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New polyimidazole ligands against subclass B1 metallo-β-lactamases: Kinetic, microbiological, docking analysis

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ABSTRACT

Beta-lactam antibiotics are one of the most commonly used drug classes in managing bacterial infections. However, their use is threatened by the alarming phenomenon of antimicrobial resistance, which represents a worldwide health concern. Given the continuous spread of metallo-β-lactamases (MBLs) producing pathogens, the need to discover broad-spectrum β-lactamase inhibitors is increasingly growing. A series of zinc chelators have been synthesized and investigated for their ability to hamper the Zn-ion network of interactions in the active site of MBLs. We assessed the inhibitory activity of new polyimidazole ligands *N,N′*-bis((imidazol-4-yl) methyl)-ethylenediamine, N,N,N′-tris((imidazol-4-yl)methyl)-ethylenediamine, N,N,N,N′-tetra((imidazol-4-yl-methyl)-ethylenediamine toward three different subclasses B1 MBLs: VIM-1, NDM-1 and IMP-1 by in vitro assays. The activity of known zinc chelators such as 1,4,7,10,13-Pentaazacyclopentadecane, 1,4,8,11-Tetraazacyclotetradecane and 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid was also assessed. Moreover, a molecular docking study was carried to gain insight into the interaction mode of the most active ligands.

1. Introduction

β-Lactam antibiotics are widely used in antimicrobial chemotherapy because of their excellent activity and low toxicity [1]. Production of β-lactamases (BLs) is the most common mechanism of β-lactam resistance among pathogenic bacteria and significantly limits the success of antibiotic chemotherapy against bacterial infections [2]. According to their amino acid sequences, β-lactamases are classified into four molecular classes: A, B, C and D [3]. The molecular classes A, C and D are serine-β-lactamases (SBLs). Class B β-lactamases are metallo-β-lactamases (MBLs) which exhibit a very broad activity spectrum making the producing bacteria resistant to all β-lactams, except monobactams [4,5]. MBLs use one or two zinc ions at the active site for catalysis [6–8]. According to amino acid sequence homology and substrate profile, MBLs are categorized in subclasses B1, B2, B3 [9]. The B1 and B3 enzymes need two zinc ions for catalysis, whereas B2 MBLs are monozinc enzymes [9]. The active site of MBLs is a hydrophobic cavity including Zn1 coordinated to H116, H118, and H196, the Zn2 coordinated to D120, C221 and H263 (BBL numbering). A specific feature of MBLs is the presence, around the active site, of loops implicated in the zinc ions coordination, stability and substrate specificity [4]. MBLs are rapidly spreading worldwide among *Enterobacterales* and non-fermenting gram-negative bacteria (i.e. *Acinetobacter spp*, *Pseudomonas aeruginosa*). Subclass B1 includes the most numerous and clinically important MBLs such as NDM-, VIM- and IMP-variants [10]. Presently, 97 IMP-, 83 VIM- and 44 NDM-variants have been reported in several countries (http://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/, last update 2022-10-11).

Abbreviations: AVI, Avibactam; AZT, Aztreonam; BL, β-lactamase; BLI, β-lactamase inhibitor; CLSI, Clinical and Laboratory Standards Institute; Cyclam, 1,4,8,11-Tetraazacyclotetradecane; DOTA, 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid; IM, Imidazole; IMP, Imipenemase; MBL, Metallo-β-lactamase; MEM, Meropenem; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDM, New Delhi metallo-beta-lactamase; PBMC, Normal peripheral blood mononuclear cell; Penta-AZA, 1,4,7,10,13-Pentaazacyclopentadecane; SBL, Serine-β-lactamse; VIM, Verona integron-encoded metallo-beta-lactamase.

