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IN VITRO AND *IN VIVO* EVALUATION OF METAL- CHELATING AGENTS AS NOVEL METALLO BETA-LACTAMASE INHIBITORS AGAINST CARBAPENEM -RESISTANT *ENTEROBACTERIACEAE*.

2018

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A thesis submitted to the School of Health Sciences, College of Health Science, University of KwaZulu-Natal, Westville, for the degree of Master of Medical Science.

This is the thesis in which the chapters are written as a set of discrete research publications that have followed the International Journal of Antimicrobial Agents format with an overall introduction and final summary. Typically these chapters will have been published in internationally recognized, peer-reviewed journals.

This is to certify that the contents of this thesis is the original research work of Ms. Kehinde Foluke OMOLABI , carried out under our supervision at the Catalysis and Peptide Research Unit, Westville campus, University of KwaZulu-Natal, Durban, South Africa.

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Co-Supervisor:

Signed: ---------- Name: **Prof. H G Kruger** Date: ----------

DECLARATIONS

DECLARATION 1 - PLAGIARISM

I, **OMOLABI, Kehinde Foluke** declare that

1. The research report in this thesis, except where otherwise indicated, is my original work.
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LIST OF MANUSCRIPT

DETAIL OF CONTRIBUTION TO PUBLICATION that form part and/or include research presented in this thesis (include publication in preparation, submitted and give details of the contributions of each author to the experimental work)

1. Kehinde F. Omolabi, Mbongeni Shungube, Nakita Reddy, Sipho Mdanda, Sphamandla Ntshangase, Hendrik G. Kruger, Thavendran Govender, Tricia Naicker, Sooraj Bajinath.

***In vitro* and *in vivo* evaluation of metal chelating agents as potential metallo beta-lactamase inhibitors against carbapenem resistant *Enterobacteriaceae*.(Submitted) 2018**

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Contribution of each author:

Kehinde F. Omolabi conducted all experimental work including animal experimentation, mass spectrometric analysis, data analysis and the preparation of the manuscript.

Mbongeni Shungube synthesized 1,4,7- tris (2-picolinyl)-1,4,7-triazacyclononane (NO3PY)

Nakita Reddy assisted with animal experimentation

Sipho Mdanda assisted with mass spectrometric analysis.

Sphamandla Ntshangase assisted with mass spectrometric analysis.

The remaining authors (Hendrik G. Kruger, Thavendran Govender, Tricia Naicker and Sooraj Bajinath) are supervisors.

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DEDICATION

To Paul Odeniran; the air under my wings.

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LIST OF ABBREVIATIONS

Abbreviations

BFCA	: Bifunctional chelating agents.
NOTA	: 1,4,7-triazacyclononane-1,4,7 triacetic acid.
NO3PY	:1,4,7- tris (2-picolinyl)-1,4,7-triazacyclononane
LC-MS	: Liquid chromatography- mass spectrometry.
MBLIs	: Metallo beta-lactamase inhibitors.
CREs	: Carbapenem resistant *Enterobacteriaceae*.
MHA	: Mueller Hinton agar.
CFU	: Colony forming unit.
PBS	: Phosphate buffered saline.
MIC	: Minimum inhibitory concentration.
DNA	: Deoxyribonucleic acid
WHO	: World Health Organization
EDTA	: Ethylenediaminetetraacetic acid
NDM	: New Delhi Metallo beta-lactamase
IMP	: Imipenemase
KPC	: *Klebsiella pneumonia*e carbapenemase
OXA	: Oxacillinase
MeOH	: Methanol

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ABSTRACT

Infectious diseases remain one of the leading causes of death worldwide, despite the discovery of new and improvements on existing antibiotics. Bacteria are constantly developing sophisticated mechanisms of resisting the effects of antibiotics, this in turn has increased their pathogenicity and virulence. Drugs belonging to the beta-lactam class of antibiotics are most commonly prescribed as they display a broad-spectrum activity against both gram-positive and gram-negative bacteria. Carbapenems which are a member of this class is regarded as the last line of defence against bacterial infections. Resistance to carbapenems is on the increase especially by bacterial strains that are capable of producing metallo-beta lactamase enzymes.

Infections caused by carbapenem resistant *Enterobacteriaceae* are deadly especially those mediated by metallo beta-lactamases. Efforts are being made to synthesize compounds that can inhibit these enzymes. Thus far little progress has been made as a clinically available metallo beta-lactamase inhibitor has not yet emerged, hence the scourge of carbapenem resistant infections rages on. Therefore, the main aim of this study was to evaluate the *in vitro* and *in vivo* activities of metal chelating agents NO3PY and NOTA as potential metallo beta-lactamase inhibitors against carbapenem resistant *Enterobacteriaceae*.

The metal-chelating agents used in this study were NOTA and NO3PY. *In vitro* analysis was performed to determine the minimum inhibitory concentrations by broth microdilution of meropenem alone and when co-administered with the chelators against resistant bacterial strains. The strains used in this study were *Escherichia coli* NDM-1, *Klebsiella pneumoniae* 449, *Escherichia coli* IMP-1 and *Enterobacter cloacae* NDM-1. Time kill kinetics was also evaluated at graded concentrations of MIC, 1*MIC, 2*MIC, 4*MIC, 8*MIC and 16*MIC. For the *in vivo* pharmacokinetics were determined using LC-MS/MS analysis. Forty-eight healthy male Balb/c mice were divided into two groups; meropenem+NO3PY group and meropenem+NOTA group. Both groups received intraperitoneal doses at 10 mg/kg of meropenem and the MBLIs. Thereafter, the in vivo efficacy of meropenem co-administered with NOTA (100 mg/kg each) in a murine thigh infection was determined.

Both chelators were able to restore the efficacy of meropenem to a concentration as low as 0.06 µg/ml. The time kill kinetics also showed that both compounds were able to significantly extend the killing time of meropenem. *In vivo* pharmacokinetic analysis revealed that NO3PY may not

be a suitable candidate for *in vivo* efficacy study as the MBLI was not bioavailable in plasma at 10mg/kg. NOTA on the other hand was bioavailable at the same concentration as NO3PY. The former was able to potentiate the effect of meropenem *in vivo* in a murine thigh infection model. It was evident by a significant reduction of colony forming unit counts in groups treated with meropenem co-administered with NOTA when compared to infected controls

Further preclinical work such as *in vitro* and in vivo cytotoxicity tests, post beta-lactamase inhibitor effects among others are recommended for NOTA to further ascertain its suitability as a potential clinical metallo beta-lactamase inhibitor.

CHAPTER ONE

1.0 Introduction

Worldwide, infectious diseases remain one of the major causes of death even with easy access to antibiotics [1]. In a global population of 6.2 billion, infectious diseases cause 15 million out of nearly 57 million total deaths annually [2]. Over the years, the discovery, design and administration of antibiotics had witnessed profound successes. In 1900, before the discovery of antibiotics, whenever a patient was diagnosed with systemic infectious disease, death was almost inevitable [3, 4]. Then, the three leading causes of deaths were pneumonia, tuberculosis and diarrhea. These diseases caused about one third of all deaths of which 40% were among children younger than five years (USDCL, 1909). The discovery of antibiotics changed the face of modern medicine as infections that claimed multitudes of lives in the past are now in check [6].

However, these successful therapies by antibiotics were short-lived as infectious organisms have developed various resistance mechanisms to antibacterial agents thereby resulting in the emergence of resistant infections with possession of new genes that exacerbate their virulence [7, 8, 9, 10].

The discoveries of antibiotics have dated back to the first decade of the 20th century. The next section highlights the timeline of these discoveries.

1.1 History of Antibiotic Discovery

In 1909, Paul Erlirch discovered the first antibiotic; Salvarsan; a compound whose antibacterial activity is mediated by the oxidation of its arsenic-bonded species [11]. The discovery of other antibiotics then followed (Figure 1).

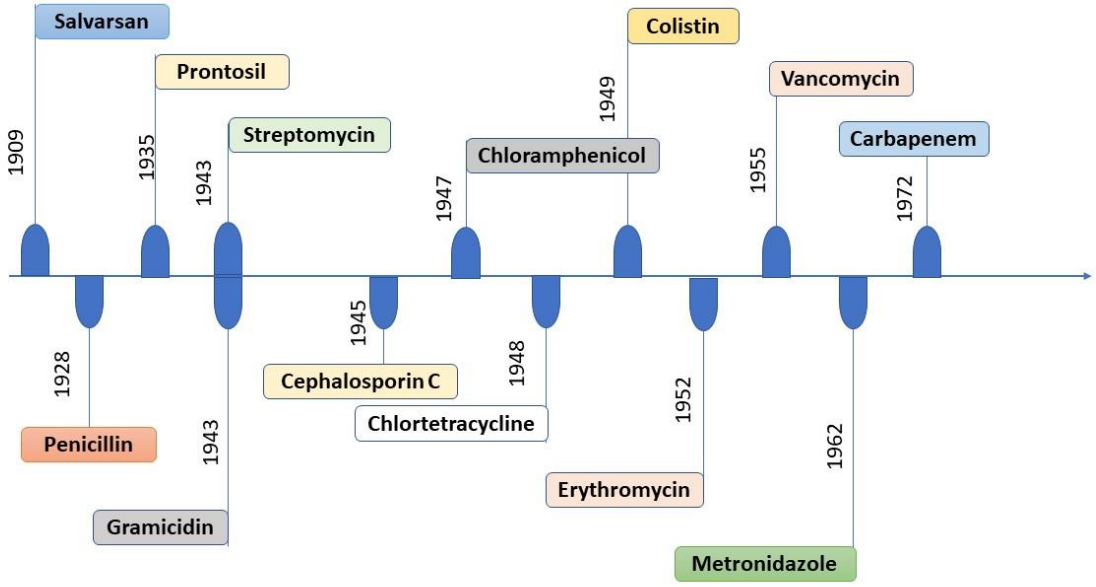


Figure 1. 1: History of antibiotic discovery.

Carbapenem, a beta-lactam antibiotic discovered by the pharmaceutical company Merck was proposed to be the drug of last resort as it was able to combat infections caused by some bacteria that hitherto had resisted treatment by other beta-lactam drugs [12,13, 14,15]. Unfortunately, the potency of this drug class is under threat by multi drug resistant bacteria that are emerging continually [16]. Therefore, it has become expedient for research to be intensified in the development of newer and more efficacious antibiotics else the most basic infections can become fatal.

The effects of antibiotics and the ways exert these on specific parts of their target, differ from one to the other, hence the next section and five subsequent sub-sections will review these various ways.

1.2 Classification of Antibiotics According to their Function and Mechanism of Action

Clinically employed antibiotics act selectively on bacteria without affecting host cells and tissues. This property makes them unique among all other drugs. Based on their function, antibiotics can either be;

1. Bacteriostatic: These classes of antibiotics function in inhibiting the growth of pathogenic bacteria, then allow the body to develop natural defenses e.g. immune system to finally eliminate the organism [18,19].

2. Bactericidal: These classes of antibiotics kill the pathogenic bacteria [18,19].

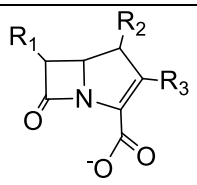
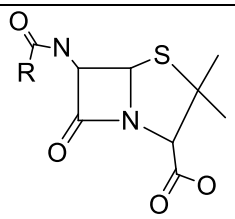
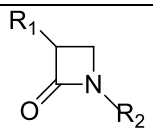
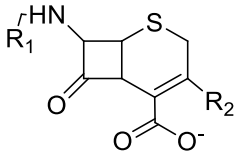
Antibiotics have been further classified into five major classes based on their mechanism of action (Figure 1). These are cell wall synthesis inhibitors, DNA synthesis inhibitors, protein synthesis inhibitors, antimetabolite and membrane function compromisers. [20-27]

1.2.1. Cell wall synthesis inhibitors

Peptidoglycan is the principal component of the bacterial cell wall and it supports the maintenance of the cell shape [28,29]. It consists of alternating strands of glucosamine and muramic acid units crosslinked by short peptides usually pentapeptides. Two enzymes are principally responsible for the synthesis of peptidoglycan. (i) Carboxy-peptidases, cleave the terminal D-ala from the pentapeptide, releasing ATP and exposing the amino acid to be crosslinked. (ii) Transpeptidases, which performs the crosslinking reaction to the glycan backbone [30].

