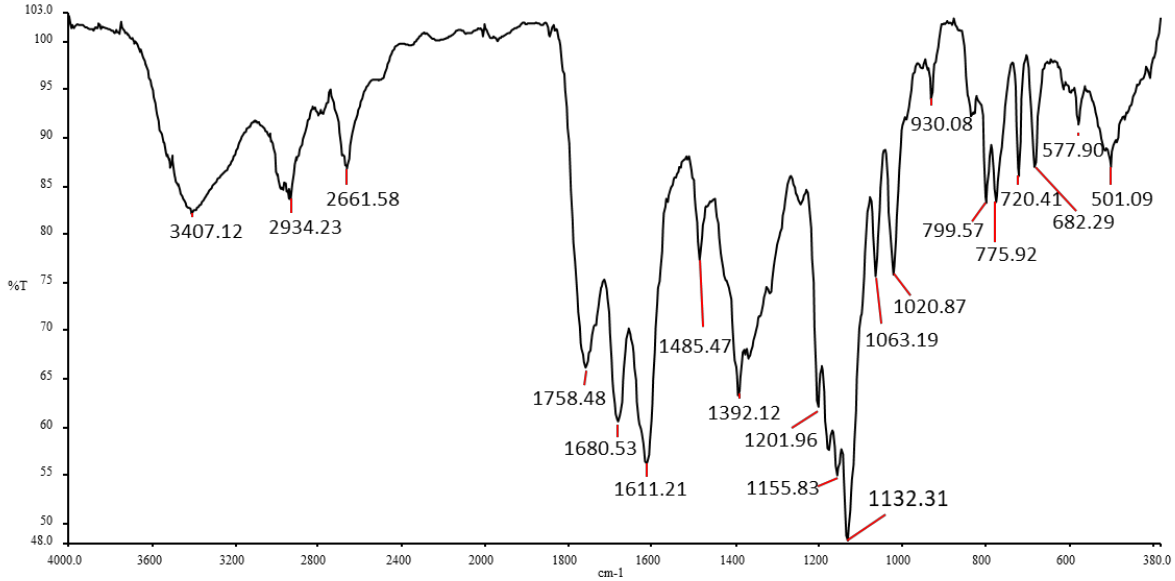
HRM spectrum of **4**

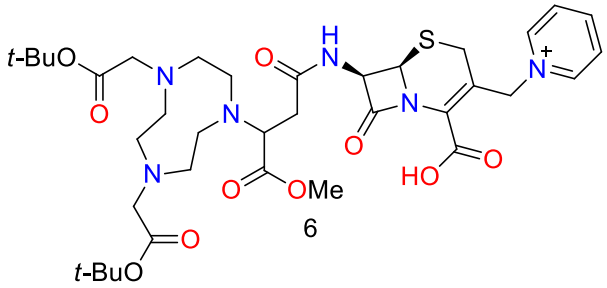


Chromatogram of **4**


mAU
220nm,4nm (1.00)
750
500
250
0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min