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A successful strategy to overcome the ever-increasing burden of BLs-mediated resistance has been to develop β-lactamase inhibitors (BLIs) administered in association with alongside β-lactam antibiotics [11,12]. The most successful inhibitors are clavulanic acid and the penicillanic acid sulphone, which have proved helpful in clinical practice to overcome BL-mediated resistance in several instances [11,12]. Almost half a century later, in 2015, avibactam, a new BLI, entered the market in combination with ceftazidime [13]. From then on, the interest in discovering new non-β–lactam containing BLI constantly raised, new compounds were approved, and others are under development [14–19]. However, these compounds are active only on some enzymes, and most of them are ineffective against MBLs. Based on the recent interest in zinc chelators and our results on polypyridine ligands [20], we designed new polyimidazole ligands based on the ethylenediamine backbone. The polyimidazole ligands, similarly polypyridine ligands, can form Zn complexes. Imidazole moiety is a constituent of biomolecules such as proteins. Furthermore, many imidazole derivatives have been investigated in medicinal chemistry [21]. Specifically, we synthesized *N,N′*-bis ((imidazol-4-yl)methyl)-ethylenediamine (BisIM), N,N,N′-tris((imidazol-4-yl)methyl)-ethylenediamine (TrisIM), N,N,N,N′-tetra((imidazol-4-yl)-methyl)-ethylenediamine (TetraIM) to study their activity as MBL inhibitors (Fig. 1). The inhibitory activity of these new polyimidazole ligands was evaluated toward subclass B1 MBLs, particularly against NDM-1, VIM-1 and IMP-1 enzymes via in vitro analysis. We also studied known zinc chelators such as 1,4,7,10,13-Pentaazacyclopentadecane (Penta-AZA), 1,4,8,11-Tetraazacyclotetradecane (Cyclam) and 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). DOTA is used in DOTAREM drug and has $\log K_{ZnL} = 21.1$. Cyclam, widely studied in inorganic medicine [22] has $\log K_{ZnL} = 15.6$ and Penta-AZA is in SC52608 drug [23] and has $\log K_{ZnL} = 19.1$ [24].

Furthermore, molecular docking was carried out to explain the mode of action of the most active compounds.

2. Experimental section

2.1. Materials

Imidazole-4-carboxaldehyde, Cyclam, ethylendiamine, DOTA were purchased from TCI (TOKYO CHEMICAL INDUSTRY CO., Tokyo, Japan). 1,4,7,10,13-Pentaazacyclopentadecane (Penta-AZA) (Fig. 1S) was purchased from DBL Pharm (Shangai, China).

RPMI 1640 medium, fetal bovine serum glutamine, penicillin and streptomycin were from Euroclone (Milan, Italy). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma–Aldrich (Milan, Italy). Phytoemoagglutinin was from Remel Europe Ltd. (Dartford, Kent, UK).

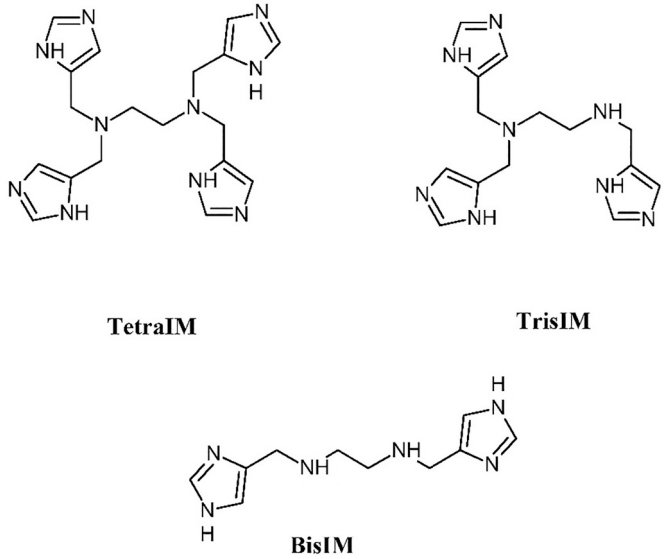

Fig. 1. Polyimidazole ligands investigated.

2.1.1. Synthesis of N,N′-Bis(4-imidazolylmethyl)-ethylenediamine (BisIM)

Ethylenediamine (0.70 ml, 10 mmol) was added to imidazole-4-carboxaldehyde (2.0 g, 20 mmol) in 30 ml of methanol. The solution was heated under reflux, and a white solid was formed. The suspension was cooled at 25 °C and $NaBH_4$ (0.76 g, 20 mmol, 10 ml of methanol) was added after 5 h. The solution obtained was heated under reflux overnight. The solvent was evaporated, and the product was isolated by CM-Sephadex C-25 (NH_4^+ form) column and a linear gradient $H_2O \rightarrow NH_4HCO_3$ (0 → 0.4 M) as the eluent. Yield 52%.TLC: Rf = 0.72 (PrOH/AcOEt/H_20/NH_3 5:2:3:1).

^{1}H NMR (500 MHz, D_2O) *δ*: 7.60 (s, 2H, H-2 Im), 6.94 (s, 2H, H-5 Im), 3.65 (s, 4H, CH_2Im), 2.65 (s, 4H, CH_2NH).

^{13}C NMR (125 MHz, D_2O) *δ*: 135.0 (C-2 Im), 125.7 (C-4 Im), 117.5 (C-5 Im), 75.0 (CH_2Im), 46.6 (CH_2NH).

ESI-MS: $m/z = 221.80\ [M + H]^+$.