Antibiotics that inhibit cell wall synthesis do so by inhibiting the transpeptidase enzyme. They act as false transpeptidase substrate by imitating the terminal D-ala of the pentapeptide [31,32]. Beta-lactams are commonly employed antibiotics worldwide [33,34]. Beta-lactam of clinical importance includes penicillin, cephalosporins, carbapenems, and monobactams [31, 35-37] (Table 1).

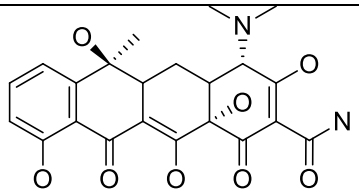
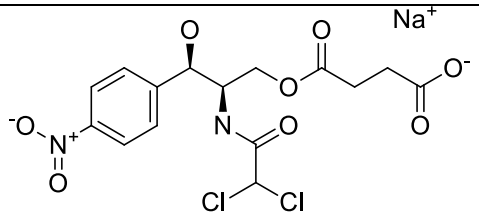
Table 1. 1: An example of the core structures of antibiotics, which inhibit bacterial cell wall

Name	Structure
Carbapenems [12]	
Penicillin Example; Ampicillin [38]	
Monobactam: Example; Aztreonam [37]	
Cephalosporins Examples: cefoxitin [39]	

1.2.2 Inhibition of protein synthesis

Proteins are very important in cellular structure and function [40]. Enzymes and hormones are crucial to bacterial cell structure, replication and survival are all proteins. Tetracyclines, aminoglycosides, macrolides, lincosamides, chloramphenicol, (Table 2) are all inhibitors of protein synthesis in the bacteria [41-44]. Protein synthesis is mediated by ribosomes and some cytoplasmic factors. Antibiotics can either interrupt the ribosome while some, the cytoplasmic factors [45].

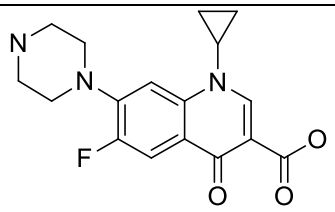
Table 1. 2: An example of structures of drugs inhibiting protein synthesis

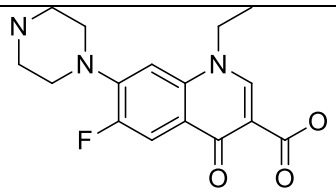
Name	Structure
Tetracycline [46]	
Chloramphenicol [47]	

1.2.3 DNA synthesis inhibitors

Ciprofloxacin, nalidixic acid and norfloxacin (Table 3) are all examples of drugs that inhibit DNA synthesis [48-49]. The mechanism of action is the inhibition of some key enzymes in DNA replication and transcription, which are DNA polymerase, topoisomerases, gyrase, helicase and RNA polymerase [50-53]. This inhibition eventually causes cell death [54].

Table 1. 3: An example of the structure of drugs, which inhibit bacterial DNA synthesis

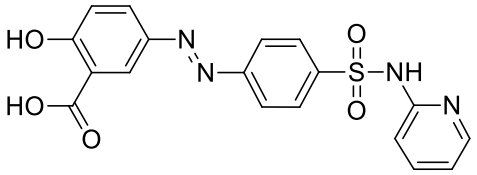
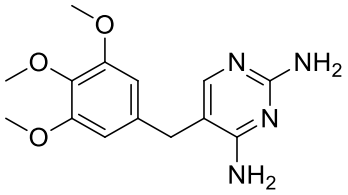
Name	Structure
Ciprofloxacin [55]	

Norfloxacin [56]	

1.2.4 Disruption of metabolic pathways

The general mechanism of this class of antibiotics is the disruption of folic acid metabolism [57]. Tetrahydrofolate is important in the synthesis of bacterial cell wall protein and nucleotides. The precursor of tetrahydrofolate is folic acid [58]. Bacteria synthesize their folic acid from para aminobenzoic acid (PABA). Sulfasalazine (Table 4) inhibits the enzyme responsible for the conversion of PABA to folic acid whereas Trimethoprim impedes the conversion of folic acid to tetrahydrofolate [59,60].

Table 1. 4: An example of the structure of drugs, which inhibit bacterial metabolic pathway

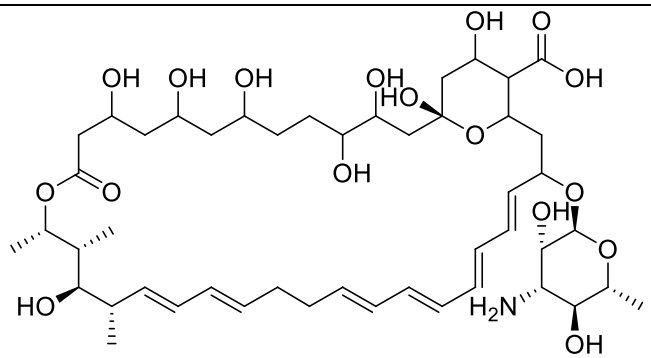
Name	Structure
Sulfasalazine [61]	
Trimethoprim [62]	

1.2.5 Membrane function compromisers

The cytoplasmic membrane encompasses the bacterial cell [63]. It is responsible for the selective permeability of substances into the cell, maintenance of osmotic balance, active

transport among others [64]. Colistin, daptomycin], and nystatin (Table 5) are examples of antibiotics that compromise the membrane function [65-70]. These antibiotics bind to the bacterial membrane, disrupting its structure by making it more permeable. The increased permeability allows other compounds to enter the cells thereby affecting the osmotic balance that eventually leads to cell death [71].

Table 1. 5: An example of the structure of a drug, which inhibits bacterial membrane function

Name	Structure
Nystatin [72]	

Myriads of factors have allowed bacteria to develop different means of resisting the effects of the different classes of antibiotics discussed above. This various mechanism will be examined in the next section.

1.3 Mechanisms of Bacterial Resistance to Antibiotics

Antimicrobial resistance has emerged as a grave problem affecting human and animal health. The Center for Disease Control and Prevention in the United States of America has declared antimicrobial resistance as the second – most significant threat to health in 2014 [73]. Yearly, in the European Union alone, WHO reports that Antimicrobial resistant infections incur no less than $1.5 billion in healthcare expenses [74]. It has also been projected that by 2050, antimicrobial resistant infections will cause 10 million deaths per annum, the largest number of this deaths will occur in Africa and Asia and the financial burden will rise to $100 trillion [75].

The ability of bacteria to evade the effects of an antibiotic is called antibiotic resistance [76]. A bacterium is said to have become resistant if its growth fails to be inhibited in spite of the availability of an antibiotic at therapeutic levels [77]. Several factors have been implicated for the increase of antibiotic resistance few of which are:

1. Use, overuse and misuse of antibiotics [78,79].
2. A large amount of the world's antibiotic has been used in the treatment of animals both nutritionally and therapeutically. The unchecked usage leads to the evolution of antibiotic resistant- bacteria in farm animals, which can then also be transferred to humans through the food chain [80].
3. Poor infection control practices [81].
4. Poor sanitary practices
5. Prolong hospital stay most especially in the intensive care units etc. [82].

The mechanism of antibiotic resistance is broadly classified as [83,84]:

1. Intrinsic/ Natural Resistance

2. Acquired Resistance

1.3.1 Intrinsic resistance

Some bacteria are naturally resistant to a specific class of antibiotic whether there has been a prior exposure to it or not [85]. This may be due to their structural composition such as the innate resistance of gram-negative bacteria to vancomycin, which is quite large to cross the outer membrane therefore, it is only used for the treatment of infections mediated by gram-positive organisms [86, 87]. Metronidazole needs an anaerobic environment to be activated to its active form; this property makes aerobic bacteria to be intrinsically resistant to metronidazole [88, 89].

1.3.2 Acquired resistance

An organism is said to have acquired resistance when it is no longer susceptible to an antibiotic at clinically achievable concentrations [90]. There are different established ways by which resistance can be acquired by different bacteria.

1. Blockage of the entry of the drug into the organism's cell altogether or availability at just limited concentrations. This is achieved by efflux pump increase and influx pump decrease [91,92].

2. Target site modification, alteration or elimination [93].

3. Resistant gene acquisition [94].

4. Enzymatic inactivation of the drug [95]

1.3.2.1 Increase in the expression of efflux pumps

Efflux pumps function in the transportation of substances from inside the cell to the outside environment. Most of the substances they extrude are toxic wastes and antibiotics [96]. Some of these pumps are specific for one antibiotic while others can extrude different classes of antibiotics with the latter being a very strong factor in multidrug resistance [97]. Mutation in any of the regulatory proteins can lead to over-expression of these pumps consequently leading to a decreased concentration of the antibiotic intracellularly [98].

1.3.2.2 Target site modification, alteration and elimination

The target molecule that an antibiotic bind to in the bacterial cell is normally very specific [99]. A little modification in the target molecule will therefore affect the binding of an antibiotic to it. Some bacteria have found a way around this by genetically mutating their target site without altering cellular functions [93]. For example, beta-lactam antibiotics interact specifically with penicillin binding proteins (PBPs) in *Streptococcus pneumonia.* Alteration in the structure of the PBPs most especially PBP2b leads to decreased affinity to beta lactam antibiotics consequently giving rise to resistance [100-102].

1.3.2.3 Resistant gene acquisition

Point mutations, gene rearrangements or deletions are the main genetic mechanism by which bacteria acquire resistance [103]. These resistance- causing mutations may not only be transferred to daughter cells (vertical gene transfer) but also between species (horizontal gene transfer) [104]. Horizontal gene transfer can either occur through any of these mechanisms: Bacterial Transduction, Conjugation or Transformation [105-108].

1.3.2.4 Enzymatic inactivation of the drug

The production of a hydrolyzing enzyme called beta-lactamase is the principal mode of resistance to beta-lactam antibiotics [109]. They are produced by *Enterobacter sp., Heamophilus sp., Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* etc. [110] and they all vary in their activity spectrum. The mechanism of action and classification of beta-lactamases will be discussed in the next two sub-sections.

1.3.3. Mechanism of Action of Beta-lactamases

When beta-lactamase binds to a beta-lactam, the serine/zinc in the active site of the beta-lactamase forms a covalent bond with the beta lactam leading to the opening of the beta-lactam ring [111]. Hydrolysis of the covalent bond occurs by the introduction of an activated water molecule into the bond [112]. The result is that the beta-lactam antibiotic gets inactivated whereas, the beta lactamase is fully recovered and functional [113].

1.3.3.1. Classification of beta lactamases

Ambler classification is most commonly used in classifying beta-lactamases structurally [114]. According to the amino acid sequence, Ambler grouped beta-lactamases into four classes, which are: A, B, C and D. Classes A, C and D have serine on their active site [114]. The serine residue is a nucleophilic agent that attacks the beta-lactam ring eventually forming a covalent acyl enzyme adduct [115]. Class C is also known as ‘AmpC’ beta-lactamases [116] and class D as OXA beta-lactamases [117]. Class B has a divalent zinc ion in their active site, which when coordinates to an activated water molecule that produces a nucleophile facilitating the hydrolysis of the beta-lactam ring [118,119]. They are known as metallo beta-lactamases. Metallo beta-

lactamases or Class B carbapenemases can hydrolyze almost all the beta-lactam drugs except monobactams [120]. Hitherto, they were not thought to constitute serious problems because they were mainly expressed by non-pathogenic bacteria [121,122] but with time, widespread invasion of gram negative pathogens occurred [123,124]. Based on their amino acid sequence, MBLs are divided into three sub-classes (B1, B2 B3) [125]. Sub-class B1 contains the largest number of MBLs of clinical importance, they include IMP-1 [126], VIM-2 [127], VIM-7 [128], NDM-1 [129,130]. *Enterobacteriaceae* producing NDM-1 cause infections such as septicemia, pulmonary infections, peritonitis etc. [131,132]. It was first isolated from a Swedish patient who had been hospitalized in New Delhi, India [133]. Over the years, the spread of beta-lactamases have necessitated different steps to be taken to stem its menace. If successful, the world's antibiotics arsenal will be preserved.

