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In Vitro Efficacy of Biocompatible Zinc Ion Chelating Molecules as Metallo-β-Lactamase Inhibitor among NDM Producing *Escherichia coli*

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Abstract

Objective This article assesses the effectiveness of captopril, tetracycline, and ciprofloxacin as metallo-β-lactamase (MBL) inhibitors against New Delhi metallo-β-lactamase (NDM)-producing *Escherichia coli.*

Materials and Methods Twenty-four well-characterized carbapenem-resistant *E. coli* isolates which produced NDM ($n = 21$) and Oxa-48-like enzymes ($n = 3$) were used to assess the inhibitors. The positive control organism was designed by cloning the NDM gene into pET-24a plasmid and transforming it into expression vector *E. coli* BL21. All the proposed inhibitors were assessed for their interaction with MBLs using checkerboard minimum inhibitory concentration (MIC) assay with imipenem and meropenem. The fractional inhibitory concentration (FIC) index was calculated to assess the activity of molecules.

Results The *E. coli* BL21 (DE3) pET-24a-bla_{NDM} showed carbapenem resistance upon isopropyl β-D-1-thiogalactopyranoside induction and had MIC of 32 µg/mL for both imipenem and meropenem. For the test isolates, ΣFIC values of imipenem and meropenem with ethylenediaminetetraacetic acid (EDTA) ranged from 0.039 to 0.266 and 0.023 to 0.156, respectively. At a 256 µg/mL concentration, captopril had ΣFIC index value for imipenem and meropenem as 0.133 to 0.375 and 0.133 to 0.188, respectively. The tetracycline and ciprofloxacin in combination with meropenem/ imipenem showed indifferent results.

Conclusion Among the three molecules tested, captopril had MBL inhibitory activity, but the concentration required for inhibition was beyond the therapeutic safety levels. Ciprofloxacin and tetracycline had weak or no MBL inhibitory activity. Checkerboard MIC of EDTA with carbapenem antibiotic and control organism with NDM enzyme production helped us create a reference system for comparing and assessing the results of potential MBL inhibitors in future.

Keywords

- MBL inhibitors
- New Delhi Metallo-β-lactamases
- tetracycline
- captopril
- ciprofloxacin

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Introduction

Beta-lactamase production is the most common and efficient mechanism of carbapenem resistance.[1,2] Mechanistically, there are two types of carbapenemases: serine β-lactamases (SBLs, Ambler class A, C, and D) and metallo-β-lactamases (MBLs, Amblers class B).[3] Based on the differences in amino acid sequences, MBL enzymes are subclassified into B1, B2, and B3. The B1 and B3 have two Zn^{2+} ions and B2 has one Zn^{2+} ion at their active site.[3] Two Zn^{2+} ions of B1 enzymes coordinate with water molecules to attack the C-N bond of the β-lactam ring, making the antibiotics ineffective. Genes for B1 enzymes are located on mobile genetic elements and are easily transmissible among Gram-negative bacteria, and hence they are clinically important.[3–5]

The activity of β-lactam antibiotics can be restored after combining them with β-lactamase inhibitors. Mechanism-based SBL inhibitors are already in clinical use, such as clavulanic acid, sulbactam, tazobactam, avibactam, and vaborbactam.[3] These inhibitors, on the other hand, have little or no effect on MBL enzymes. MBL inhibitors are urgently needed as MBL enzymes act on a wide range of β-lactam substrates limiting treatment options.

A potent MBL inhibitor should be stable in vivo, should not affect host metabolism, and should completely restore the activity of β-lactam antibiotics. The development of universal MBL inhibitors is difficult. This is attributed to the differences in mechanisms of β-lactam hydrolysis, and the design of the catalytic site.[2] The molecules containing thiol or thiocarbonyl, small bicyclic compounds, aspergillomarasmine A, bismuth compounds, dicarboxylates, hydroxamates, aryl sulfonamides, tetrazole-based molecules, and strong chelating agents have been assessed as potent MBL inhibitors.[3,6] But many of them have an off-target effect on metalloenzymes involved in human metabolism, and some of the molecules are metabolized in the body to make them ineffective.[2] Some of these molecules have entered into phase III trials but the search for newer molecules continues.