2.1.2. Synthesis of N,N,N′-Tris(4-imidazolylmethyl)-ethylenediamine (TrisIM)

Imidazole-4-carboxaldehyde (1.0 g, 10.4 mmol) was added to BisIM (2.30 g, 10.4 mmol) in MeOH (20 ml) and the solution was stirred at 25 °C for 12 h protected with a drying tube ($CaCl_2$). After 5 h, $NaBH_4$ (0.39 g, 10.4 mmol) was added. The solution was stirred at room temperature with $CaCl_2$ protection. After 24 h, the solvent was evaporated and the solid was purified by CM-Sephadex C-25 column and a linear gradient $H_2O \rightarrow NH_4HCO_3$ (0 → 0.4 M) as the eluent. Yield 55%. TLC: Rf = 0.66 (PrOH/H_2O/NH_3 5:2:1).

^{1}H NMR (500 MHz, D_2O) *δ*: 7.58 (s, 1H, H-2 Im), 7.57 (s, 2H, H-2 Im), 6.91 (s, 2H, H-5 Im), 6.89 (s, 1H, H-5 Im), 3.57 (s, 2H, CH_2Im), 3.51 (s, 4H, CH_2Im), 2.54 (m, 2H, CH_2NH), 2.49 (m, 2H, CH_2NH).

^{13}C NMR (125 MHz, D_2O) *δ*: 135.0 (C-2 Im N′), 132.8 (C-2 Im N), 124.0 (C-4 Im N), 124.0 (C-4 Im N′), 119.6 (C-5 Im N), 117.2 (C-5 Im N′), 77.1 (CH_2Im N), 74.8 (CH_2Im N′), 44.5 (CH_2NH), 43.6 (CH_2NH).

ESI-MS: $m/z = 301.20\ [M + H]^+$.

2.1.3. Synthesis of N,N,N′N′-tetra(4-imidazolylmethyl)-ethylenediamine (TetraIM)

TetraIM was synthesized as reported for TrisIM, starting from Imidazole-4-carboxaldehyde (2.0 g, 20.8 mmol), BisIM (2.30 g, 10.4 mmol) and $NaBH_4$ (0.78 g, 20.8 mmol). The product was isolated by column chromatography as reported for TrisIM. Yield 50%. TLC: Rf = 0.68 (PrOH/H_2O/NH_3 5:2:1).

^{1}H NMR (500 MHz, D_2O) *δ*: 7.46 (s, 4H, H-2 Im), 6.70 (s, 4H, H-5 Im), 3.35 (s, 8H, CH_2Im), 2.28 (s, 4H, CH_2NH).

^{13}C NMR (125 MHz, D_2O) *δ*: 133.0 (C-2 Im), 124.0 (C-4 Im), 119.6 (C-5 Im), 78.0 (CH_2Im), 49.9 (CH_2NH).

ESI MS $m/z = 381.25\ [M + H]^+$.

2.2. Instrumentation

^{1}H and ^{13}C NMR spectra were recorded at 25 °C with a Varian UNITY PLUS-500 spectrometer at 499.9 and 125 MHz, respectively. NMR spectra were obtained by using standard pulse programs from the Varian library. ESI mass spectra were acquired with an API 2000– ABSciex spectrometer.

2.3. Docking study

Rigid docking studies were performed using GOLD 2022 CCDC Software Ltd. (www.ccdc.cam.ac.uk), choosing default parameters for the genetic algorithm and the CHEMPLP scoring function to rank the best poses of ligands in the protein active site. 3D structure of NDM-1, VIM-1 and IMP-1 were retrieved from Protein Data Bank (PDB,

https://www.rcsb.org) with PDB codes: 5ZGR, 5N5I and 1JJT, respectively. All compounds were docked at the substrate binding site. Ligand structures were obtained from the Cambridge Structural Database (CSD) and modified by Mercury software of Cambridge Crystallographic Data Center CCDC (CSD code: SUTFIZ for BisIM, CAJCOJ for Cyclam, GOYBUR for DOTA, MATGOH for Penta-AZA). Low-energy ligand structures were obtained by the Conformer Generator module of Mercury. During docking calculations, a maximum of 10 poses for each compound was generated. The solvent and substrate were deleted. The best-scored docking pose of each ligand was chosen for the analysis and evaluated with respect to the complementarity with the corresponding binding site and the presence of hydrogen bonds as well as hydrophobic and electrostatic interactions existing with key amino acid residues and cofactors.

2.4. Bacterial strains

The *E. coli* AQ/5 harboring $bla_{\text{VIM-1}}$, *E. coli* AQ/7 harboring $bla_{\text{NDM-1}}$, *E. coli* AQ/41 harboring $bla_{\text{IMP-1}}$ and $bla_{\text{CMY-2}}$ and *K. pneumoniae* AQ/17 harboring $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-181}}$ clinical strains were from the collection strains of Clinical Biochemistry and Clinical Molecular Biology, University of L'Aquila. Clinical strains used in the present study were resistant to amoxicillin, meropenem, cefepime, ceftazidime, amoxicillin-clavulanate and ceftazidime-avibactam [19].