1.4 Combatting Beta- Lactamase Mediated Resistance

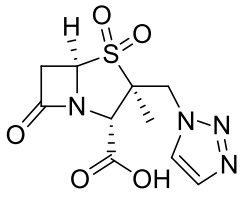
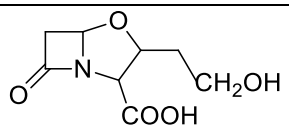
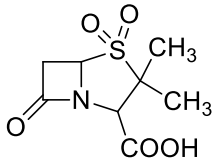
In combating antimicrobial resistance mediated by beta-lactamases, two approaches have been in operation. (i) Modification of the structure of the available beta-lactam drugs, so that this alteration will prevent hydrolysis of antibiotic drugs by beta-lactamases [112]. (ii) Co-administration of beta-lactam drugs with beta-lactamase inhibitors [134]. The inhibitor acts as a protector of the beta-lactam drug [112]. It inhibits the beta-lactamase enzymes (serine and zinc) consequently allowing the normal action of the beta-lactam drug. The next and subsequent sub-sections will dissert both the different classes of beta-lactamase inhibitors.

1.4.1 Serine beta-lactamase inhibitors

Clavulanic acid, Sulbactam and Tazobactam are beta-lactamase inhibitors, which have structural similarity to penicillins [135-138] (Table 6). They are the earliest discovered inhibitors before the advent of new ones. Clavulanic acid was isolated from Streptomyces clavuligenus [139]. When combined with beta-lactam drugs, it successfully inhibits the growth of class A beta-lactamase producing organisms [140]. Sulbactam and tazobactam are penicillanic acid sulfones synthesized in 1978 and 1980 respectively [136,137]. Sulbactam inhibits class A beta-lactamase producing organisms but not as strongly as clavulanic acid and tazobactam [141]. Generally, all

this class of inhibitors are very effective against class A beta-lactamase but quite weak in inhibiting class C and D beta lactamases [142-144].

Table 1. 6: Structure of serine beta-lactamase inhibitors

Name	Structure
Tazobactam [135]	
Clavulanic acid [135]	
Sulbactam [145]	

Clavulanic acid, sulbactam and tazobactam are traditional serine beta-lactamase inhibitors. In recent times other inhibitors that do not have a beta-lactam ring as their core have been discovered. Three of which are discussed next.

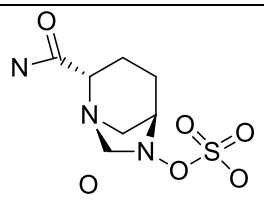
1.4.2. Newer serine beta lactamase inhibitors

1.4.2.1. Avibactam

Avibactam (Table 7) is a non-beta lactam-beta lactamase inhibitor belonging to the diazabicyclooctane family whose mechanism of inhibition is mediated by reactive urea [146-

147]. It is active against beta lactamases of Ambler class A (ESBL and KPC), class C (AmpC) and some of the class D group (OXA-48) [148,149]. Avibactam is a reversible inhibitor with a half-life of 16 min for TEM-1 beta–lactamase [146]. It is not an inducer of beta-lactamase production unlike clavulanic acid, tazobactam and sulbactam [149].

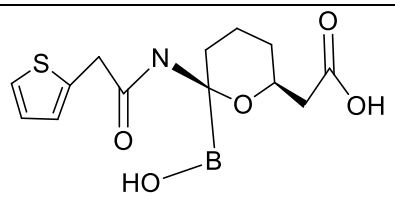
Table 1. 7: Structure of avibactam

Name	Structure
Avibactam [150]	

1.4.2.2. Varbobactam (formerly known as RPX7009)

Prior to this period boronic acid has been known to be a very efficient inhibitor of serine- beta lactamase [151]. Like avibactam, varbobactam is a non-beta lactam beta-lactamase inhibitor but with a boronic acid core (Table 8) [152]. It is able to inhibit Ambler class A and C enzymes but not class B metallo-beta-lactamase [152]. Meropenem when co-administered with varbobatam *in vitro* elicited potent activity against multi-drug resistant carbapenemase producing strain of *Enterobacteriaceae* strain and KPC-producing *K. pneumoniae* [153].

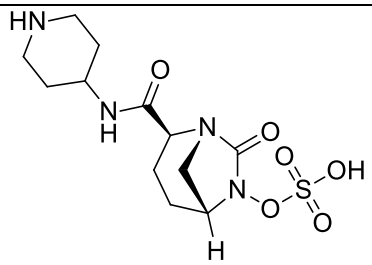
Table 1. 8: Structure of Varbobactam

Name	Structure
Varbobactam [153]	

1.4.2.3. Relebactam

Relebactam (Table 9) formerly known as MK-7655, is known for its broad-spectrum activity against class A and C beta-lactamase inhibitors and KPC-producing strains [154]. It has a remarkable similarity to avibactam in terms of activity [153]. The effects of its co-administration with imipenem and cilastatin are still being investigated clinically [155].

Table 1. 9: Structure of Relebactam

Name	Structure
Relebactam [153]	

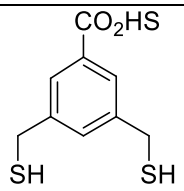
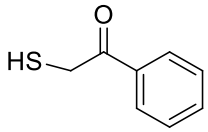
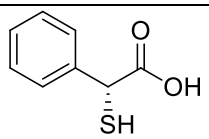
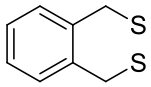
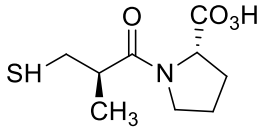
There are also various synthetic compounds that have been tested as potential inhibitors for class B beta-lactamases (metallo beta-lactamase). They are examined in the next section.

1.4.3 Some metallo-beta lactamase inhibitors

Thiol-based Inhibitors

Metallo-beta-lactamase inhibitors potentiate their activity through their zinc moiety [114]. Sulfur has a good affinity for zinc and this property has been exploited in designing thiol-based inhibitors [156] (Table 10). A library of mercaptoacetic thiol esters have been synthesized and tested against several MBLs. It was discovered to restore the efficacy of beta-lactams [157-159].

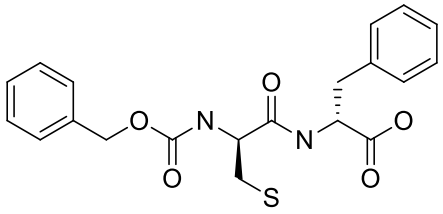
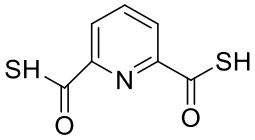
Table 1. 10: Structure of some thiol-based metallo-beta lactamase inhibitors

Name	Structure
3,5-bis(mercaptomethyl) benzo(thioperoxoic)S-acid [160]	
2-mercapto-1-phenylethanone [160]	
(*R*)-Thiomandalic acid [158]	
Benzenedimethanethiol [160]	
(*R*)-Captropil [160]	

Peptide and pyridine dicarboxylates

N-carbobenzoxy-D-cysteinyl-D-phenylalanine [126] (Table 11) elicited a remarkable activity against MBL-producing *B. cereus* [161]. Dithioacid was also reported to be active against MBL-producing *B. fragilis* and *S. maltophilia* [162].

Table 1. 11: Structure of peptide and pyridine derivatives

Name	Structure
N-carbobenzoxy-D-cysteinyl-D-phenylalanine [126]	
Dithioacid [163]	

Calcium EDTA (Ca-EDTA)

EDTA, though a very active metal-chelator with antimicrobial activity has a limited use clinically due to its toxicity [164]. However, Calcium-disodium EDTA (Ca-EDTA) exhibit minimal toxicity and has been approved for treating lead poisoning [165,166]. Ca-EDTA is very active against MBLs such as IMP-1, VIM-2 and NDM-1 [167,168]. However, the potential toxicity of Ca-EDTA includes nephrotoxicity, neurotoxicity and hypocalcemia [167].

The various potential metallo beta-lactamase reviewed above have their limitations, hence infections caused by carbapenem resistant organism still remain a significant public health challenge.

1.5 Why is Carbapenem Resistance a Significant Health Problem?

Enterobacteriaceae is a large family of gram-negative bacteria, which are normal resident of the human intestinal flora [169]. They can become pathogenic, causing infections such as meningitis, septicemia, pneumonia [170] etc. Examples of bacteria belonging to this class include: *E. coli*, *K. pneumoniae*,*Salmonella*, *Shigella* [170] etc. *Enterobacteriaceae* are also prime factors in causing health-care related (nosocomial) infections such as bloodstream bladder, lungs and skin infections [171-173]. *Enterobacteriaceae* can become carbapenem resistant in patients exposed to long hospital stay especially in intensive care units, transfer from one health-care facility to another, usage of in-dwelling catheter, mechanical ventilation etc [174]. The Center for Disease Control also classified Carbapenem resistant *Enterobacteriaceae* (CRE) as any bacteria belonging to the *Enterbactriaceae* family with a susceptibility to etrapenem ≥ 2mg/ml or ≥4mg/ml to meropenem, imipenem and doripenem [175]. Infections caused by CREs have contributed significantly to the morbidity and mortality rate worldwide [176]. A report by China-based Antibiotic Resistance Surveillance "The CHINET", reveals that majority of cases of CRE infections is caused by *K.pneumoniae*,which is resistant to both imipenem and meropenem [177]. The employment of carbapenems for the treatment of these infections is now being threatened due to the production of carbapenem hydrolyzing enzymes by this group of bacteria.

South Africa as a country has not been spared from the escalating menace of infection caused by CREs. In a comprehensive review of the current state of resistance to antibiotics of last resort in South Africa, Sekyere revealed that out of 2315 cases of carbapenem resistant infections reported between January 2000 and May 20 2016, 1,220 cases were from Guateng and this constituted the majority followed by the 515 cases from KwaZulu-Natal [178]. The most common resistant isolate identified was *Klebsiella pneumonia* while the most described carbapenemase was New

Delhi Metallo beta-lactamase (NDM) followed by OXA-48 [178]. Several studies have further documented that many South Africans who contract infections caused by CREs eventually die [179-181].

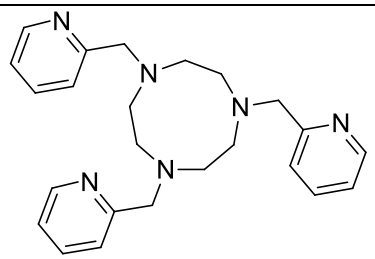
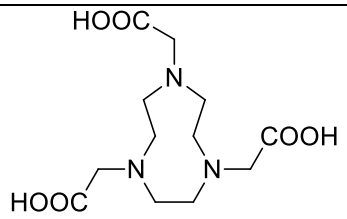
Inhibitors for other classes of beta-lactamases are clinically and commercially available as earlier reviewed. A challenge still remains for the design of compounds that will effectively inhibit class B cabapenemases (metallo-beta lactamase). None of the potential metallo-beta lactamases earlier mentioned has made it to the clinical stage as they can only inhibit the metallo-beta lactamases *in vitro.* This may be due to the toxicity of the compounds, its non-bioavailability among other factors. In stemming the scourge of infections mediated by metallo beta-lactamase producing bacteria, this study is aimed at exploring some bi-functional chelating agents (BFCAs) as potential metallo beta-lactamase inhibitors *in vitro* and most especially *in vivo*.

1.6. Bifunctional Chelating Agents

Bi-functional chelating agents (BFCAs) are very vital in their use for radio-imaging and radiotherapy [182]. BFCAs have been used in complexing radionuclides/small metal ions with high thermodynamic stability and kinetic inertness. Examples are ^{68}Ga, ^{64}Cu, ^{44}Sc, ^{86}Y for positron emission tomography (PET), ^{67}Ga, ^{99m}Tc, ^{111}In, ^{177}Lu for single photon emission computed tomography (SPECT) [183,184]. These metal ions are conveyed to tumor sites by targeted therapy. It is assumed that given its function of chelating metal ions, it will be able to chelate Zn^{2+} that are employed by metallo-beta lactamases facilitating the hydrolysis of beta-lactams. Complexation of the chelator will inhibit the activity of the metallo-beta lactamase, thereby restoring the efficacy of carbapenems. In this study, two macrocyclic BFCAs were investigated in order to determine if they will effectively inhibit metallo-beta lactamase *in vitro*

and *in vivo*. The chelating agents were 1,4,7-triazacyclononane-1,4,7 triacetic acid (NOTA) and 1,4,7-Tris(2-picolinyl)-1,4,7-triazacyclononane (NO3PY) [185-186] (Table 12). NOTA has been studied extensively and it is regarded as one of the best chelators for radiocopper owing to the high stability with which it binds radionuclides and its commercial availability [187]. NO3PY like NOTA, exhibits high stability when in reducing conditions and rapid complexation with radionuclides [188]. NO3PY was synthesized by the Catalysis and Peptide research Unit of the University of KwaZulu Natal, Westville, Durban, South Africa for the purpose of this study

Table 1. 12: Structure of NOTA and NO3PY

Name	Structure
1,4,7-Tris(2-picolinyl)-1,4,7-triazacyclononane (**NO3PY**) [186]	
1,4,7-triazacyclononane-1,4,7 triacetic acid (**NOTA**) [185]	

To determine the *in vitro* and *in vivo* efficacy of both metal chelating agents as potential metallo beta-lactamase, the methodology examined in the next section was employed.