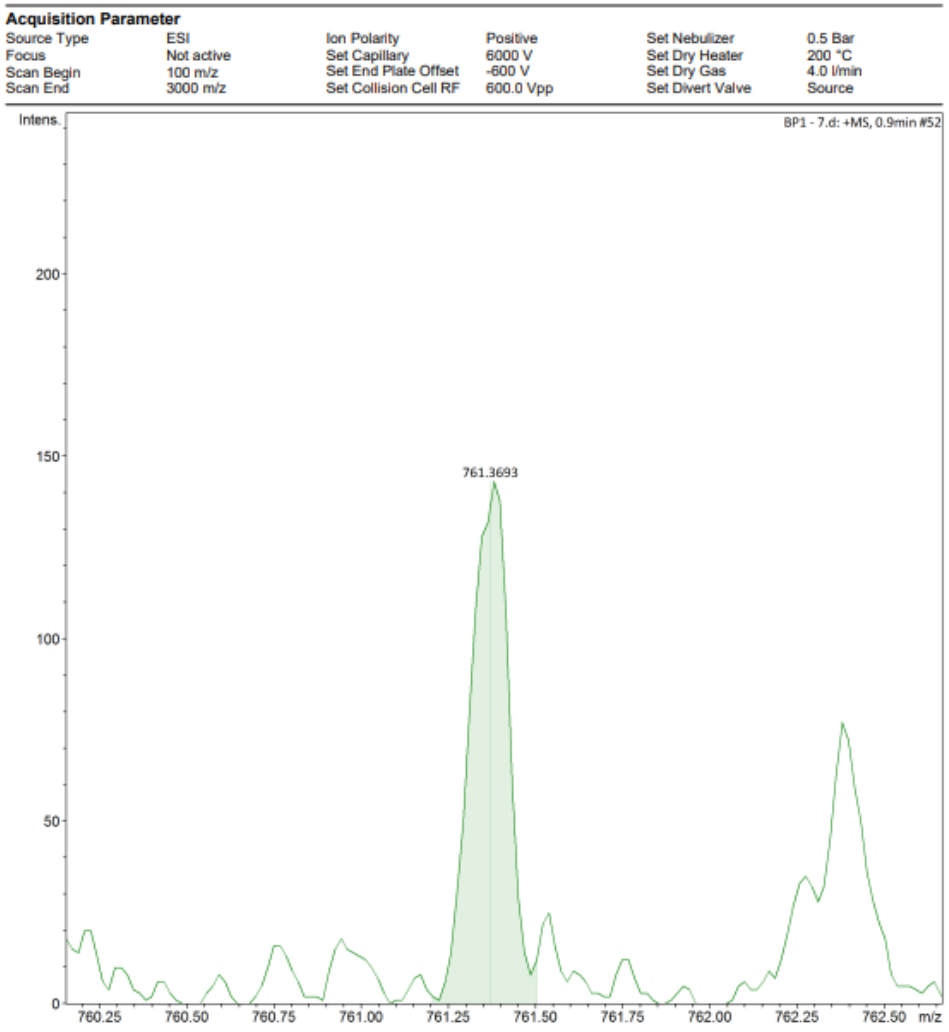
IR spectrum of **6**



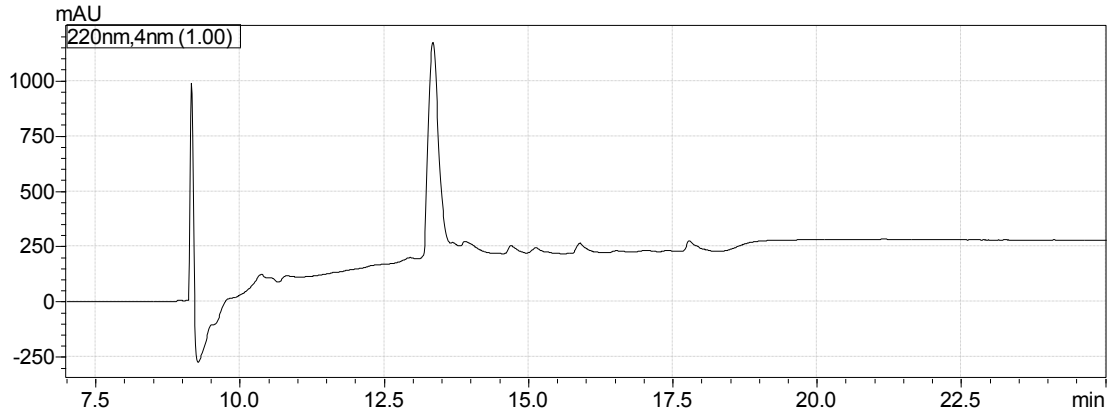


6

HRM spectrum of **6**



Chromatogram of **6**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220nm,4nm (1.00)
1000
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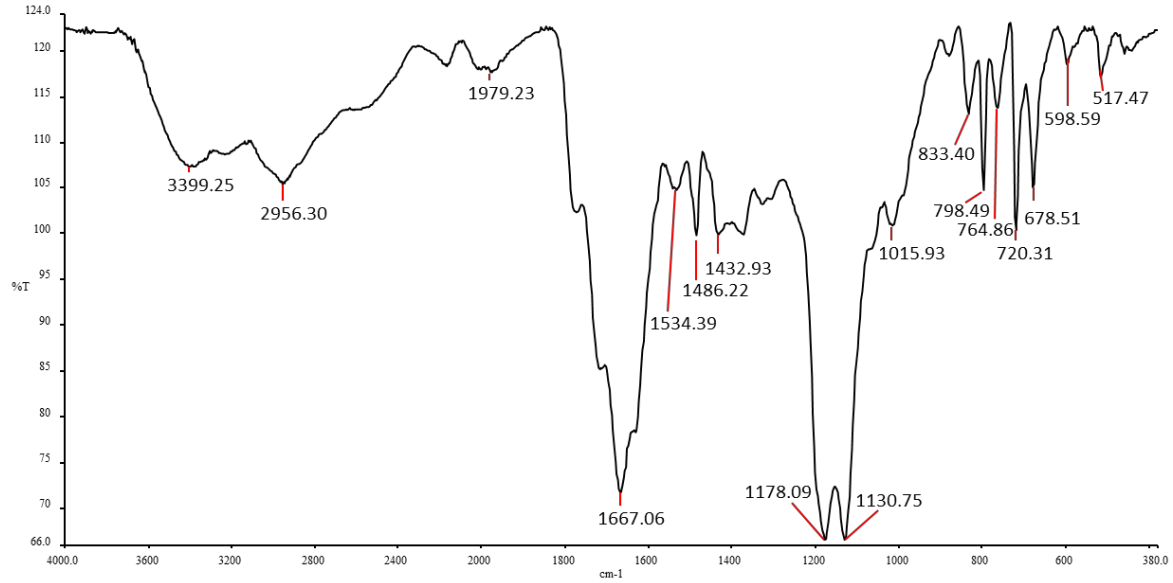
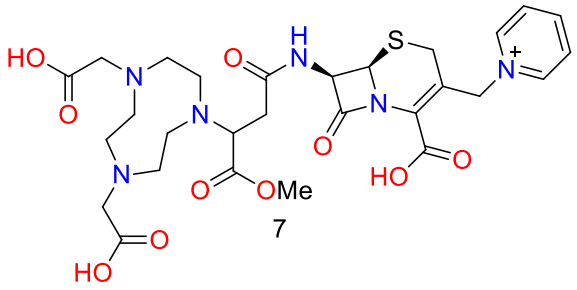
500
250
0
-250
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min