2.5. Metallo-β-lactamase purification

The NDM-1, VIM-1 and IMP-1 enzymes were purified from overnight cultures of *E. coli* DE3/pET24-NDM-1, *E. coli* DE3/pET-24-VIM-1 and *E. coli* DE3/pET-24-IMP-1 following procedures previously reported [25,26].

2.6. Kinetic parameters determination

Purified NDM-1, VIM-1 and IMP-1 variants were used to determine, by kinetic assays, the K_i and IC_{50} values against BisIM, TrisIM, TetraIM, Penta-AZA, Cyclam and DOTA. Steady-state kinetic experiments were carried out under initial-rate conditions using Hanes plot linearization. Competitive inhibition assays with all compounds were directly monitored using 100 μM meropenem ($\lambda = 273$ nm, $\Delta\varepsilon_M^{273} = -6500\ M^{-1}\ cm^{-1}$) as the reporter substrate and 30–80 nM of each enzyme. The inhibition experiments were performed by pre-incubating the enzyme with each ligand for 5 min at 25 °C. The K_i values were calculated using the following equation: $v_0/v_i = 1 + (K_m\ x\ I)/(K_m + S)\ x\ K_i$, where v_i and v_0 represented the initial rates of hydrolysis of meropenem with or without inhibitor, respectively; I was the concentration of inhibitor or poor substrate; K_i was the inhibition constant; K_m was the Michaelis-Menten constant; and S was the reporter substrate concentration. The plot v_0/v_i versus [I] yielded a straight line of slope $K_m/(K_m + S) \times K_i$ [27,28].

2.7. Antimicrobial susceptibility testing

The antimicrobial susceptibility for each strain of meropenem, BisIM, TrisIM, TetraIM, Penta-AZA, Cyclam and DOTA was evaluated by microdilution method using a bacterial inoculum of 5×10^5 CFU/mL, according to Clinical and Laboratory Standards Institute (CLSI) performance standards [29]. The compound MIC range was ≤0.06 mg/L to ≥256 mg/L. The compounds were also used in combination with meropenem at a fixed concentration of 4 mg/L. Aztreonam-avibactam combination was used as a comparator. Three experiments were performed using cation-adjusted Mueller Hinton broth (CAMHB). A concurrent quality control procedure was performed by testing *E. coli* (ATCC® 25922TM) as a reference strain.

2.8. Human peripheral blood mononuclear cells (PBMCs) isolation

Normal peripheral blood mononuclear cells (PBMCs) were obtained from heparinized human whole blood of healthy adults and isolated by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich, St. Louis, MO, USA). The collected PBMCs were washed twice and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. PBMCs were preactivated with 90 μg/ml phytohemagglutinin for 24 h at 37 °C in a humidified atmosphere of 5% CO_2; afterward, stimulated lymphocytes were seeded 5×10^4 cells/well, at a density of 5×10^5/ml in 96-well plates and exposed to compounds.

2.9. PBMC cytotoxicity assay

The effect of the compounds on PBMC viability was evaluated by the MTT colorimetric method that quantifies cellular metabolic activity [30]. Stimulated PBMCs were treated with increasing concentrations of compounds, from 12.5 μM to 100 μM, for 48 h, at 37 °C in a humidified atmosphere of 5% CO_2. At the end of treatment, the MTT solution was added to each well at a concentration of 0.5 mg/mL and cells were incubated at 37 °C for a further 4 h. The MTT-formazan crystals were dissolved in acidified isopropanol (0.04 M HCl in isopropanol), and the absorbance at 570 nm was estimated by a microplate reader (Biorad, Model 550). The percentage of cell survival was obtained by comparing the absorbance of the treated groups with that of the untreated cells, the viability of which was taken as 100%.

3. Results and discussion

3.1. Synthesis and characterization

BisIM, TrisIM and TetraIM have been synthesized by a reductive amination reaction, with a similar procedure to that reported for polypyridine ligands [20] and BisIM [31]. ^1H and ^{13}C NMR spectra confirmed the identity of polyimidazole ligands (Fig. 2S-4S). Polyimidazole ligands can form metal ion complexes, similar to polypyridine ligands. We calculated $\log K_{ZnL}$ for polyimdazole ligands and we found $\log K_{ZnL}$ is 11.38 for BisIM, $\log K_{ZnL}$ is 13.88 for TrisIM and 16.3 for TetraIM. The calculation of $\log K_{ZnL}$ values of the new ligands was based on the donor group additivity rule reported successfully elsewhere [32].