1.7. Analytical and Biophysical Techniques for Measuring *In vitro* And *In vivo* Efficacy

1.7.1. Synergy Testing and Time Kill Kinetics

One of the strategies to combat the growing trend of antibiotic resistance is the application of drug combinations [189-191]. There are several ways in which two or more test drug can interact when co-administered, the effect can either be synergistic/ additive, or antagonistic and suppressive [192]. Synergy means that the activities of both compounds are improved when co-administered compared to their individual effects. Drug combination/ interaction is not only limited to two drugs or antibiotics, it can be between an antibiotic and a compound that has no antibacterial activity such as an inhibitor of beta lactamases [193]. Here, the synergistic mechanism is such that the inhibitor inactivates the antibiotic hydrolyzing enzymes thereby allowing the antibiotic to work normally. The checkerboard method or fractional inhibitory concentration (FIC) [194] is used in measuring the effects of drug combination [195,196]. Its methodology is very similar to that used in determining minimum inhibitory concentration (MIC).

The FIC for a drug is the minimal inhibitory concentration (MIC) of the drug in combination divided by the MIC of the drug used alone. If the FIC index is ≤ 0.5, the antibiotic combination is interpreted as being synergistic; FIC index >0.5 and ≤ 1.0 as additive, between 1 and 4 as indifferent and > 4 as antagonistic [197-198].

Time-kill kinetics is a pharmacological function used to evaluate the rate at which different concentrations of antibiotics reduces bacterial growth over a period of time [199]. It can be used to determine whether the different classes of antibiotics exert their effects in a time-dependent or a concentration-dependent manner [200]. In vitro time kill-kinetics gives information about dosing intervals [201]. It involves incubating a specific density of bacterial inoculum with a known concentration of antibiotic and monitoring the rate at which the antibiotic kills the bacteria per unit time [199]. Determining the efficacy of potential beta-lactamase inhibitor can be taken a step further by evaluating if such inhibitor will be able to restore the efficacy of carbapenem *in vivo.* Murine thigh infection model is one of the techniques that can give this information.

1.7.2. Murine Thigh Infection Model.

In vivo efficacy testing is very crucial in drug research. It is a bridge that links the *in vitro* susceptibility test and clinical trials. As the basis of clinical trials, combination therapies or an entirely new drug must be evaluated in animal models [202,203]. One of its many advantages is that it allows individual effects to be monitored separately when certain parameters are varied [204]. The limitation however is the difference in the pharmacokinetic profiles of animal and actual human subjects. The rate of drug elimination is much faster in animals than humans [205,206].

Murine thigh infection model represents an uncomplicated, sensitive and highly reproducible approach for evaluating the *in vivo* efficacy of a drug while measuring at the same time, the drug pharmacokinetics either in the plasma or in the tissue of the infected animals [205,207]. In the 1940s, the first murine thigh infection as used by Eagle and his associate to evaluate the effects of penicillin on Streptococcus growth [208]. This model was then improved upon by Craig and his co-workers in the 1970s. The improvement involves suppressing the immune system of the mice by making them neutropenic before the introduction of the bacterial inoculum [209,210]. Induction of neutropenia involves the administration of immunosuppressant drug at 150mg/kg and 100mg/kg on days 1 and 4 respectively. Then, a 0.2ml volume of bacterial suspension containing 10^5-10^6 cfu can be inoculated into the mice thigh.

For a single dose study, after two hours of inoculating the mice (ideally, at this time the organism will be in the logarithm growth phase), 0.2ml of a known concentration of antibiotic is administered. Animals can then be euthanized at different time points for the collection of blood and thigh removal. The viable bacterial cell count of the treated mice thigh is evaluated and compared with that of infected control [207]. Infection by different microorganisms have been studied using this model e.g. Pseudomonas *aeruginosa*, Enterobacter *cloacae*, Escherichia *coli*, *K. pneumoniae* among others.

It is important to determine the *in vivo* bioavailabity and pharmacokinetic parameters of any potential drug candidate. This will give an insight into its half-life, LC_{50}, T_{max} among other important variables. Often times, the Liquid-Chromatography Mass Spectrometry (LC-MS) is used to achieve this procedure.

1.7.3. Liquid Chromatography Mass-Spectrometry (LC-MS)

Mass spectrometry is an analytical tool that enables the production of ions and the separation of it based on their charge to mass ratio [211,212]. Using this technique, analytes can be quantified and important information about their chemical composition is obtained [213-215]. Basically, it is made up of five parts, which are: sample introduction, ionization, mass analysis, ion detection, data treatment. In principle, the analyte is introduced as a small quantity and admitted to the ionization source after chromatographic separation, which transforms it into ions. The resultant ions pass through the mass analyzer where they are separated according to their mass-to-charge ratio [216,217]. Lenses resident in the mass-analyzer focus the ions to the detector in the form of electrical signals. The electrical signals that reach the detector are directly proportional to the number of ions formed. There are several ionization techniques involved in mass spectrometry, which are classified as either hard or soft [218,219]. Example of a hard ionization technique is electron ionization [220,221]. Chemical ionization, atmospheric pressure chemical ionization and fast atom bombardment are all examples of soft ionization techniques. Electron spray ionization and matrix-assisted laser desorption ionization are examples of soft ionization techniques [222-228].

Coupling of liquid chromatography, which is a type of chromatographic separation technique to mass spectrometry results in a powerful, sensitive, selective and high-speed analytical technique [229]. LC-MS has become a preferred tool for analyzing drugs and different metabolites. It allows more specific identification of a compound, which is impossible with liquid chromatography alone. Apart from the pharmacokinetic analysis of drugs, it is being used for the analysis of mixture of complex proteins, biological fluids (serum, urine etc) and natural products [230-233].

1.8 Aims and Objectives of The Study

The aim of this study is to evaluate the *in vitro* and *in vivo* efficacy of metal chelating (NO3PY and NOTA) agents in combination with meropenem as potential metallo- beta lactamase inhibitors.

SPECIFIC OBJECTIVES OF THE STUDY

1. To pre-screen both compounds (NO3PY and NOTA) *in vitro* for antibacterial activity using the CLSI broth microdilution and checkerboard methods. The compounds are co-administered with meropenem

2. To develop a simple, sensitive, specific and reproducible LC-MS/MS method for the detection and quantification NO3PY and NOTA in different biological matrices.

3. To quantify the *in vivo* bio-availability i.e. free concentrations of the compounds in the blood (i.e. plasma) at different time points.

4. To determine if the compounds restore the efficacy of meropenem *in vivo* (Thigh infection in a murine model).

5. To determine if plasma concentrations of meropenem co-administered with NO3PY and NOTA are above the MIC for Carbapenem resistant *enterobacteriaceae*

1.8.1. Thesis Outline

This thesis comprises of Chapter 1, which is an introduction and review on antibiotics, antibiotic resistance, metallo beta-lactamase inhibitors and techniques utilized herein. Chapter 2 presents the methodology involved to determine the *in vitro* and *in vivo* activities of the NO3PY and NOTA as inhibitors against metallo beta lactamase producing enzymes. The results obtained in this chapter were submitted for publication in a high impact factor journal (International Journal of Antimicrobial Agent. Impact Factor: 4.253). Chapter 3 concludes the thesis and provides future directions for the study.

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CHAPTER TWO

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

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Running title: Potential MBL inhibitors against carbapenem resistant *Enterobacteriaceae*

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Highlights

- This study presents the *in vitro* and *in vivo* activities of two metal chelators (NOTA and NO3PY) against carbapenem resistant *Enterobacteriaceae.*
- Both chelators were able to return the activity of meropenem, resulting in MICs as low as 0.06 mg/ml.
- NO3PY showed poor bioavailability in pharmacokinetic experiments, therefore only NOTA was used for in vivo efficacy tests.
- NOTA showed excellent antibacterial activity and was able to effectively reduce bacterial CFUs.

ABSTRACT

Herein we compared the *in vitro* and *in vivo* activities of two metal chelators (NOTA and NO3PY) as potential metallo beta-lactamase inhibitors (MBLIs). The minimum inhibitory concentration (µg/ml) of meropenem co-administered with metal chelators against meropenem resistant strains was determined. These resistant bacterial strains include; *Escherichia coli* NDM-1, *Klebsiella pneumoniae* 449, *Escherichia coli* IMP-1 and *Enterobacter cloacae* NDM-1. Also, the time kill kinetics over a 24-hour period was evaluated. Both MBLIs restored the efficacy of meropenem against all bacteria tested. Bonferroni's pairwise comparison test showed significant differences between 8* MIC and 16* MIC when compared to the meropenem control in *E. coli* NDM-1 for NOTA and only 16 *MIC for NO3PY. Overall, there were no major differences in the *in vitro* efficacy of the MBLIs. A validated liquid chromatography- mass spectrometric method (LC–MS) for the quantification of meropenem and each chelator, in mouse plasma was developed. Forty-eight healthy male Balb/c mice were divided into two groups; meropenem+NO3PY group and meropenem+NOTA group. Both groups received intraperitoneal doses at 10 mg/kg of meropenem and the MBLIs. NO3PY showed poor bioavailability at the selected doses. NOTA was bioavailable and its *in vivo* efficacy was determined. The co-administration of meropenem and NOTA (100 mg/kg each) in a murine thigh infection model brought about a significant decrease in the colony forming unit counts of *K. pneumoniae* 449 over an 8-hour period. The findings suggest that NOTA holds strong potential for use as a metallo-beta lactamase inhibitor in the treatment of carbapenem-resistant *Enterobacteriacae* infections.

Keywords:

Metallo beta-lactamase, Chelating agents, Enterobacteriaceae, Infection modeling

2.1. Introduction

Beta-lactams are the most widely prescribed class of antibiotics throughout the world [1,2]. Its broad-spectrum activity makes it suitable for the treatment of a wide range of infections caused by gram positive and gram-negative bacteria [3]. Carbapenems are beta-lactam drugs that are regarded as the last line of defense against infections caused by resistant bacteria [4,5]. Unfortunately, resistance to carbapenems is on the increase [6]. Infections caused by carbapenem resistant *Enterobacteriaceae* (CREs) have contributed significantly to the morbidity and mortality rate globally [7]. The main resistance mechanism employed by *Enterobacteriaceae* against carbapenems is the production of various forms of hydrolyzing enzymes, called beta-lactamases [8].

Beta-lactamases are able to hydrolyze the antibiotic beta-lactam ring through a series of reactions which ultimately lead to the destruction of the antibiotic [9]. The beta-lactamase enzymes are either categorized as serine beta-lactamases or metallo beta-lactamases according to their structural configuration and mode of action [10]. The former has serine at its active site while the latter has zinc [10]. The synthesis of beta-lactamase inhibitors capable of overcoming resistance to beta-lactam drugs, is a thriving area of research [11]. Serine beta-lactamases have been successfully inhibited by beta-lactamase inhibitors which are clinically available, these include, clavulanic acid, avibactam, sulbactam and tazobactam [12]. The mechanism of action of metallo beta-lactamase inhibitors is to covalently bind to zinc within the beta lactamase enzyme, as a result the metallo beta-lactamase enzyme is truncated and the efficacy of the beta-lactam drug is restored [13]. Attempts have been made to synthesize metal-chelating agents that can function as

metallo beta-lactamase inhibitors by chelating the zinc in the active site of beta-lactamase enzyme [14,15].