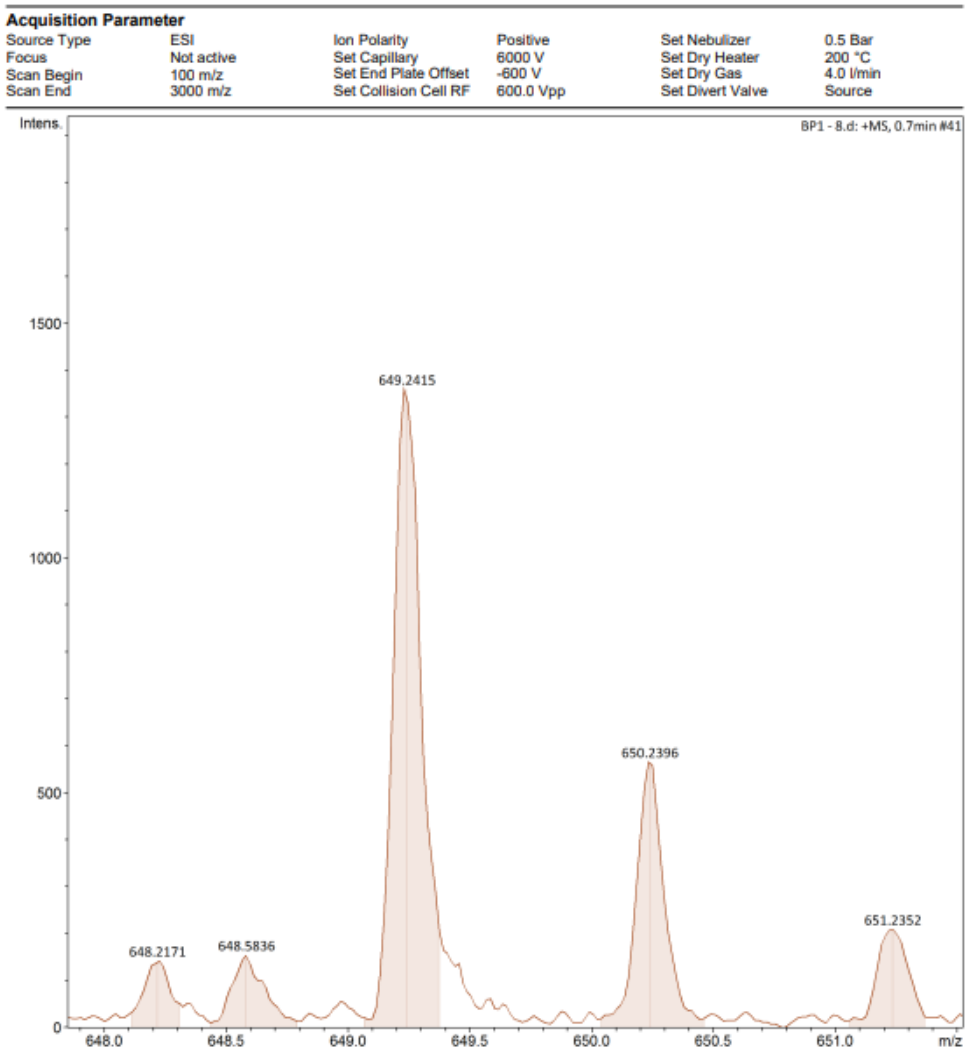
IR spectrum of **7**



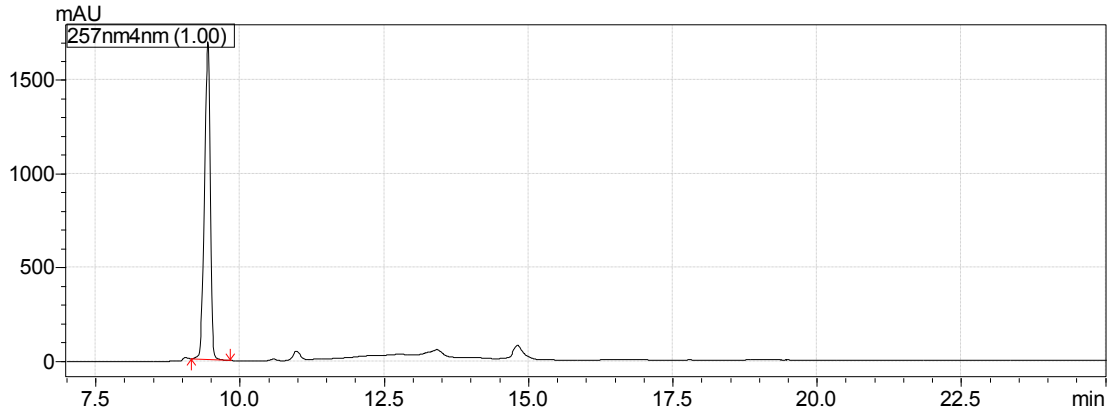


7

HRM spectrum of **7**



Chromatogram of **7**


mAU
257nm4nm (1.00)
1500
1000
500
0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min

Appendix five: Chapter five

Biological Activity

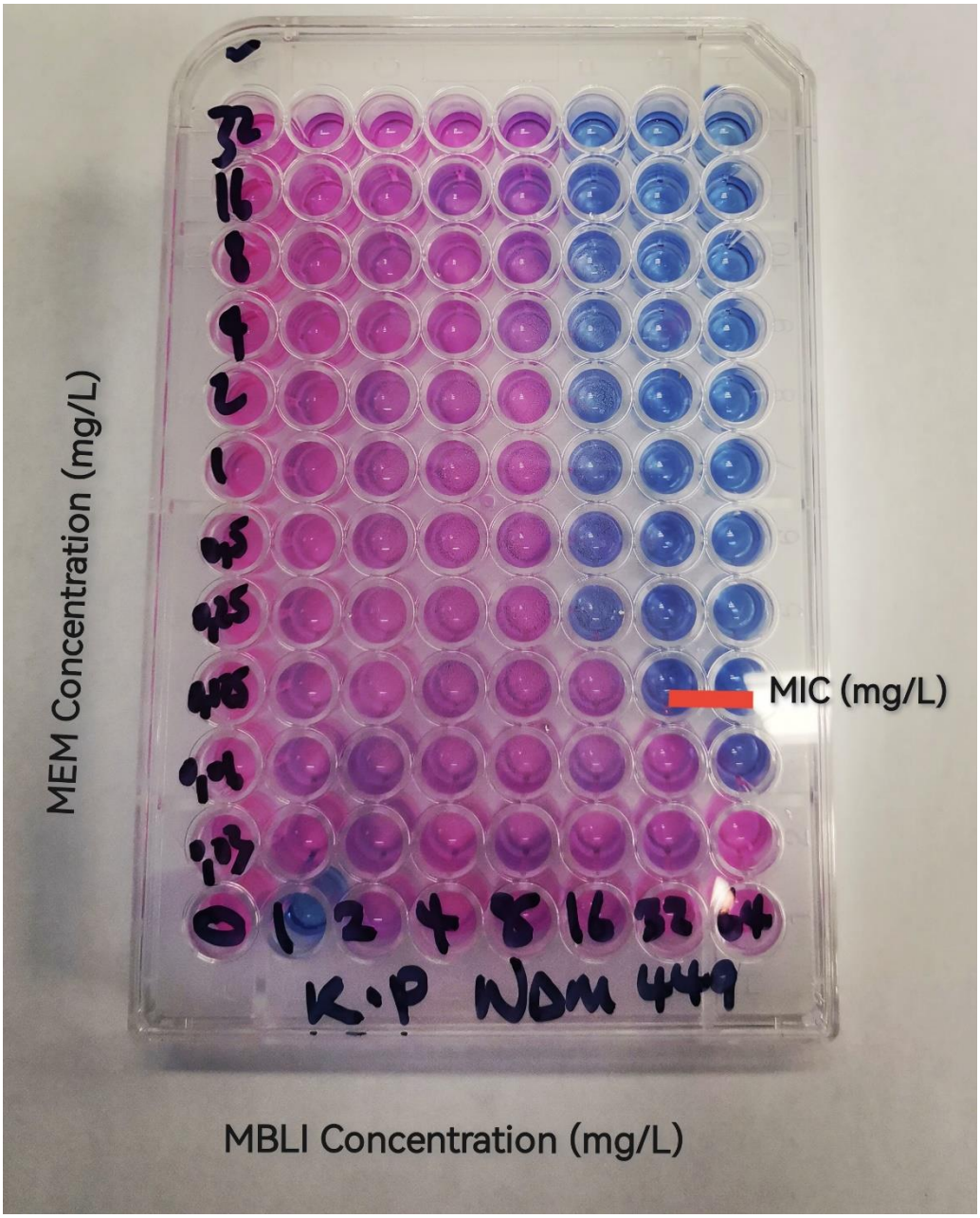


Figure S16: Checkerboard results from the combination of BP10+meropenem. MEM (meropenem), MBLI (BP10). Pink wells = no inhibition, purple wells = partial inhibition and blue wells = inhibition. MIC = lowest concentration of MBLI correlating to lowest meropenem concentration. *K. pneumoniae* NDM is inhibited in this assay