3.2. Kinetic determination of K_i and IC_{50}

The newly synthesized compounds BisIM, TrisIM, TetraIM and the commercial Penta-AZA, Cyclam and DOTA were tested against the most common subclass B1 MBLs including NDM-1, VIM-1 and IMP-1. Using meropenem (MEM) as a reporter substrate, all compounds acted as competitive inhibitors of the β-lactamase activity. They were slow-binding inhibitors because they needed almost 5 min of pre-incubation to inhibit NDM-1, VIM-1 and IMP-1. All tested compounds efficiently inhibited the NDM-1 activity with K_i and IC_{50} values in the range of 0.28–1.51 μM and 0.7–9.4 μM, respectively (Table 1). The VIM-1 enzyme was well inhibited by BisIM and TetraIM with K_i values of 2.0 and 3.2 μM, respectively. TrisIM inhibited VIM-1 with a K_i value of 19 μM. Penta-AZA, Cyclam and DOTA were used against VIM-1 at high concentrations and the K_i values were estimated to be higher than 500 μM. The IMP-1 was the most resistant enzyme to the action of the tested compounds. BisIM, Cyclam and DOTA exhibited $K_i > 500$ μM, whereas the K_i values of 73, 320 and 380 μM were determined for TetraIM, TrisIM and Penta-AZA, respectively. The IMP-1 enzyme is the most resistant MBL to the recently approved inhibitors. In particular, IMP-1 is resistant to avibactam, vaborbactam and relebactam [33]. On the basis of recently published data, IMP-1 was well inhibited by cyclic boronic acid QPX7728 with an IC50 of 0.610 μM [34].

Table 1
Inhibition kinetic parameters determined for NDM-1, VIM-1 and IMP-1 against BisIM, TrisIM, TetraIM, Penta-AZA, Cyclam and DOTA.

Compounds	Subclass B1 MBLs					
	NDM-1		VIM-1		IMP-1	
	K_i μM	IC_{50} μM	K_i μM	IC_{50} μM	K_i μM	IC_{50} μM
BisIM	0.28 ± 0.05	0.7 ± 0.1	2.0 ± 0.1	7.0 ± 0.5	>500	>500
TrisIM	0.54 ± 0.01	9.4 ± 0.5	19.0 ± 0.5	75 ± 2	320 ± 15	470 ± 40
TetraIM	0.78 ± 0.09	1.4 ± 0.2	3.2 ± 0.2	8 ± 1	73 ± 5	180 ± 8
Penta-AZA	0.76 ± 0.03	4.5 ± 0.1	>500	>500	380 ± 15	450 ± 35
Cyclam	1.51 ± 0.01	3.3 ± 0.1	>500	>500	>500	>500
DOTA	0.53 ± 0.09	1.7 ± 0.1	>500	>500	>500	>500

3.3. Docking analysis

All compounds were docked on the binding site of NDM-1, VIM-1 and IMP-1 enzymes to clarify their interaction mode with these MBLs via different interaction networks (Table 2). The BBL numbering was used for VIM-1 and IMP-1, while for NDM-1, the NDM-1 numbering was used to facilitate the reader comparison with other published studies on NDM-1.