Bifunctional chelating agents (BFCAs) are routinely used in radio-imaging and radiopharmaceuticals [16-18]. They are well known for their ability to bind small molecules and radionuclides with a high thermodynamic stability [19,20]. The metal ions include, Mn^{2+}, Gd^{3+}, Cu^{2+}, Fe^{2+}, Al^{2+} among others [21-28]. BFCAs have two moieties, one is a strong metal binding unit used in complexing a radionuclide of interest while the other binds a carrier biomolecule (e.g antibodies, peptides) that serves to transport the complexed radionuclides to the target site *in vivo* [29-30]. Examples of BFCAs include, 1,4,7-triazacyclononane-1,4,7 triacetic acid (NOTA), 1,4,7,10 tetraazacyclododecane (DOTA), diethylenetri-aminepentaacetic acid (DTPA) etc. [29,31-32]. The strong affinity of BFCAs for metal ions have been exploited for their ability to bind zinc which is essential for metallo beta-lactamase action [10, 14-15].

Reports by Somboro *et al.* suggested that the co-administration of NOTA and meropenem (Supplementary file 1), against metallo beta-lactamase producing CREs was successful in producing a bacteriostatic effect *in vitro* [33]. However, this study neither investigated the time-kill kinetics nor the *in vivo* efficacy of this combination. 1,4,7- tris (2-picolinyl)-1,4,7-triazacyclononane (NO3PY), a BFCA has so far only been used for radiochemistry [34]. This compound was synthesized in our laboratory and used as a potential MBLI for this study. Using time kill kinetics , the activities of NOTA and NO3PY (Supplementary file 1) when co-administered with meropenem against MBL- producing CREs (*E. coli* NDM-1, *K. pneumoniae* 449, *E. cloacae* NDM-1 and *E. coli* IMP-1) were evaluated. This was to determine the rate at which these compounds at different concentration reduce the growth of MBL- producing CREs over a period of time. We went further to investigate the *in vivo* pharmacokinetics and efficacy

of these inhibitors in combination with meropenem. This study, is to the best of our knowledge the first time that a BFCA, NO3PY will be tested for metallo beta-lactamase inhibition when co-administered with meropenem against a panel of metallo-beta lactamase producing bacteria. This study is also the first to investigate the *in vivo* pharmacokinetics and efficacy of NOTA as a metallo beta-lactamase inhibitor.

2.2. Methods

2.2.1. Bacterial source

Metallo beta-lactamase producers belonging to the family of *Enterobaceriaceae* were purchased from Patrice Nordmann at the Institut National de la Santé et de la Recherche Médicale (U914), Paris, France [35]. The bacterial strains used were; *Escherichia coli* NDM-1, *Klebsiella pneumoniae* 449, *Escherichia coli* IMP-1and *Enterobacter cloacae* NDM-1. These bacterial strains were selected based on their varying degrees of susceptibility to meropenem. Bacterial stock solutions were preserved in Trypticase soy agar and glass beads (4mm) at -80^{o}C. *Escherichia coli* ATCC 25922 was used as the control.

2.2.2. Antibiotics and inhibitors

Meropenem was obtained from Sigma-Aldrich (Schnelldorf, Germany) and NOTA from Macrocyclics, Texas, United States of America. NO3PY was made available by the synthesis group of Catalysis and Peptide Research Unit, University of KwaZulu Natal, South Africa. The structure was confirmed by nuclear magnetic resonance (NMR) as reported in literature [34]. Distilled water was used for preparing meropenem and NOTA stock solutions while phosphate buffered saline was used for NO3PY. Meropenem stock solution was stored at -80ºC.

2.2.3 Susceptibility testing

The broth microdilution method, according to the Clinical and Laboratory Standards Institute, 2014 [36], was used to determine the susceptibility profile of meropenem alone and in combination with NO3PY and NOTA using the checkerboard method [37]. Mueller–Hinton broth (MHB) was used as the growth medium for the study. A 0.5 McFarland standard for each bacterial suspension was used. The experiment was conducted in ninety-six well microtitre plates. Thereafter, the plates were incubated at 35ºC for 24 h. The minimum inhibitory concentration was recorded at the antibiotic-inhibitor drug concentration that showed no visible growth in the presence of the resazurin dye. The test was conducted at three independent times to confirm results (Table 1).

2.2.4. Time kill assay

An initial inoculum density of 10^7cfu/ml of 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 inhibitors were added at a fixed concentration of 8 µg/ml. Meropenem and growth control groups were included. For the former control group, the exact MIC of meropenem alone against the test organism was used (32 µg/ml for *E. cloacae* NDM-1 and 16 µg/ml for *E. coli* IMP-1) except for *K. pneumoniae* 449 and *E. coli* NDM-1 where only clinically achievable concentrations were used (32 µg/ml). Aliquots of 100ul in duplicate were removed for colony counts at hours of 0, 2, 4, 6, 8, 10 and 24. Viable counts were determined by the serial dilution method and plated on Mueller Hinton agar (MHA). MHA- plates were incubated at 35ºC for 24 h and plate counts were done after 24 h of incubation. Antimicrobial agents were considered bactericidal at the lowest concentration, which reduced the original inoculum by ≥3

$\log_{10}$ CFU/ml (99.9%). Values for each time point is generated from the mean±SD values of the duplicate CFU/ml count from a single experiment (Figures 1-4).

2.3 Pharmacokinetic study

Male Balb/c mice (average weight 26 ± 2 g) were obtained from 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. Animals were given a 10 mg/kg.b.w dose of meropenem and 10 mg/kg.b.w of each inhibitor intraperitoneally. 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 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 was analysed using liquid chromatography mass spectrometry (LC-MS).

2.3.1 Sample preparation for LC-MS/MS analysis

During sample preparation, 100 µl of the biological sample was spiked with 20 µl of 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 13 000 g for 15 min at 4ºC. The supernatants were filtered through an SPE cartridge [DSC-18 (50mg)] suitable for the sample. The filtrate was then collected into auto-sampler vials and vortexed briefly, before injecting into the LC-MS/MS system. The calibration curve was constructed, following 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 achieve chromatographic separation increasing from 70% A to 30% B. The injection volume was 5µl and the total run time was 10 mins. The MS acquisition parameters were: positive ion polarity; end plate offset was 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 radio-frequency was 500 Vpp; collision energies were 1eV for NO3PY, NOTA, meropenem and ampicillin (internal standard). Data Analysis 4.0 SP 5 (Bruker Daltonics) was used to further process the data (Figure 5).

2.4 In vivo murine thigh infection model

A thigh infection protocol was performed as described by Michail *et al.* [38]. Briefly, six-week old, pathogen free-specific, male Bagg inbred albino c-strain (BALB/c) mice weighing 20-25 g (n=40), were rendered neutropenic (neutrophils $<100/mm^3$) by administration with cyclophosphamide, intraperitoneally (IP) at 4 days (150 mg/kg) and 1 day (100 mg/kg) before infection. The left thigh was infected using a 100ul intramuscular injection containing 10^7-10^8 CFU/ml. This procedure was done two hours before the treatment with meropenem + NOTA (100 mg/kg.b.w each) combination, commenced. The mice were randomly separated into two

groups, the infected control and the treated group. Mice were humanely euthanized, by halothane overdose, at 2h, 4h, 6h and 8h post treatment. The left thigh muscle was then aseptically removed and homogenized in 5ml of phosphate buffered saline (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 quantitatively enumerated using colony forming units (CFU), the titer was then expressed as log10 CFU/thigh muscle (Figure 6).

2.5 Statistical analyses

Experimental data generated from the time-kill kinetic study were analysed using GraphPad Prism version 5.0 (GraphPad Inc., San Diego, CA, USA). The bacterial density was represented using $\log_{10}$ cfu/ml units and was plotted against time in hours for each bacterium. The kill rate was determined at different time intervals using a linear regression model to find the slope for each transformed concentration. Thereafter, a non-linear regression analysis (dose-response) was used to determine the sigmoidal model for the evaluation of the pharmacodynamic relationship between the antibiotic concentration and bacterial growth or death. The 50% inhibitory concentration, Hill's slope and r^2 were also determined. A comparative analysis of the kill rate of each of the beta-lactamase inhibitor in combination with meropenem was assessed using the two-way analysis of variance (ANOVA). Bonferroni's pairwise comparison test was used to compare the effectiveness of growth control, meropenem control and kill rate of both beta-lactamase inhibitors for each bacteria organism [39].

2.6. Results

2.6.1. Susceptibility test results

The minimum inhibitory concentration (MIC) of meropenem alone and in combination with NOTA and NO3PY against carbapenem resistant *Enterobacteriaceae* was determined. The results (Table 1) show that *E. coli* NDM-1, *K. pneumoniae* 449, *E. coli* IMP-1 and *E. cloacae* NDM-1 were highly resistant to meropenem. Excellent activity was achieved by NO3PY in restoring the efficacy of meropenem at a concentration as low as 0.06 µg/ml for all organisms except *K. pneumoniae* 449 for which the MIC was 0.125µg/ml. Also, meropenem when co-administered with NOTA produced inhibition at a concentration as low as 0.06 µg/ml for all organisms except *E. coli* NDM-1 and *K. pneumoniae* 449 for which the MIC was 0.125 µg/ml.

Table 2. 1: The MICs of meropenem only and in combination with NOTA and NO3PY (n=3)

Organism	**Minimum Inhibitory Concentration (µg/ml)**		
	[a] [1]	[a+ b] [2]	[a+ c] [3]
Escherichia coli NDM-1	128	0.06+4	0.125+4
Klebsiella pneumonia 449	128	0.125+4	0.125+4
Escherichia coli IMP-1	16	0.06+4	0.06+4
Enterobacter cloacae NDM-1	32	0.06+4	0.06+4

- a = concentration of meropenem (µg/ml)
- b = concentration of NO3PY (µg/ml)
- c = concentration of NOTA (µg/ml)
- [1] = concentration of meropenem alone that resulted in inhibition
- [2]= concentration of meropenem and NO3PY that resulted in inhibition

- ^{3}= concentration of meropenem and NOTA that resulted in inhibition

2.6.2. Test group comparison of the resistant bacteria

E. coli NDM1 with NO3PY showed (Figure 1A) there was significant increase ($P < 0.05$) in colony forming unit (cfu) count of growth control compared to other groups, while Bonferroni's analysis revealed significant difference ($P < 0.05$) in efficacy of 16*MIC (1+8) compared to the meropenem control. No significant difference between other MICs with the comparison test (Figure 1A). *E. coli* NDM1 with NOTA shows a significant increase ($P < 0.05$) in the colony forming unit (cfu) count of growth control compared with other groups (Figure 1B). The Bonferroni's comparison multiple test revealed significant difference ($P < 0.05$) in the efficacy of 8*MIC (0.5+8) and 16*MIC (1+8) compared to the meropenem control (Figure 1B). Other MICs reveal no significant difference in the comparison tests (Figure 1B). For other organisms (*K. pneumoniae*- 449, *E. coli* IMP-1 and *E. cloacae* NDM-1), the growth control compared to other groups with both metallo beta-lactamase inhibitors showed there was significant increase ($P < 0.05$) in cfu count. However, there was no statistically significant difference between other groups in the comparison tests (Figures 2A, 2B, 3A, 3B, 4A and 4B).