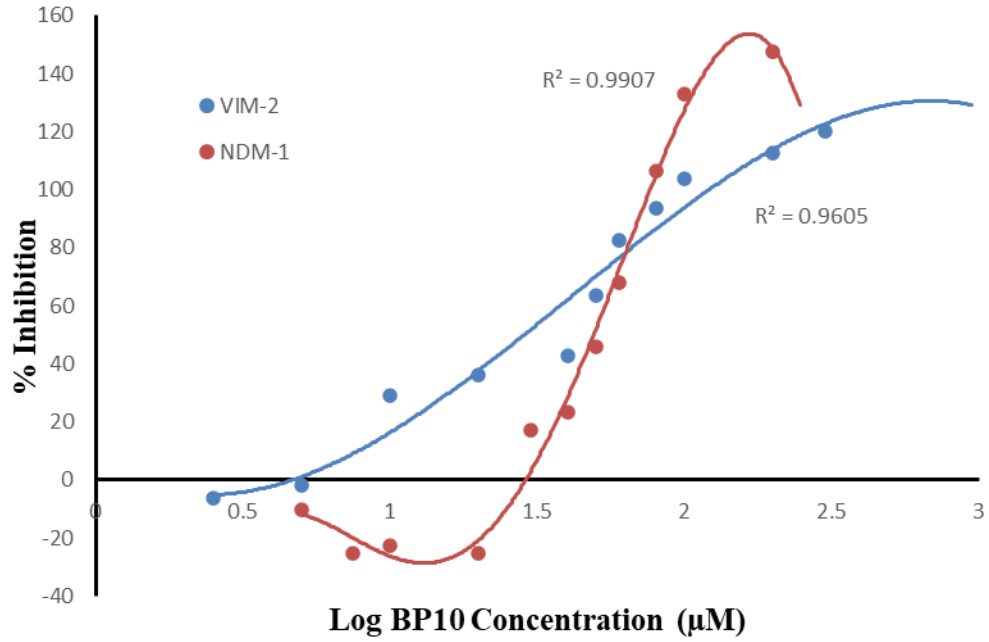


Figure S17: Sigmoidal curve used to determine the IC_{50} of BP10 for NDM-1 and VIM-2

Table S7: *Klebsiella pneumoniae* NDM infected mice receiving the placebo

Treatment Time (h)	Best Log10 Cfu/ml	Mean Log10 Cfu/ml	Std Dev	RSD (%)
2	8.1	8.7	0.8	9.7
4	11.3	11.7	0.6	5.1
6	12.6	12.6	1	8
8	12.8	13	0.7	5.3

Table S8: *Klebsiella pneumoniae* NDM infected mice receiving meropenem only treatment

Treatment Time (h)	Best Log10 Cfu/ml	Mean Log10 Cfu/ml	Std Dev	RSD (%)
2	7.2	6.8	0.4	6.5
4	8	8.3	0.5	5.5
6	8.3	8.4	0.3	3.6
8	8.3	8.2	0.2	2.1

Table S9: *Klebsiella pneumoniae* NDM infected mice receiving BP10 and meropenem treatment

Treatment Time (h)	Best Log10 Cfu/ml	Mean Log10 Cfu/ml	Std Dev	RSD (%)
2	4.9	5.5	0.4	7.2
4	4.7	4.8	0.1	2.1
6	4	4.5	0.2	5.2
8	3.9	4	0.1	2.7

Table S10: Pharmaokinetic properties of BP10 and Meropenem

Time (Hour)	BP10 (ng/mL)	Meropenem (ng/mL)	SD	SD
0	0	0	0	0
2	2674	7061	137	719
4	6969	27496	1606	3185
6	4427	14857	1686	1631
8	3486	8838	1821	1091