Overall, the presence in the molecules of imidazole moiety allows the accommodation of the ligand molecule by pi-interactions (metal-pi interactions, polar-pi interactions, pi-stacking interactions) as well as H bonding interactions within the conserved active site bordering loops. The active site of NDM-1 contains two zinc ions, Zn1 and Zn2, coordinated by His120, His122, His189 (NDM-1 numbering) and by Asp124, Cys208 and His250 (NDM-1 numbering), respectively. The NDM-1 numbering will be used for NDM-1 enzyme. In comparison to VIM-1 and IMP-1, the NDM-1 active site results wider and enclosed by several loops. The hydrophobic residues Leu65, Met67, Phe70, and Val73 of the hairpin L3 loop are responsible for the substrate binding recognition providing preferable interaction points for the substrate hydrophobic substituents, whereas the L10 loop accommodates Zn2 coordinated by Cys208 and Lys211 and Asn220 involved in the substrate binding [35]. The His122, His189, Cys208, Lys211, Asn220 and His250 of NDM-1 are conserved residues in MBLs and appear to be crucial in the substrate recognition [36] together to Zn ions, as well as the Ile35, Trp93, Gln123, Ala215 and Gly219 are assumed to stabilize protein-ligand binding through hydrophobic interactions [37]. Fig. 2 shows the best docking pose of ligands into the NDM-1 binding site. The bis-imidazole compound, BisIM, is anchored to the active site of NDM-1 by H bonds with the side chains of His122 and Asp124 through its ethylenediamine group (Fig. 2A). This latter also coordinates both Zn1 and Zn2 atoms. Further stabilization of the ligand is also provided by hydrophobic interactions occurring between Ile35 (pi-sigma interaction), His250 (pi-pi T shaped interaction) and His122 (pi-pi stacking interaction) and the imidazole rings of BisIM. In the TetraIM molecule, Zn1 binds the imidazole ring of the ligand via a pi-cation interaction (Fig. 2C). Side chains of Asp220 and Asp124 form two H bonds with nitrogen atoms of two imidazole rings of the ligand. Additional hydrophobic and electrostatic interactions with Ile35 (pi-alkyl), His250 (pi-pi stacking interaction) and Met67 (pi-sulfur interaction) help to further accommodate the ligand structure into the active site of NDM-1. TrisIM (Fig. 2B) compound differs from TetraIM for the presence in this latter of a further imidazole ring. In the TrisIM molecule, the H bond with Asp124 through its imidazole ring was also observed. The binding pose fits well into the active site of the target molecule thanks to the coordination of Zn2 ion by the N atom of the ethylenediamine group, along with hydrophobic interactions occurring between Ile35, Val73 (pi-alkyl interactions), His122 (pi-pi stacking interaction) and imidazole rings of the ligands. Moreover, His250 is H bonded (unconventional H bond, N…HC) to the ligand. As for macrocycle compounds, a different trend was found for DOTA in comparison to Cyclam and Penta-AZA molecules. Better scored DOTA (Fig. 2D) establishes electrostatic interactions through the carboxylate groups with His122 (pi-anion interaction) and both Zn ions. Moreover, two carboxylate groups of the ligand are H bonded to the main chain of Gln123 and the side chain of Cys208, contributing to also stabilizing the molecule into the binding site of NDM-1. On the other hand, Cyclam and Penta-AZA show lower binding scores, probably due to less extensive interaction with NDM-1. In particular, Cyclam (Fig. 2F) coordinates both Zn ions and is H bonded to the side chain of Asp124 and His250. Similarly, Penta-AZA (Fig. 2E) displays Zn2—N interaction and a conventional H bond with the side chain of Asp124.

Moving to the imidazole series on VIM-1 (Fig. 3), more stable binding modes were provided after docking calculation of BisIM, TrisIM and TetraIM than macrocycle compounds. Two Zn1—N interactions were found in the BisIM compound with the N of the imidazole and ethylenediamine linker (Fig. 3A). The ligand is also stabilized via H-bonding interactions with His240, Asp117 and Asp118. Furthermore, electrostatic interactions (pi-sulfur) with Cys198 and hydrophobic interactions with His240 (pi-pi T shaped interaction) and His179 (pi-pi stacking interaction) guarantee a favorable fitting between the ligand and the protein. Similarly, the TrisIM (Fig. 3B) molecule interacts with Zn1 (pi-cation) and Asp118 by electrostatic interactions (pi-anion interaction). An H bond occurs between the main chain of Asp117 and the nitrogen of the imidazole moiety of the ligand. Further stabilization is provided by hydrophobic interactions between Phe62, His116, Trp87, His240 (pi-pi stacking interactions) and Pro68 (pi-Alkyl interaction) with imidazole rings. All four imidazole rings in TetraIM are favorably accommodated into the active site of VIM-1 (Fig. 3C). An imidazole nitrogen was predicted to directly coordinate the Zn2 ion. Many carbon-hydrogen bonds involving residues Asn210, Pro68, Glu202, His201, Ser205, Tyr67 and His240 were also observed in this ligand. Furthermore, electrostatic bindings with Asp118 (pi-anion interaction), Cys198 (pi-sulfur interaction), as well as hydrophobic interactions with Pro68 (pi-alkyl interactions) and Tyr67 (pi-pi stacking interaction) provide further stabilization of the ligand into the binding site of the enzyme. The less active compound Penta-AZA in VIM-1 is unable to bind Zn ions, but interacts only with His240 by H bonding (data not shown). A different trend was observed for DOTA molecule that shows a higher affinity score not in accordance with the experimental results. However, even if the binding of the ligand concerns a more extended area of the target molecule, a bump with Asp63 could disfavor the ligand fitting process (data not shown).

According to IC_{50} values, docking of polyimidazole ligands with IMP-1 reveals that TetraIM appears to be the best scored molecule, demonstrating the highest binding affinity to the target IMP-1 with respect to other polyimidazole ligands. In TetraIM a binding with the Zn1 ion takes part in the interaction network with the enzyme. Moreover, H bonding interactions were predicted to occur between the imidazole moieties of the ligand and the residues His197 and Asp81, as well as a pi-sulfur interaction with Cys158 contributes to stabilizing the TetraIM molecule

Table 2
Docking score of top ranked compounds with NDM-1, VIM-1 and IMP-1 MBLs.