Drugs currently in clinical use and have Zn^{2+} ion binding, or chelating characteristics can be repurposed as MBL inhibitors. Such compounds' kinetic and dynamic properties are already known, and if verified, they can be used as MBL inhibitors. And we can cut short the time taken for the development of new molecules. Fluoroquinolones are known to be combined with metal cations with varying affinity ($Al^{3+} > Cu^{2+} > Zn^{2+} > Fe^{3+}$).[7] Tetracyclines are known to bind Zn^{2+}-dependent matrix metalloproteinases and prevent their action.[8] The captopril and its isomers that inhibit angiotensin-converting enzyme (metalloenzyme) can extend their action toward MBL enzymes. All three chemical compounds could bind or chelate Zn^{2+} ions and have an inhibitory activity on metalloproteinases.[9] Hence, the study aimed to assess the effectiveness of captopril, tetracycline, and ciprofloxacin as MBL inhibitors against New Delhi metallo-β-lactamase (NDM)-producing *Escherichia coli.*

Methods

A total of 24 well-characterized clinical isolates of carbapenem-resistant *E. coli* were included in the study. The multiplex polymerase chain reaction (PCR) was performed with previously published protocols for ESBL, AmpC, and carbapenemase genes.[10–12] Twenty-one isolates had the bla_{NDM} gene with the ESBL gene and/or AmpC gene, and three isolates had the $bla_{Oxa\text{-}48\ like}$ gene (►**Table 1**). The minimum inhibitory concentration (MIC) of all the 24 test isolates for imipenem, meropenem, ciprofloxacin, tetracycline, captopril, and ethylenediaminetetraacetic acid (EDTA) was determined by broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (►**Table 1**). To investigate the effect of inhibitors, we designed a control organism that produces only NDM enzymes, lacks other β-lactamases and other mechanisms of carbapenem resistance.

The NDM Plasmid Deoxyribonucleic Acid Extraction[13]

The plasmid deoxyribonucleic acid (DNA) was extracted from NDM producing clinical isolates of *E. coli* using alkali lysis method. Briefly, the NDM producing *E. coli* was grown overnight in Luria-Bertani broth at 37°C in an orbital shaker incubator. A 1.5 mL of overnight culture was centrifuged and supernatant was removed. To the pellet 100 µL prechilled solution I and 200 µL of alkaline lysis solution II were added. The tube was gently inverted 4 to 5 times, and 150 µL of alkaline lysis solution III was added and centrifuged at 13,000 revolutions per minute (rpm) for 30 minutes at 4°C. An equal volume of phenol:chloroform mixture was added to the supernatant and centrifuged at 13,000 rpm. Note that 1/10th volume sodium acetate and 2.5 volume absolute ethanol were added to the aqueous layer. The tube was maintained at –20°C overnight. The next day it was centrifuged and to the pellet, 200 µl of 70% ethanol was added and recentrifuged. The supernatant was discarded and pellet was air-dried. Twenty microliter of sterile distilled water was added to the pellet and the concentration of the DNA was estimated using nanodrop.

Amplification and Purification of Full Length NDM Gene for Cloning[14]

The amplification of bla_{NDM} gene was done using forward primer (5′ttatattaa**catatg**gaattgcccaatattatgcac3′) and reverse primer (5′ttt**gaattc**tcagcgcagcttgtcggccatg3′) as described previously. The forward and reverse primers had restriction sites for NdeI and *Eco*RI enzymes, respectively. The PCR was performed on a BioRad C1000 gradient thermal cycler with 25 µL volume under the following protocol: 95°C, 1 minute followed by 25 cycles of 95°C, 30 seconds 64°C, 45 seconds 72°C, 1 minute 30 seconds final elongation at 72°C, 10 minutes; and cooling the mixture to 4°C. The PCR product was electrophoresed in 1% agarose gel. According to the manufacturer's protocol, the amplified PCR product was purified using a DNA purification kit (Macherey Nagel, Germany). The purified product was used for cloning into expression vector.