Computational Results

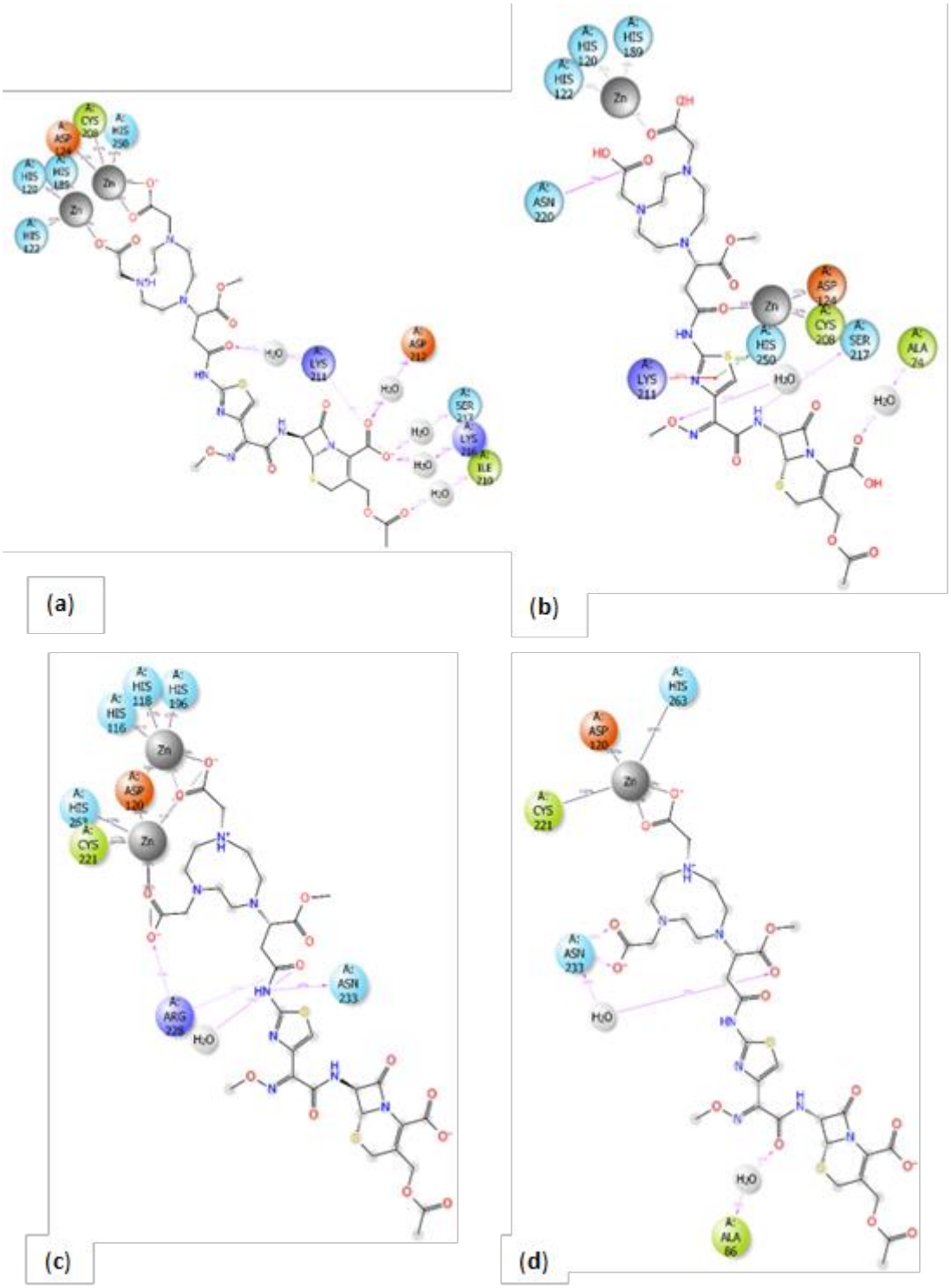


Figure S18: Interactions diagrams of the investigated systems (a) NDM-1—BP10_SR, (b) NDM-1—BP10_SS, (c) VIM-2—BP10_SR, and (d) VIM-2—BP10_SS

Table S11. Docking scores and the binding free energies for NDM-1—BP10 and VIM-2—BP10 complexes

Complexes	Docking scores (KJ/mol)	ΔG_{bind} (KJ/mol)
NDM-1—BP10_SR	-27.20	-40.79
NDM-1—BP10_SS	-25.94	-53.76
VIM-2—BP10_SR	-29.71	-22.43
VIM-2—BP10_SS	-26.78	-72.34