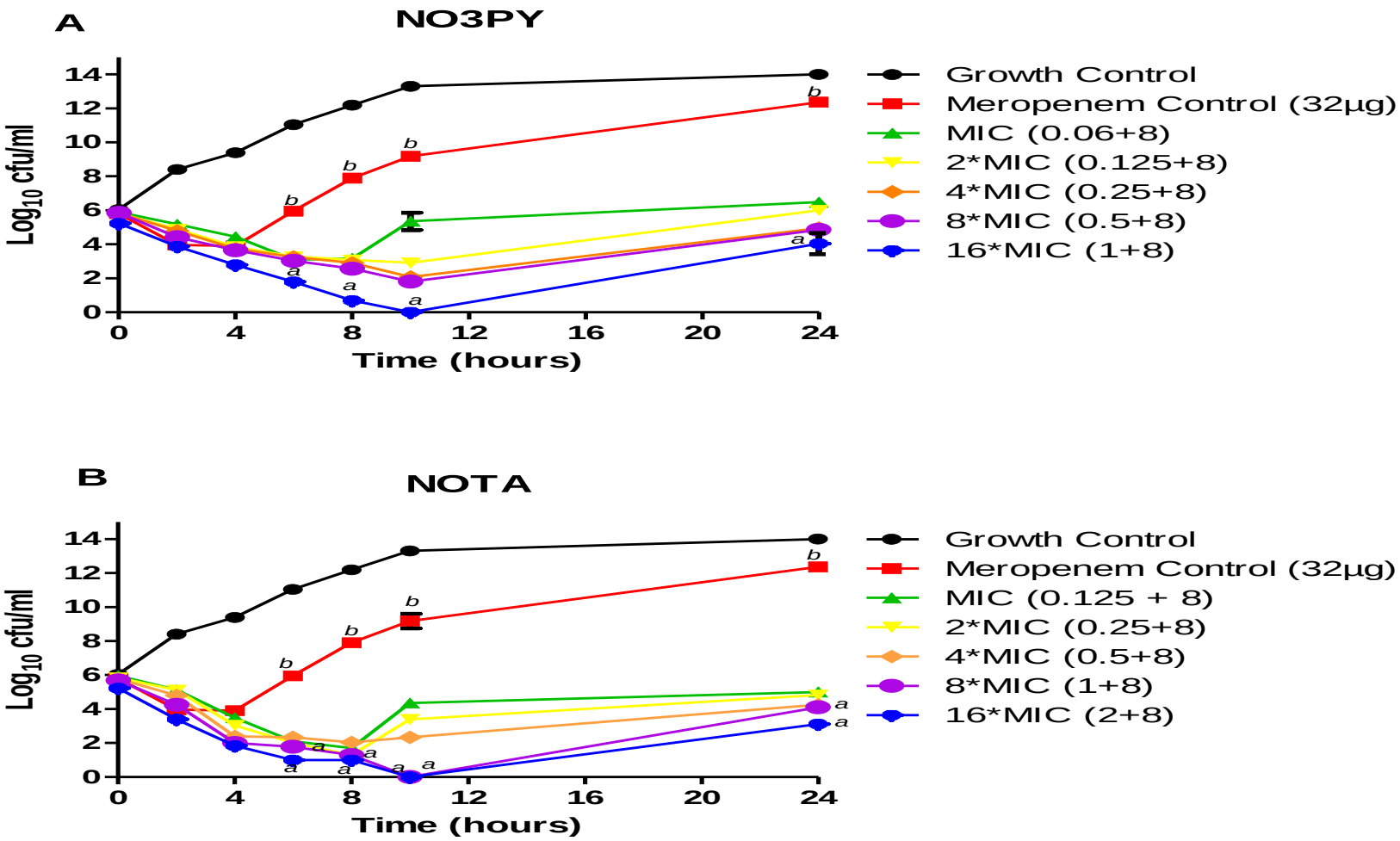

Figure 2. 1: ***E. coli*** **NDM-1 exposed to meropenem co-administered with NO3PY (A) and NOTA (B) at different MICs. NO3PY indicates significant difference (*P* < 0.05) between meropenem control and 8*MIC (1+8). NOTA shows significance (*P* < 0.05) between meropenem control and 8*MIC (1+8) and 16*MIC (2+8). Symbol *a* indicates significant increase in the rate of kill as compared to *b*. Mean values of duplicate cfu/ml count are plotted.**

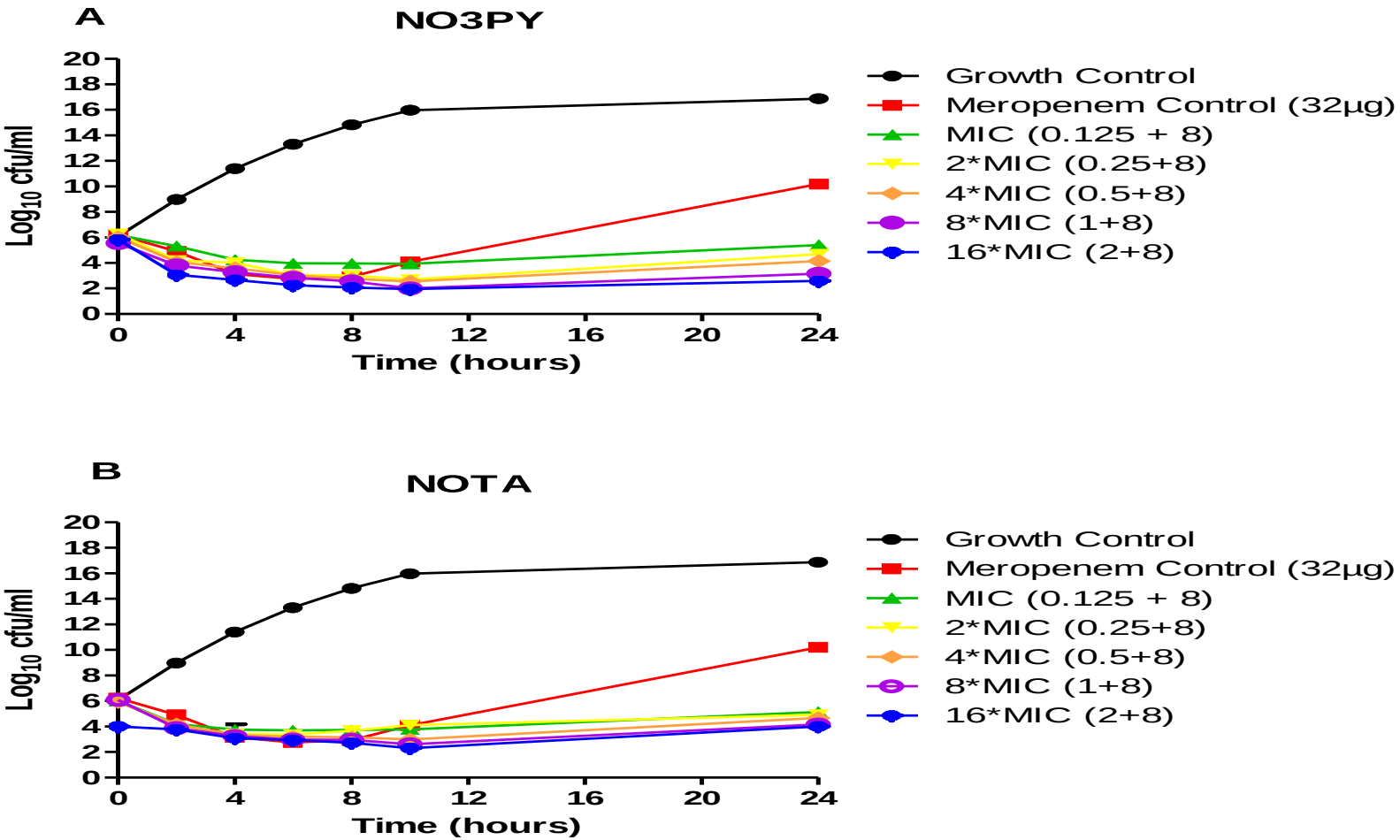

Figure 2. 2: ***K. pneumoniae*****-449 exposed to meropenem co-administered with NO3PY (A) and NOTA (B) at different MICs. No significant difference (*P* < 0.05) between the treatment groups. Mean values of duplicate cfu/ml count are plotted.**

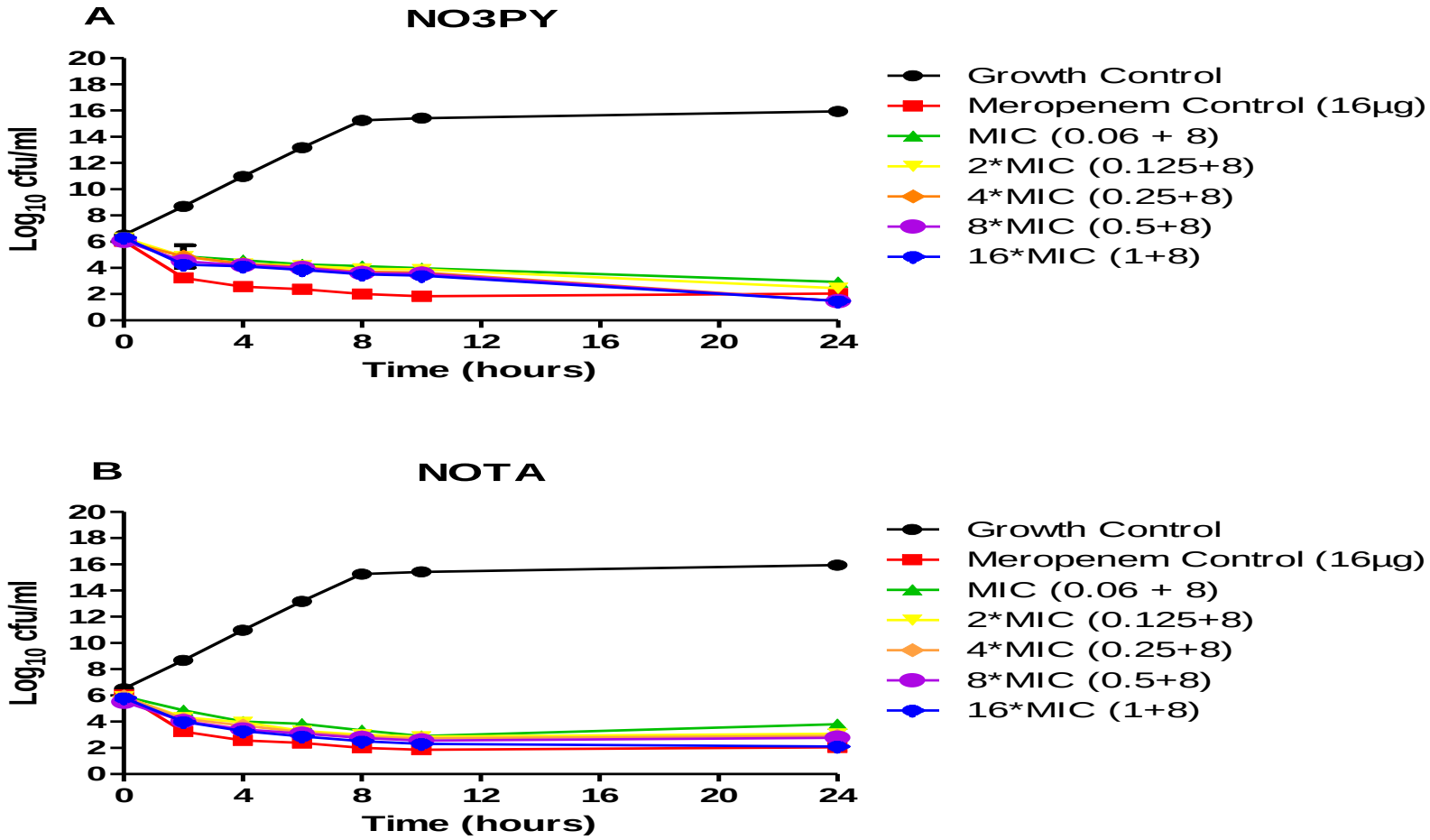

Figure 2. 3: ***E. coli*** **IMP-1 exposed to meropenem co-administered with NO3PY (A) and NOTA (B) at different MICs. No significant difference (*P* < 0.05) between the treatment groups. Mean values of duplicate cfu/ml count are plotted**