Table 1 The test strains included in the study with their β-lactamase content and MIC

Strain number	β-lactamase content	Minimum inhibitory concentration (μg/mL)					
		Imipenem	Meropenem	Ciprofloxacin	Tetracycline	Captopril	EDTA
2	NDM + TEM+ CIT +CTXM-1	16	8	128	256	2,048	2,048
3	NDM + TEM	16	4	64	128	2,048	2,048
4	OXA-48-like + TEM+ CIT +CTXM-1	4	0.25	256	256	2,048	2,048
6	NDM + TEM + CTXM-1	32	32	256	256	2,048	2,048
8	NDM + TEM+ CTXM-1 + CTXM-8	8	8	128	128	2,048	2,048
9	NDM+ CTXM-1	64	16	256	256	2,048	2,048
10	NDM	32	16	64	128	2,048	2,048
16	NDM + TEM + CTXM-1	64	16	128	256	2,048	2,048
21	NDM + TEM+ CIT	16	8	64	128	2,048	2,048
22	NDM + CIT	16	64	128	64	2,048	2,048
42	NDM + TEM+ CIT +CTXM-1	16	16	512	128	2,048	2,048
44	NDM + TEM + CTXM-1	16	16	512	512	2,048	2,048
45	NDM + TEM+ CIT	32	16	128	256	2,048	2,048
46	NDM + TEM + CTXM-1	8	8	256	256	2,048	2,048
77	NDM + OXA + TEM+ CIT +CTXM-1	16	16	256	128	2,048	2,048
109	NDM + TEM+ CIT	16	16	128	128	2,048	2,048
114	NDM+ CIT +CTXM-1	16	16	256	256	2,048	2,048
137	OXA-48-like	2	< 0.125	128	256	2,048	2,048
142	NDM + TEM+ CTXM-1 + CTXM-25	8	2	128	128	2,048	2,048
162	OXA-48-like + TEM+ CIT +CTXM-25	1	< 0.125	128	128	2,048	2,048
174	NDM + TEM+ CIT +CTXM-1 + CTXM-25	16	16	256	256	2,048	2,048
197	NDM + TEM+ CIT +CTXM-8	8	16	512	256	2,048	2,048
209	NDM+ CIT +CTXM-8	16	64	128	256	2,048	2,048
217	NDM	16	32	512	256	2,048	2,048
ATCC *E. coli* 25922		< 0.125	< 0.125	< 0.125	< 0.125	2,048	2,048
E. coli BL21 (DE3) pET-24a-*bla*$_{NDM}$		32	32	< 0.125	< 0.125	2,048	2,048

Abbreviations: EDTA, ethylenediaminetetraacetic acid calcium disodium salt; MIC, minimum inhibitory concentration.

Cloning the NDM Gene in the pET24a (+) Vector[14]

The pET-24a (+) vector DNA was obtained from Merck Millipore. The plasmid vector and amplified NDM gene were digested with NdeI and *Eco*RI restriction enzymes by keeping the reaction at 37°C for 2 hours, separated in 1% agarose gel, and purified using a gel extraction kit, as directed by the manufacturer (Macherey Nagel, Germany). The T4 DNA ligase was used to ligate the purified vector and

insert the gene. The reaction took place in 20 µL volume with vector-to-insert ratio of 1:3 to 1:4. It was incubated for 1 hour at 25°C and then overnight (16 hours) at 16°C; subsequently, it was used to transform into competent *E. coli* DH5α. The transformed DH5α were subcultured on LB agar with kanamycin (50 µg/µL) and incubated at 37°C incubator. The pET-24a-bla_{NDM} plasmid was reextracted from *E. coli* DH5α cells and confirmed for the NDM gene by PCR and restriction digestion. The confirmed pET-24a-bla_{NDM} plasmid was transformed into competent *E. coli* BL21 (DE3) cells and transformant *E. coli* BL21 (DE3) possessing pET-24a-bla_{NDM} plasmid was confirmed for the NDM gene by restriction digestion and PCR. The *E. coli* BL21 pET-24a-bla_{NDM} was used as a positive control for NDM enzymes and the ATCC *E. coli* 25922 was used as a negative control.