Ligand	CHEMPLP (KJ/mol)		
	NDM-1	VIM-1	IMP-1
DOTA	87.16	85.07	55.76
TetraIM	62.37	74.51	80.28
TrisIM	61.99	71.80	71.76
BisIM	56.78	69.40	59.34
Cyclam	42.18	48.18	51.66
Penta-AZA	41.88	44.67	38.90

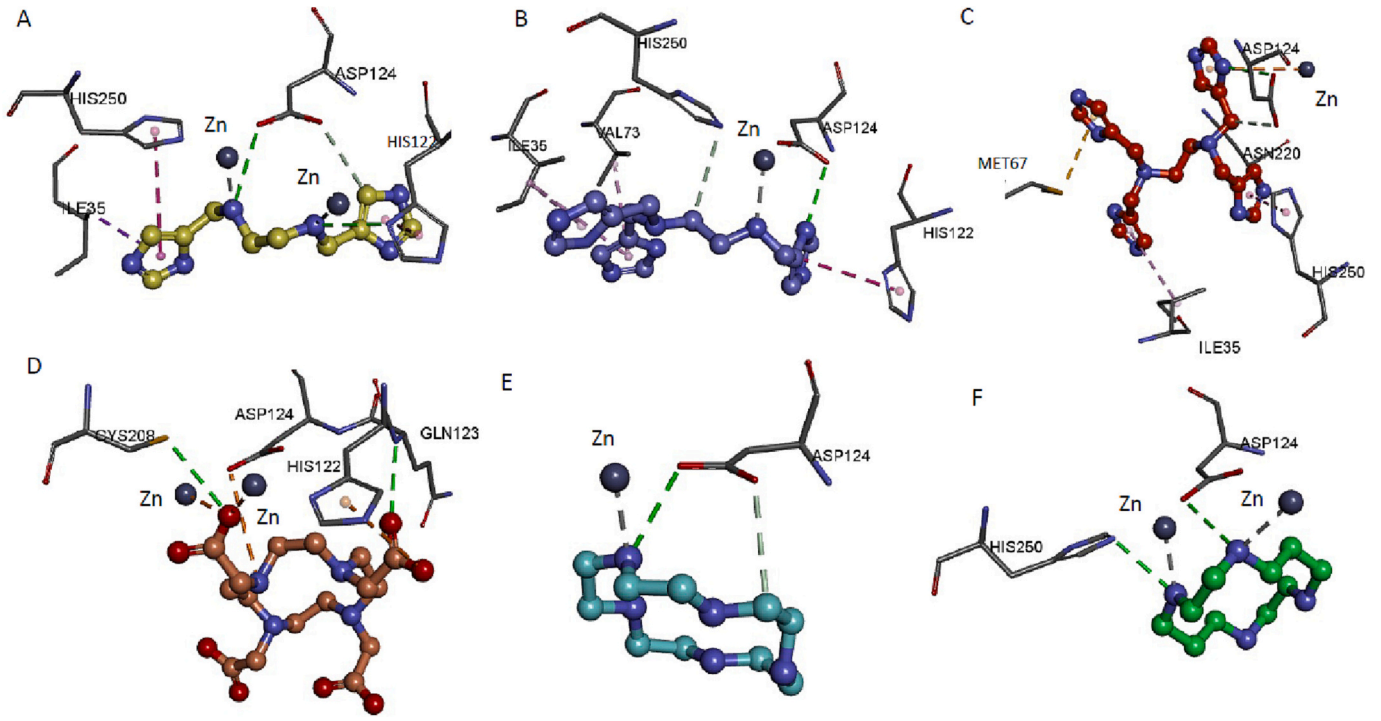

Fig. 2. Docking pose of BisIM (A), TrisIM (B), TetraIM (C), DOTA (D), Penta-AZA (E), Cyclam (F) in the active site of NDM-1. H bonding interactions and hydrophobic interactions are depicted as green and pink dotted lines, respectively. Pi-sulfur and pi-cation interactions are represented as orange dotted lines. Unconventional H bonding interactions are shown as grey dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