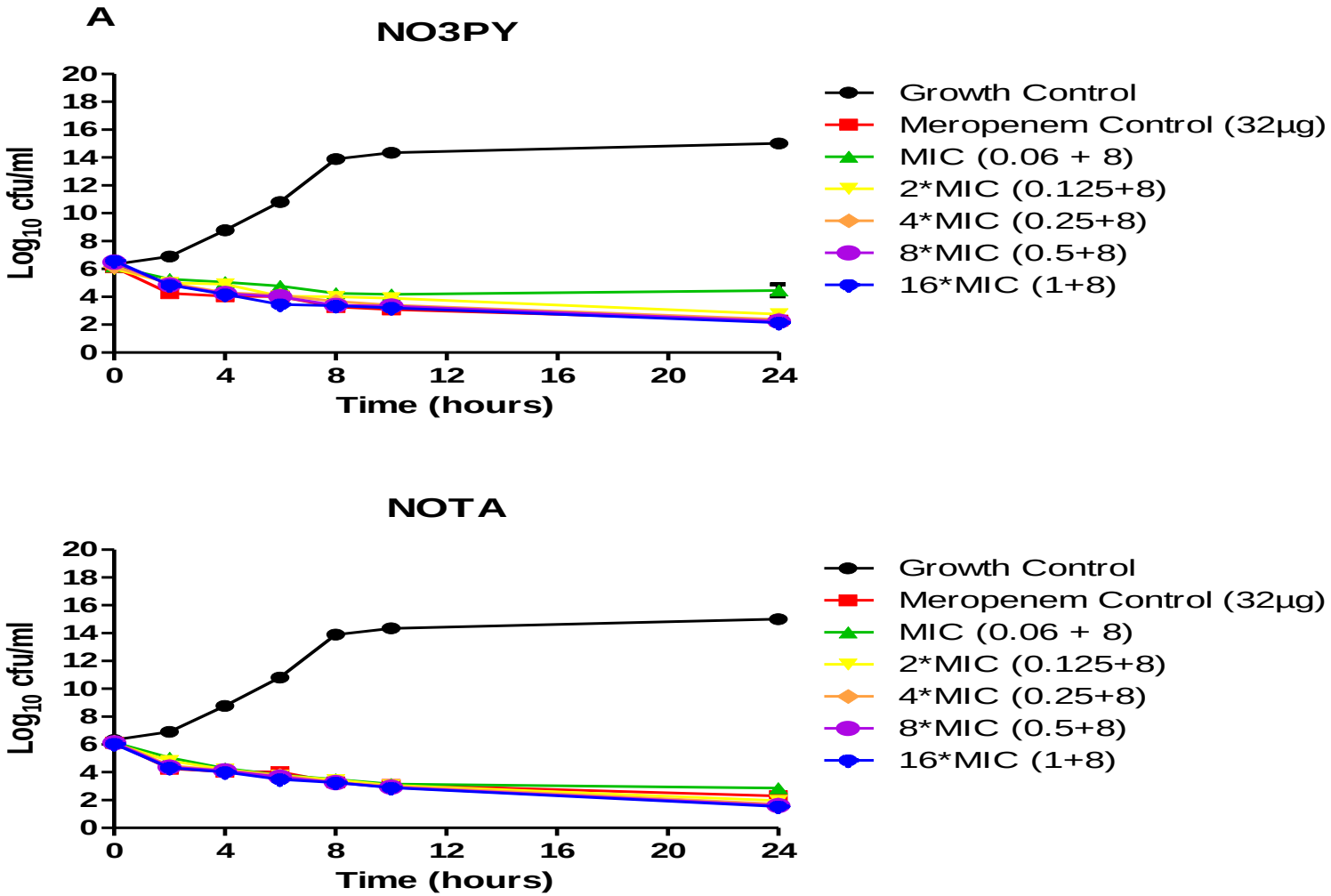

Figure 2. 4: ***E. cloacae*** **NDM-1 exposed to meropenem co-administered with NO3PY (A) and NOTA (B) at different MICs. No significant difference (*P* < 0.05) between the treatment groups. Mean values of duplicate cfu/ml count are plotted**

2.6.3. Comparison between NO3PY and NOTA against resistant bacteria

Drug efficacy against the resistant bacteria shows that there was no significant difference between the two inhibitors against two of the resistant *E. coli* NDM-1 and K. *pneumoniae*- 449. However, for *E. coli* IMP-1, there was a significant increase in the efficacy of NO3PY compared to NOTA. *E. cloacae* NDM-1 also showed significant difference between the two inhibitors (Table 2). Generally, the mean difference shows that NO3PY is slightly more effective than NOTA, although these differences were not significant

Table 2. 2: Association between NO3PY and NOTA inhibitors against metallo beta-lactamase producing CREs

Resistant organisms	**Mean square**	***F***	***P* value**
E. coli NDM-1	7.9630	1.9940	$P = 0.1956$
K. Pneumoniae -449	0.3571	0.1573	$P = 0.7021$
E. coli IMP-1	3.5080	6.2610	$P = 0.0368$*
E. cloacae NDM-1	3.5750	6.249	$P = 0.0370$*

The efficacy (reduction of the cfu per time) of the two inhibitors when co-administered with meropenem against metallo beta-lactamase producing organisms. It is calculated from the $\log_{10}$ of the colony forming unit counts. *represents $P < 0.05$.

2.6.4. IC_{50} evaluation

Notably, there were significant differences in the IC_{50} of inhibitors tested against *K. pneumonia*-449 and *E. coli* IMP-1 (Table 3). The lowest IC_{50} dose for NO3PY was against *E. coli* IMP-1 while that of NOTA was against *E. cloacae* NDM-1. It was also observed that the NO3PY inhibitor is slightly more effective than NOTA against the same organisms although it was not significant.

Table 2 3: Non-linear regression model fitted to time-kill assay data

		Meropenem	MIC	2*MIC	4*MIC	8*MIC	16*MIC
***E. coli* NDM-1 (NO3PY)**	IC_{50} (µg/ml)	15.87	8.408	8.437	8.587	8.403	8.809
	Hill slope	9.004	-216.4	-36.13	-18.24	-14.01	-14.01
	R^2	0.93	0.17	0.37	0.57	0.57	0.56
***E. coli* NDM-1 (NOTA)**	IC_{50} (µg/ml)	15.87	8.626	8.676	8.465	8.558	8.483
	Hill slope	9.004	-31.19	-36.47	-118.5	-30.87	-18.73
	R^2	0.93	0.48	0.57	0.76	0.63	0.73
***K. pneumonia*-449 (NO3PY)**	IC_{50} (µg/ml)	21.26	8.990	7.515	7.966	7.337	5.105[a]
	Hill slope	74.65	-156.8	-6.693	-7.278	-3.882	-4.188
	R^2	0.78	0.68	0.71	0.81	0.90	0.97
***K. pneumonia*-449 (NOTA)**	IC_{50} (µg/ml)	21.26	8.497	4.564	8.802	8.294	9.723[b]
	Hill slope	74.65	-41.30	-10.17	-44.56	-14.58	-15.68
	R^2	0.78	0.68	0.70	0.73	0.84	0.42
***E. coli* IMP-1 (NO3PY)**	IC_{50} (µg/ml)	0.79	0.0002[a]	0.0122[a]	0.1386	0.1293	0.0056[a]
	Hill slope	-3.550	-0.3319	-0.5994	-2.160	-0.9984	-0.4433
	R^2	0.99	0.85	0.82	0.75	0.73	0.75
***E. coli* IMP-1 (NOTA)**	IC_{50} (µg/ml)	0.79	8.139[b]	0.1026[b]	0.1637	0.1790	0.0794[b]

	Hill slope	-3.550	-0.3705	-1.475	-1.814	-1.727	-1.476
	R^2	0.99	0.92	0.98	0.99	0.99	0.99
***E. cloacae* NDM-1 (NO3PY)**	IC_{50} (µg/ml)	0.0850	0.3279	0.0010	0.026	0.035	0.014
	Hill slope	-1.546	-2.490	-0.290	-0.987	-1.830	-3.159
	R^2	0.80	0.91	0.83	0.87	0.87	0.89
***E. cloacae* NDM-1 (NOTA)**	IC_{50} (µg/ml)	0.0850	0.2422	0.0120	0.008	0.007	0.028
	Hill slope	-1.546	-2.231	-0.7139	-0.602	-1.561	-0.985
	R^2	0.80	0.96	0.87	0.82	0.81	0.81

This is the IC_{50} value of meropenem and the inhibitors at different MICs against metallo beta-lactamase producing organisms. *a* represents significant increase ($P < 0.05$) as compared to *b* in effectiveness.

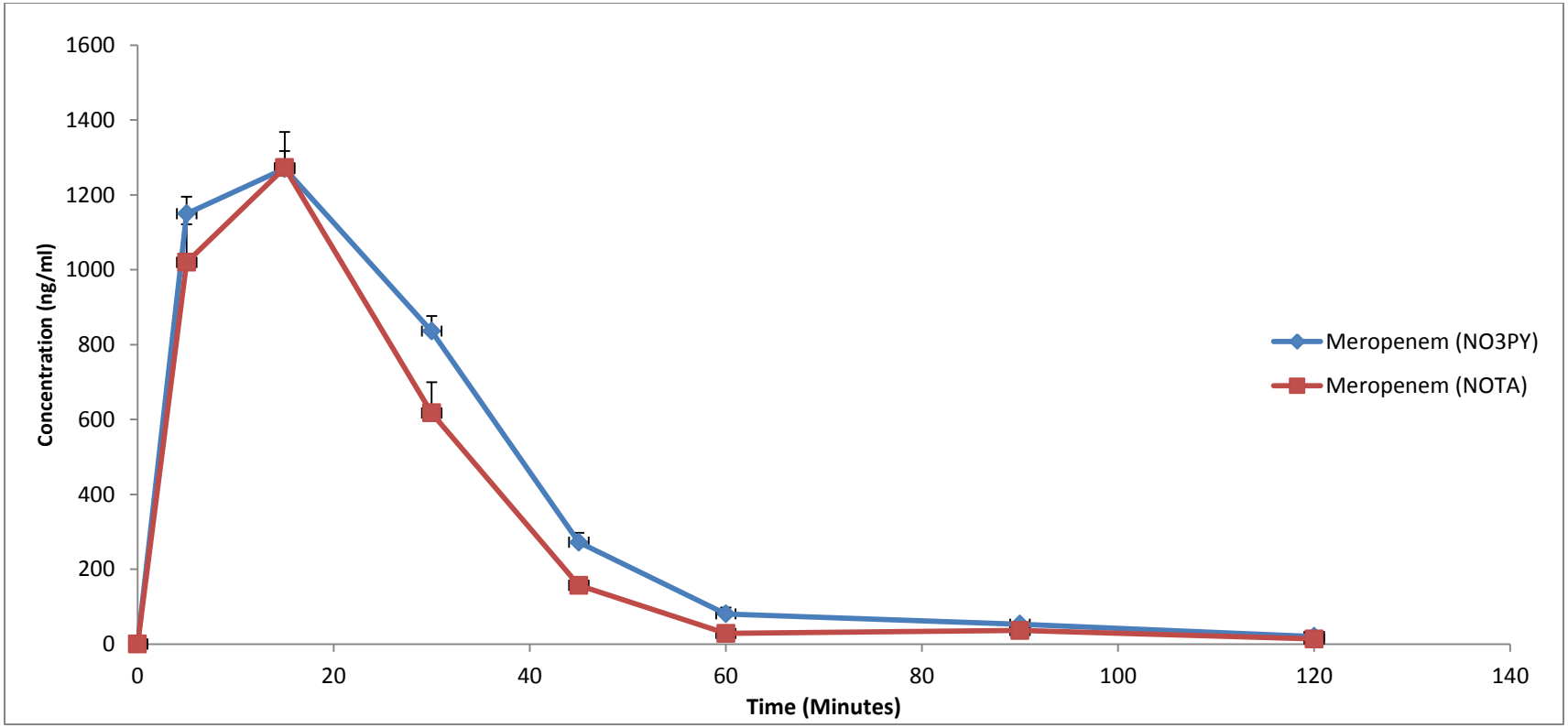

Figure 2 5: Concentrations of meropenem in plasma, following a single 10 mg kg−1 intraperitoneal dose of meropenem NO3PY and NOTA (data are represented as means ± SD, n= 3). NO3PY was below the limit of detection (10 ngml^{-1}) and could not be quantified. NOTA was above the limit of detection (10 ngml^{-1}) but lower than the limit of quantification (100 ngml^{-1}).