Drug Interaction Models

The in vitro interaction of the EDTA calcium disodium salt, captopril (Sigma code C8856–1G), tetracycline (HiMedia, Mumbai, Maharashtra, India), and ciprofloxacin (HiMedia) with meropenem (Sigma code PHR1772–500MG) or imipenem (Sigma code PHR1796–200MG) alone and in combination against the recombinant *E. coli* BL21 (DE3) pET-24a-bla_{NDM} strain and 24 test *E. coli* isolates was performed using the checkerboard assay as shown in ►**Table 2**.

Preparation of Stock Drug Solution

The stock solution was prepared at a concentration of 5120 µg/mL using the formula $W = CV/P$, where W is the weight of drug for required concentration, C is the concentration required, P is the potency of the drug, and V is the volume required. The dilutions of the drugs were made according to the CLSI M100-S30 guidelines.[15]

Checkerboard MIC Assay[5]

The dilutions of the drugs in cation-adjusted Mueller-Hinton broth alone and in combination were loaded into 96-well polypropylene microtiter plates (►**Table 2**). The organisms were grown in trypticase soy broth till they reached 0.5 McFarland turbidity and were diluted to 1:20 in sterile saline. Note that 10 µL of the diluted inoculum was added to the growth control well and other wells with the drugs alone and in combination. The inoculated plates were incubated at 35 ± 2°C for 16 to 20 hours. The lowest concentration of imipenem/meropenem alone and in combination with the proposed inhibitor was noted as the MIC. The MICs of the isolate at various combinations of the drugs were noted. The fractional inhibitory concentration index (FICI) was calculated using the formula

$$\Sigma FIC = FIC_A + FIC_B = (MIC_{AB}/MIC_A) + (MIC_{BA}/MIC_B)$$

where MIC_A and MIC_B and MIC_{AB} and MIC_{BA} are the MICs of drugs A and B when acting alone and combination, respectively. A FICI value of 0.5 or lower was considered synergy, FICI greater than 4 was considered antagonism, and FICI value between 0.5 and 4 was considered to be indifferent.[16]

Results

The cloning of the bla_{NDM} gene into the pET-24(a) plasmid vector was confirmed by PCR and restriction digestion. The PCR amplicon of 813 base pair (bp) and the restriction digestion products of 813 bp indicated the confirmation of the clone. The *E. coli* BL21 (DE3) pET-24a-bla_{NDM} strain had leaky expression of NDM gene. On induction with isopropyl β-D-1-thiogalactopyranoside (0.1 mM), the isolate showed a MIC of 32 µg/mL for both imipenem and meropenem. The MICs of the test and control organisms against all the molecules used are depicted in ►**Table 1**. Except for three OXA-48 producing *E. coli* susceptible to meropenem, all other isolates exhibited resistance to both imipenem and meropenem.

The checkerboard synergistic assay results of imipenem and meropenem with EDTA, captopril, ciprofloxacin, and tetracycline are tabulated in ►**Table 3**. The EDTA had potent NDM inhibitory activity and restored the sensitivity of both imipenem and meropenem for NDM producing *E. coli*. The imipenem MIC of the *E. coli* BL21 (DE3) pET-24a-bla_{NDM} was lowered from 32 to 1 µg/mL (fivefold reduction in MIC) with a synergistic ΣFIC value of 0.0938 and the meropenem MIC

Table 2 The layout of checkerboard assay between carbapenem antibiotic and proposed inhibitors

Inhibitor (µg/mL)		1	2	3	4	5	6	7	8	9	10	11	12
	A	512	512/0.125	512/0.25	512/0.5	512/1	512/2	512/4	512/8				
	B	256	256/0.125	256/0.25	256/0.5	256/1	256/2	256/4	256/8				
	C	128	128/0.125	128/0.25	128/0.5	128/1	128/2	128/4	128/8				
	D	64	64/0.125	64/0.25	64/0.5	64/1	64/2	64/4	64/8				
	E	32	32/0.125	32/0.25	32/0.5	32/1	32/2	32/4	32/8				
	F	16	16/0.125	16/0.25	16/0.5	16/1	16/2	16/4	16/8				
	G	8	8/0.125	8/0.25	8/0.5	8/1	8/2	8/4	8/8				
	H	GC	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Carbapenem antibiotic (µg/mL)													