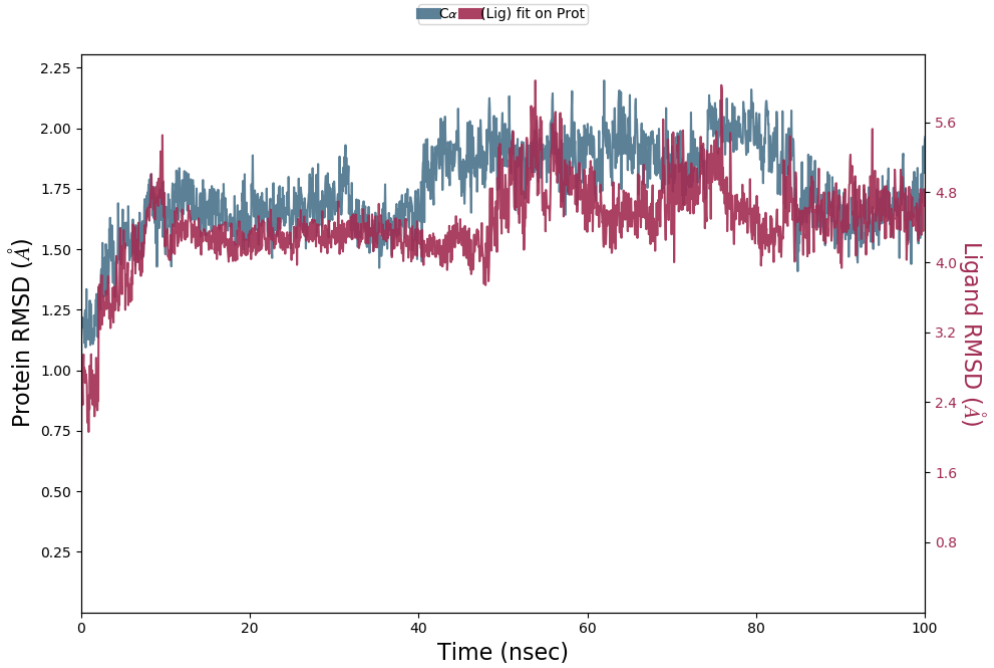


Figure S19: RMSD plot for NDM-1 Ca-backbone aligned with BP10_SR

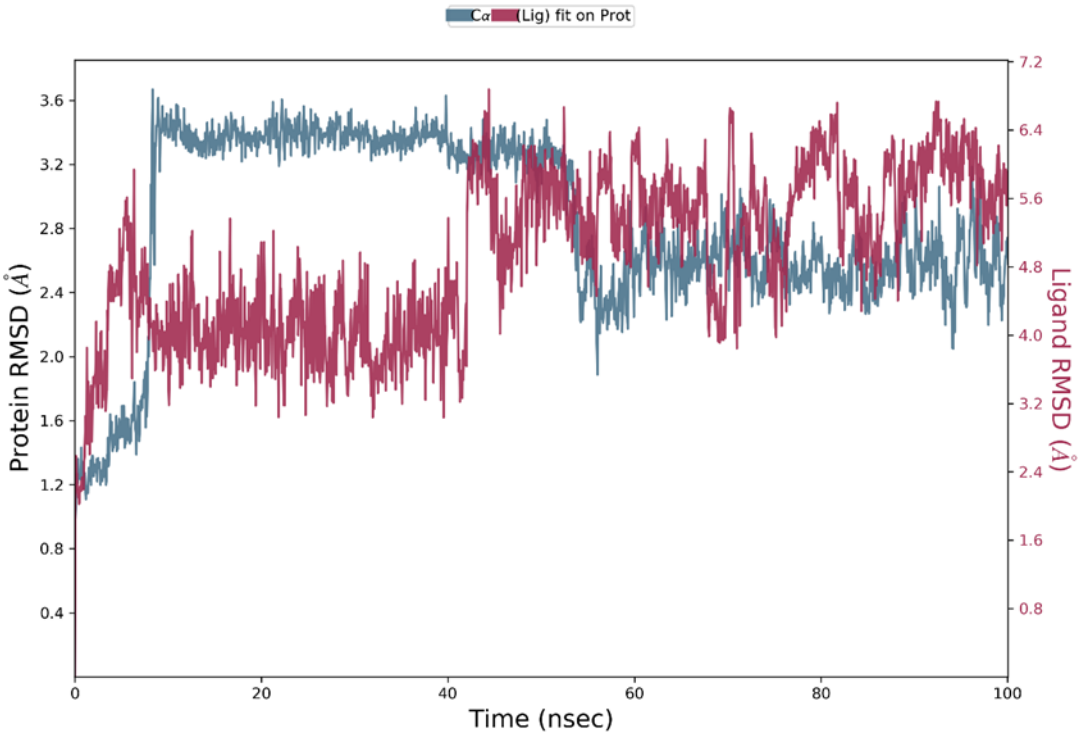


Figure S20: RMSD plot for NDM-1—BP10_SS complex