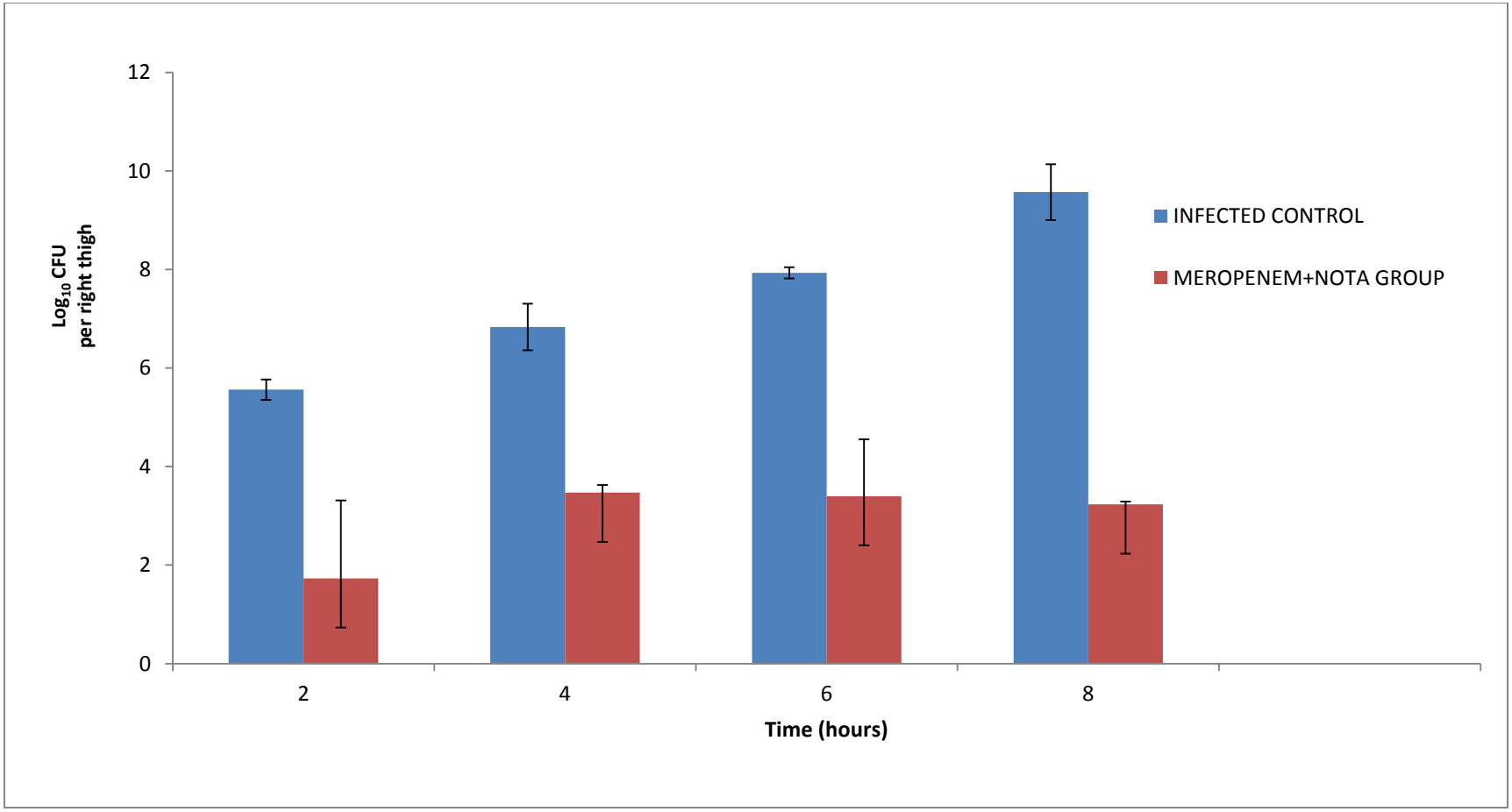

Figure 2. 6: ***In vivo*** **efficacy of NOTA when co-administered with meropenem in a murine thigh infection model (data are represented as means ± SD, n= 3). Student T-test revealed that there is significant difference (*P* = 0.0031) between the infected control and Meropenem+ NOTA treated group.**

2.7. Discussion

This study demonstrates how the co-administration of the two beta-lactamase inhibitors with meropenem was able to re-sensitize metallo-beta lactamase producing *Enterobacteriaceae* to carbapenems *in vitro*. Previously, it has been reported that NOTA was able to restore the efficacy of meropenem against *E. coli* NDM-1 and *E. cloacae* NDM-1 [33]. In this study we went further to investigate the other metallo beta-lactamase producing *Enterobacteriaceae* and to investigate the time kill kinetics of this approach. NO3PY, from the minimum inhibitory concentrations demonstrated its ability to chelate the zinc moiety present at the active site of the metallo-beta-lactamase *in vitro* thus leading to its inactivation and the eventual restoration of the potency of meropenem against CREs (Table 1).

Meropenem alone did not show a high kill effect on *E. coli* NDM-1, *K. pneumoniae* 449, *E. coli* IMP-1 and *E. cloacae* NDM-1 with time as regrowth was observed before 24 h, this could be attributed to the presence of resistant mutants. The beta-lactamase inhibitor individual analysis indicated that NO3PY exhibited the highest killing effect on *E. coli* IMP-1 while NOTA had the highest on *E. cloacae* NDM-1 with time (Table 2). Though, there were noticeable differences in the killing activity of both inhibitors on other resistant bacteria, however, this was not statistically significant. The action of meropenem is time–dependent [40,41], and this work reveals that meropenem alone appeared highly and rapidly bacteriostatic in the early logarithmic phase of growth till 4 h in *E. coli* NDM-1 before a growth relapse was observed after 6 h. Meanwhile, the MIC 2*MIC, 4*MIC, 8*MIC and 16*MIC extend their killing rate until 10 h before a weaker effect was noticed (Figures 1A and 1B), thus not only did the inhibitors restored the efficacy of meropenem, they also prolonged its duration of action. This prolonged action was also observed for *K. pnemoniae*-449 which maintained relatively bacteriostatic activity until the

10 h and 24 h for meropenem and all the MICs respectively. Greater number of colony-forming units were observed for meropenem by the 24 h (Figures 2A and 2B). The *E. coli* IMP-1 was susceptible to meropenem when co-administered with both inhibitors showing an impressive bactericidal effect on the organism (Figures 3A and 3B); the same was observed in *E. cloacae* NDM-1 (Fig 4A and 4B). The half maximal inhibitory concentration (IC_{50}) evaluation suggest an overall effectiveness of beta-lactamase inhibitors against most of the resistant organisms (Table 3).

NOTA was below the limit of quantification in the PK study (Figure 5), but it was detected, therefore we have performed the *in vivo* tests of NOTA at a much higher dose (100 mg.kg.b.w). At this concentration the chelator was able to restore the potency of meropenem by significantly reducing the colony forming unit count of *K. P* 449 when compared to the infected control (Figure 6).

Furthermore, the restoration of the potency of meropenem by NO3PY might only be limited to *in vitro* analysis. This is due to the poor bioavailability of the inhibitor *in vivo* as it was also below the limit of detection (Figure 5). A possible explanation for this is that the inhibitor's strong affinity for metal ions may have resulted in it being bound to serum cations. For the use of BFCAs for radiotherapy, a complexed metal ion is introduced into the biological system in conjugation with a vector, allowing for transport to the target site [42]. It should be noted that the chemical properties of a complex (a metal ion complexed by a chelator) is different from that of the free metal ion or the ligand, it is these differences that may enhance the bioavailability of the chelator *in vivo* thus making them desirable and applicable as radiopharmaceuticals [43]. Herein, we introduced a free chelator into the biological system. To improve its *in vivo*

bioavailability as an MBLI, it is logical that other factors such as linking it to a carrier biomolecule be considered in its development for further clinical applications.

2.8. Conclusions

The administration of NOTA and NO3PY *in vitro* was able to restore the efficacy of meropenem against *K. pneumoniae* 449, *E. coli* NDM-1, *E. coli* IMP-8 and *E. cloacae* NDM-1. From the time kill kinetics, the chelators showed similar trends in their bacterial kill rate however, NO3PY demonstrated a slightly better efficacy than NOTA though not significant. In the *in vivo* pharmacokinetics study, NO3PY had poor availability when compared to NOTA. The derivatization of NO3PY with a carrier molecule may lead to the enhancement of its bioavailability. The potency of meropenem when co-administered with NOTA was restored in a murine thigh infection model. Further preclinical work like *in vitro* and in vivo cytotoxicity tests, post beta-lactamase inhibitor effects among others are recommended for NOTA to further ascertain its suitability as a potential clinical metallo beta-lactamase inhibitor. It is also recommended that the affinity, stoichiometry and thermodynamics between these metal chelators and serum albumins and also other physiologically relevant divalent cations (Ca^{2+}, Zn^{2+}) be evaluated using isothermal titration calorimetry. Further modifications of these MBLIs are ongoing in our laboratory.

2.9. Authors contributions:

Co-conceptualized the study: KFO, TG, TN, SB. Performed the experiments: KFO, MS, NM, SM, SN. Analyzed the data: KFO. Vetting of the results: All. Wrote the paper: KFO. Undertook critical revision of the manuscript: KFO, HGK, TN, SB. Funding: TN and TG

2.10. Acknowledgements

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2.11. Declarations

2.11.1. Funding

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2.11.2. Competing interests

The authors declare that they have no conflict of interest.

2.12. Ethical approval

All animal study experiments were approved by the Institutional Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal (UKZN) (approval reference: AREC/009/018 for NO3PY PK study, AREC/013/016D for NOTA pK study and AREC/081/015D for NOTA *in vivo* efficacy study)..

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CHAPTER THREE

3.0. Conclusion

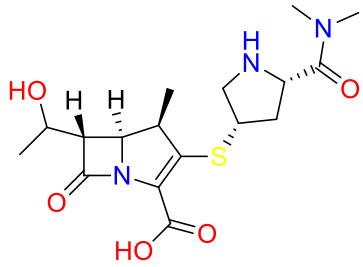
There is a growing global concern due to the increasing development of antibiotic resistance mechanisms in pathogenic bacteria, especially those mediated by beta-lactamases. Genes encoding metallo beta-lactamases are spreading rapidly, thus causing serious outbreaks which have very few treatment options. It is therefore imperative and urgent to find possible ways of combatting this scourge, by identifying agents that can be co- administered with carbapenems to inhibit the activity of these enzymes. If the activity of metallo beta-lactamase is inhibited, it will be unable to compete with carbapenems for the penicillin binding protein receptor in the bacterium. Thus, the bacterium will be resensitized to the effects of carbapenems, leading to the eradication of the infection.

This study has focused on the evaluation of the *in vitro* and *in vivo* activity of metal chelating agents NO3PY and NOTA as potential metallo beta-lactamase inhibitors. The chelators are ligands normally used in binding nuclides/ metal ions for radioimaging and therapy. The checkerboard MIC results revealed that both chelators were able to restore the efficacy of meropenem *in vitro*. This lends credence to the ability of the inhibitors to chelate zinc ions present at the active site of metallo beta-lactamase enzymes. The time kill kinetics also showed that both compounds were able to significantly extend the killing time of meropenem. A sensitive LC-MS method was developed to detect NOTA in plasma, but it was below the limit of quantification whereas NO3PY was undetectable. NOTA was also able to potentiate the effects of meropenem *in vivo* which was evident by the significant decrease of the colony forming unit count of *Klebsiella pneumoniae* 449 when compared to infected control in a murine thigh infection model. This suggests that NOTA holds a strong potential of being a clinically available metallo beta-lactamase inhibitor.

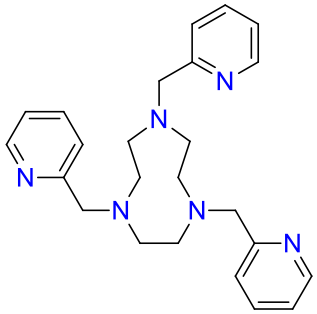
A possible way of improving the bioavailability of these chelators in the biological system is to either derivatize the structure in such a way that it will preferentially bind to the zinc molecule in the active site of the metallo beta-lactamase enzymes or if they can be targeted directly to the site of infection either by encapsulating in microspheres, nanoparticles, liposomes or linking it to a biological vector. All these approaches will prevent the chelators from binding to stray metal

ions in the body. Thus, their overall bioavailabity will increase and by extension their potency *in vivo.* As NOTA restored the potency of meropenem in a murine thigh infection, this might just be the first step in unveiling the answer to the scourge of infections mediated by MBL-producing CREs. It is therefore recommended that further *in vitro* and *in vivo* cytotoxicity assays should be carried out. The possibility of the MBLIs in restoring the potency of carbapenems *in vivo* should be tested further in primates (monkeys, apes etc) before it can be subjected to clinical trials in human subjects.

Supporting Information for Chapter Two



Structure of Meropenem



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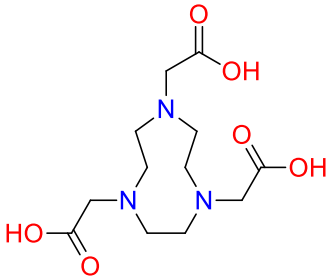

Structure of NOTA