Table 3 The ΣFIC index value of imipenem and meropenem with proposed inhibitors

Serial no.	Meropenem								Imipenem							
	EDTA		Captopril		Ciprofloxacin		Tetracycline		EDTA		Captopril		Ciprofloxacin		Tetracycline	
	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
2	32/0.125	0.031	256/0.5	0.188	64/4	0.75	256/8	2	128/1	0.125	256/4	0.375	64/8	1	256/16	2
3	64/0.25	0.094	256/0.25	0.188	32/2	1	128/4	2	32/0.5	0.047	256/1	0.188	32/4	0.75	128/16	2
4	2048/0.25	2	2048/0.25	2	128/0.125	1	256/0.25	2	2048/4	2	2048/4	2	128/1	0.75	256/4	2
6	64/0.125	0.035	256/0.5	0.141	256/32	2	256/32	2	128/1	0.094	256/4	0.25	128/8	0.75	256/4	2
8	32/0.125	0.031	256/0.5	0.188	32/4	0.75	64/4	1	64/0.125	0.047	256/0.5	0.188	32/4	0.75	128/8	2
9	32/0.125	0.023	256/0.5	0.156	64/8	0.75	256/16	2	64/0.25	0.047	256/0.5	0.133	256/64	2	256/64	2
10	32/0.125	0.023	256/0.5	0.156	32/4	0.75	128/16	2	64/2	0.094	256/0.5	0.141	64/4	1.125	64/8	0.5
16	64/1	0.094	256/1	0.188	64/4	0.75	256/16	2	64/0.5	0.039	256/1	0.141	64/32	1	256/64	2
21	32/0.5	0.078	256/0.5	0.188	32/4	1	64/2	1	32/0.5	0.047	256/0.25	0.141	32/4	0.75	128/16	2
22	64/0.5	0.039	256/4	0.188	64/16	0.75	64/64	2	32/4	0.266	256/2	0.25	32/4	0.5	64/16	2
42	64/0.5	0.063	256/0.5	0.156	128/4	0.5	128/16	2	32/0.5	0.047	256/1	0.188	256/8	1	64/8	1
44	64/0.125	0.047	256/1	0.188	256/8	1	256/8	1	64/0.25	0.047	256/0.5	0.156	256/8	1	256/8	1
45	64/0.5	0.063	256/0.5	0.156	32/4	0.5	128/8	1	64/2	0.094	256/0.5	0.141	64/16	1	256/32	2
46	32/0.125	0.031	256/0.25	0.156	32/4	0.625	256/8	2	64/0.125	0.047	256/0.5	0.188	128/4	1	128/4	1
77	64/1	0.094	256/0.5	0.156	128/8	1	64/8	1	32/2	0.141	256/2	0.25	128/4	0.75	128/16	2
109	64/0.5	0.063	256/0.5	0.156	32/8	0.75	128/16	2	64/0.5	0.063	256/1	0.188	64/4	0.75	128/16	2
114	64/0.25	0.063	256/0.25	0.141	64/4	0.5	256/16	2	32/0.5	0.047	256/0.5	0.156	64/4	0.5	128/8	1
137	–	–	–	–	–	–	–	–	–	–	–	–	64/1	2	256/2	2
142	64/0.25	0.156	256/0.125	0.188	32/0.5	0.5	512/2	2	64/0.25	0.063	256/0.25	0.156	32/4	0.75	128/8	2
162	–	–	–	–	–	–	–	–	–	–	–	–	64/05	1	128/8	2
174	64/0.5	0.063	256/0.25	0.141	32/4	0.375	256/16	2	64/0.25	0.047	256/0.25	0.136	128/4	0.75	256/16	2
197	64/0.25	0.047	256/0.5	0.156	128/4	0.5	256/16	2	64/0.25	0.063	256/0.5	0.188	256/4	1	256/8	2
209	64/1	0.047	256/0.5	0.133	32/8	0.375	128/32	1	64/0.5	0.063	256/0.5	0.156	64/4	0.75	256/16	2
217	64/2	0.094	256/2	0.188	256/16	1	256/32	2	64/2	0.156	128/2	0.188	256/8	1	128/8	1
E. coli ATCC 25922	< 0.125	–	< 0.125	–	< 0.125	–	< 0.125	–	< 0.125	–	< 0.125	–	< 0.125		< 0.125	–
E. coli BL21 (DE3) pET-24a-blaNDM	32/0.125	0.0195	256/0.5	0.141	–		–		128/1	0.094	256/4	0.25	–		–	