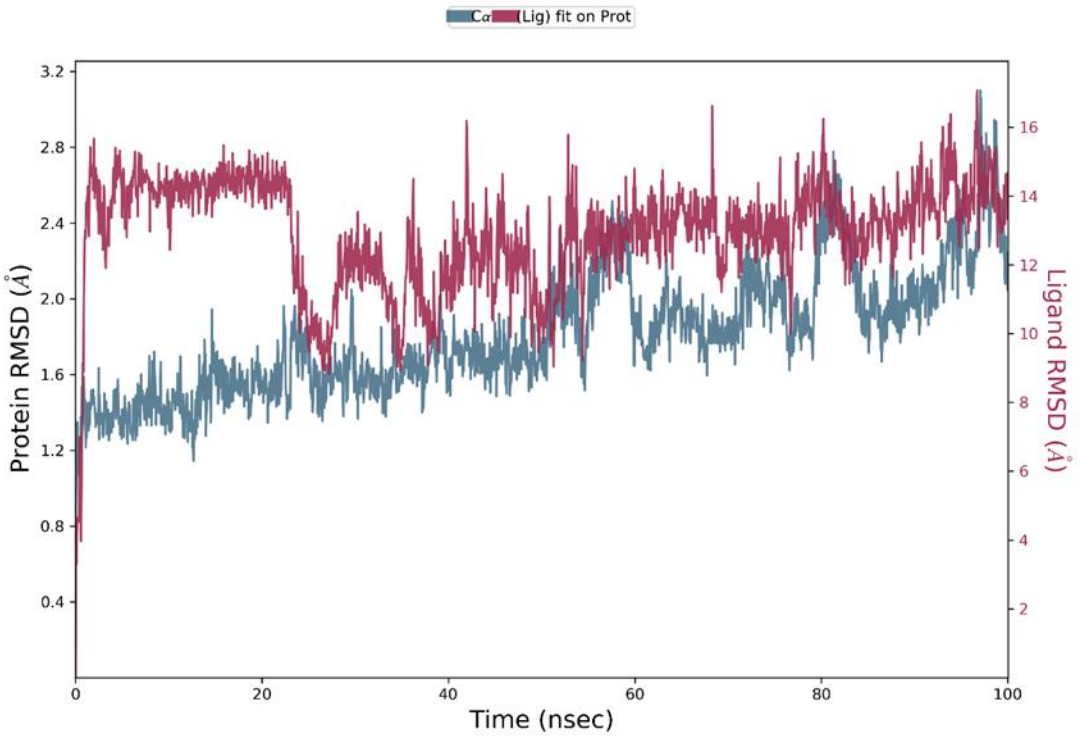


Figure S21: RMSD plot for VIM-2—BP10_SR complex

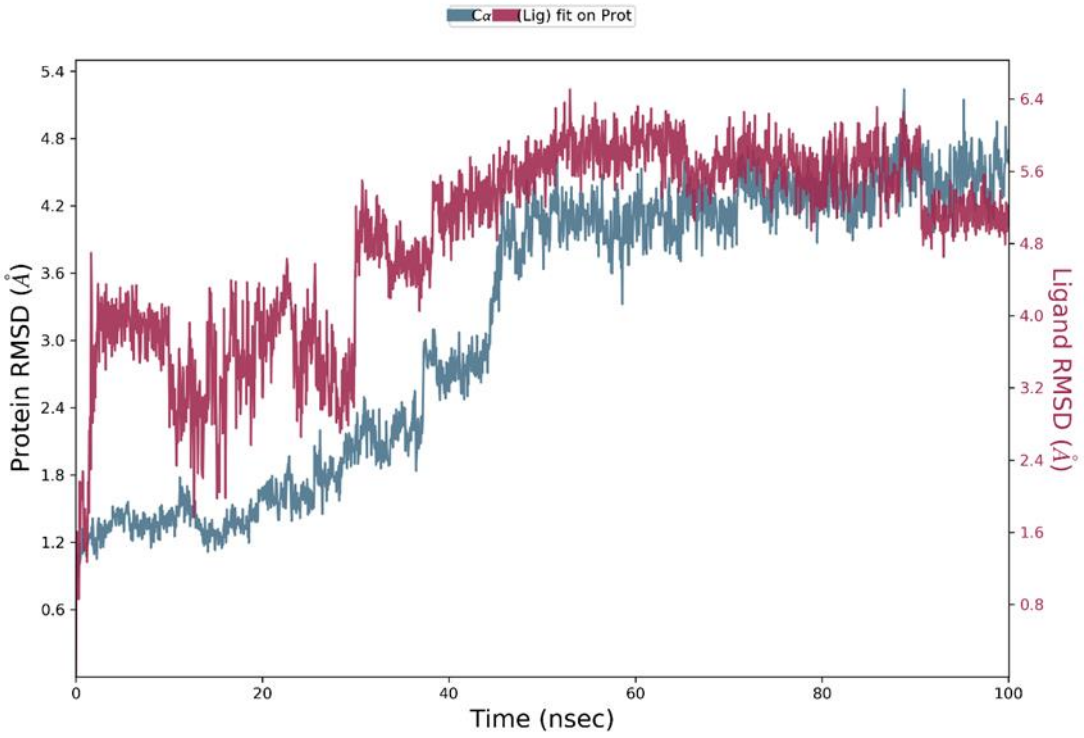


Figure S22: RMSD plot for VIM-2—BP10_SS complex