Abbreviations: ΣFIC, fractional inhibitory concentration index; EDTA, ethylenediaminetetraacetic acid calcium disodium salt; MIC, minimum inhibitory concentration.

was lowered from 32 to 0.125 µg/mL with a synergistic ΣFIC value of 0.0195. Interestingly, for NDM producing clinical isolates, there was a three- to eightfold reduction in imipenem and meropenem MIC in combination with EDTA, and sensitivity of the isolates to both the antibiotics was restored. The synergistic activity of the EDTA started at 32 µg/mL, and best results were obtained at 64 µg/mL. The highest concentration of EDTA at which synergistic activity was seen was 128 µg/mL. For test isolates, ΣFIC values of imipenem with EDTA and meropenem with EDTA ranged from 0.039 to 0.266 and 0.023 to 0.156, respectively. All the clinical isolates of *E. coli* which produced NDM carbapenemase have a synergistic ΣFIC values less than 0.5 at a 64-µg/mL concentration of EDTA for both imipenem and meropenem. Also, all ΣFIC index values of the EDTA in combination with meropenem and imipenem against NDM positive isolates were less than 0.5. However, the *E. coli* strains with OXA-48-like enzyme production did not reduce the MIC of imipenem and meropenem when combined with EDTA. Such isolates had ΣFIC index values of 2 suggesting indifferent results. Therefore, the checkerboard synergy assay results of EDTA and carbapenem antibiotic mixtures were considered as a reference for assessing the results of the other three inhibitors.

The checkerboard synergy assay showed captopril lowers the MIC of imipenem and meropenem against NDM-producing *E. coli*. Captopril at 256 µg/mL reduced the imipenem MIC of *E. coli* BL21 (DE3) pET-24a-bla_{NDM} from 32 to 4 µg/mL (ΣFIC 0.25) and meropenem MIC from 32 to 0.5 µg/mL (ΣFIC 0.141). The captopril inhibited the NDM enzymes of test isolates at a 256-µg/mL concentration. A four- to sixfold reduction in MIC of both the carbapenem antibiotics was noticed. The ΣFIC index value of imipenem and meropenem with captopril (256 µg/mL) ranged from 0.133 to 0.375 and 0.133 to 0.188, respectively. Captopril at 256 µg/mL showed a ΣFIC index value of greater than 0.5 for both the antibiotics. Captopril did not synergize with imipenem or meropenem for OXA-48-like enzyme producing *E. coli*.

The tetracycline and ciprofloxacin in combination with meropenem/imipenem showed indifferent results on checkerboard MIC for all isolates. The ΣFIC value of ciprofloxacin with meropenem ranged between 0.375 and 2. There were one- to twofold reductions in meropenem MIC in combination with ciprofloxacin. Only two NDM producing isolates (no. 174 and 209) had a synergistic ΣFIC value of 0.375, the rest of the isolates had ΣFIC value of greater than 0.5. For isolate number 174, the MIC of meropenem was reduced from 16 to 4 µg/mL, and for isolate 209 the MIC was reduced from 64 to 8 µg/mL in combination with ciprofloxacin. But for the same isolates, the imipenem and ciprofloxacin combinations had ΣFIC value of 0.75. The ΣFIC value of ciprofloxacin with imipenem ranged between 0.5 and 2.

Discussion

There are no effective MBL inhibitors in the clinic, and designing an efficient inhibitor which can act on various types of MBL enzymes is challenging. Attempts have been made to identify the potential inhibitors of the MBLs and various compounds have been designed to inhibit MBL enzymes. The molecules mimicking substrate (β-lactam), transition state analogs (an intermediate form of the substrate when interacting with enzyme), hydrolysis products of β-lactam antibiotics (such as moxalactam and cefoxitin), MBL-product complexes, and strong metal ion chelating agents have been used to inhibit MBL enzymes.[5,6,14] However, the efficacy and safety of these inhibitors in vivo is not known.

An organism with only NDM production without other carbapenem resistance mechanisms was required to assess the efficacy of MBL inhibitors. The presence of other β-lactamases like ESBL/AmpC or presence of another mechanism of resistance may mask the inhibitory property of the proposed inhibitors. Hence, *E. coli* BL21 (DE3) pET-24a-bla_{NDM} was created by cloning the NDM gene. When induced, it displayed carbapenem resistance solely by MBL production, and molecules which can inhibit MBL enzymes could be easily assessed.

The EDTA is a known inhibitor of MBLs. We assessed EDTA as a potent NDM inhibitor, and results of other inhibitors were compared with the results of EDTA synergy. All the ΣFIC index values of the EDTA in combination with meropenem and imipenem against NDM positive isolates were less than 0.5. The EDTA could bring back the sensitivity of the isolates to imipenem and meropenem. EDTA calcium disodium salt is used as a chelating agent for treatment of lead poisoning.[6] NDM producing bacterial infections can be treated by combining EDTA with β-lactam antibiotics. But cytotoxic effect, potential complications, and high concentration required to inhibit MBL enzymes, as noted in our study, may limit its use.[17] The checkerboard synergy assay results of EDTA and carbapenem antibiotic mixtures can be used to assess the efficacy of potential MBL inhibitors in the future.

Captopril is an angiotensin-converting enzyme inhibitor used for the treatment of hypertension. It resembles hydrolyzed penicillin and was explored as BcII and CphA inhibitor.[18] The checkerboard synergy assay showed captopril lowers the MIC of imipenem and meropenem to sensitive levels against test and control organisms. The captopril efficiently inhibited the NDM enzymes at a concentration of 256 µg/mL. This concentration is beyond the therapeutic dosage levels of captopril for human use. When compared with the synergistic action of EDTA, captopril was a weak inhibitor of NDM enzymes. The stereoisomers of captopril, especially D-enantiomer have shown good potency to inhibit VIM-2, IMP-1, and BcII enzymes.[9,18] But their utility in NDM enzymes was not assessed previously.

Quinolones, tetracycline, and their derivatives can chelate di- or trivalent metal ions like magnesium and zinc.[7,8] The tetracycline and ciprofloxacin in combination with meropenem or imipenem showed indifferent results on checkerboard MIC. They have either weak or no inhibitory activity against NDM enzymes. Though there was one- to twofold reduction in MIC of imipenem, the sensitivity was not restored as compared with EDTA and captopril. The combination of tetracycline with meropenem and imipenem had an indifferent ΣFIC value of 1 or 2. The MIC of the carbapenem antibiotics was not reduced or reduced only onefold in combination with tetracycline. The results indicate that

ciprofloxacin and tetracycline could not chelate the zinc present at the active site of the MBL enzymes even on repeated testing.

There was no difference in the efficacy of the inhibitors for isolates producing only NDM and NDM with ESBL and/or AmpC enzymes. But further studies are required to assess the efficacy of these molecules against isolates having multiple mechanisms of carbapenem resistance.

Conclusion

The cloning of the NDM gene into pET24(a) vector plasmid and transforming it into the expression vector *E. coli* BL21 gave us a control organism that could express only the NDM gene. We created a model that can screen and study future MBL inhibitors. The checkerboard synergy of EDTA with carbapenem antibiotic created a reference system for comparing and assessing the results of potential MBL inhibitors. Captopril could completely revert the sensitivity of the carbapenem antibiotics for NDM producing *E. coli*. The therapeutic utility of captopril in combination with carbapenem antibiotics against NDM-producing organisms can be evaluated by in vivo studies in animal models. At the same time, ciprofloxacin and tetracycline displayed no appreciable synergy with carbapenem antibiotics against NDM producing *E. coli* isolates.

Ethical Approval
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Conflict of Interest
None declared.

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