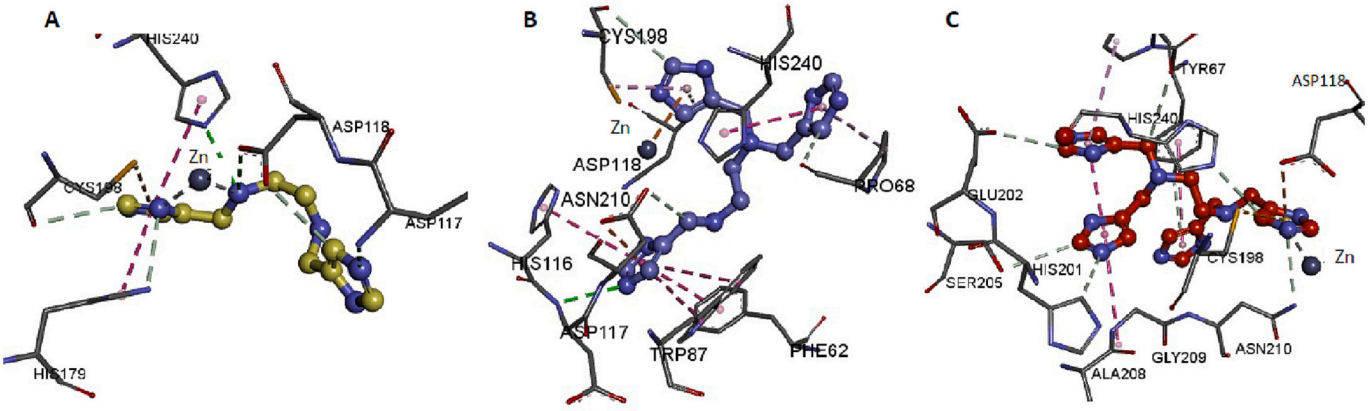

Fig. 3. Docking pose of BisIM (A), TrisIM (B), TetraIM (C) in the active site of VIM-1. H bonding interactions and hydrophobic interactions are depicted as green and pink dotted lines, respectively. Pi-sulfur and pi-cation interactions are represented as orange dotted lines. Unconventional H bonding interactions are shown as grey dotted lines.

into the active site of IMP-1. Yet, the ligand pose is well anchored to the protein through hydrophobic interactions with Pro32, Val31, Val25 (pi-alkyl interactions), and Gly166 (pi-sigma interaction) (Fig. 5S). As for the other polyimidazole ligands the lowest activity of these compounds against IMP-1 is most probably due to the interactions with residues not in direct contact with zinc coordination network. TetraIM seems to be the most active compound against IMP-1 because of its interaction with Zn-1 binding site.

3.4. MIC determination

The antibacterial activity of meropenem in combination with BisIM, TrisIM, TetraIM, DOTA, Penta-AZA and Cyclam at a fixed concentration of 4 mg/L was assessed in four *Enterobacterales* clinical isolates, including three *E. coli* and one *K. pneumoniae*. As shown in Table 3, all clinical isolates produced almost one MBL (VIM-1, IMP-1 or NDM-1) and they were resistant to meropenem (MIC range, 16–128 mg/L) and aztreonam (MIC>128 mg/L). According to CLSI guidelines, the breakpoints for meropenem are MIC≤1 mg/L (Susceptible), MIC = 1 mg/L (Intermediate) and MIC≥4 mg/L (Resistant). The clinical strains selected for this study showed resistance to all tested compounds with MICs ranging from 128 mg/L to ≥256 mg/L. Using these compounds in association with meropenem, we observed that TetraIM was able to restore the susceptibility of meropenem only in VIM-1 producing *E. coli* AQ/5 with a MIC value of ≤0.06 mg/L. However, TetraIM was able to decrease the MIC value for meropenem of 4-fold dilution in NDM-1 producing *E. coli* AQ/7. Penta-AZA restored the susceptibility to meropenem in VIM-1 producing *E. coli* AQ/5 with a MIC value of 0.5 mg/L

Table 3
Antibacterial activity of meropenem in combination with BisIM, TrisIM, TetraIM, DOTA, Penta-AZA and Cyclam at a fixed concentration of 4 mg/L.

Clinical Strains	*E. coli* AQ/7 (NDM-1)	*E. coli* AQ/5 (VIM-1)	*E. coli* AQ/41 (IMP-1)	*K. pneumoniae* AQ/17 (NDM-1 OXA-181)
MEM	64 (R)	16 (R)	128 (R)	128 (R)
BisIM	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)
MEM + BisIM	16 (R)	0.25 (S)	64 (R)	64 (R)
TrisIM	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)
MEM + TrisIM	64 (R)	16 (R)	128 (R)	128 (R)
TetraIM	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)
MEM + TetraIM	4 (R)	≤0.06 (S)	64 (R)	64 (R)
DOTA	128 (R)	128 (R)	128 (R)	128 (R)
MEM + DOTA	64 (R)	16 (R)	128 (R)	128 (R)
Penta-AZA	128 (R)	≥256 (R)	≥256 (R)	≥256 (R)
MEM + Penta-AZA	16 (R)	0.5 (S)	64 (R)	32 (R)
Cyclam	≥256 (R)	≥256 (R)	≥256 (R)	≥256 (R)
MEM + Cyclam	32 (R)	2 (I)	64 (R)	32 (R)
AZT	>128 (R)	>128 (R)	>128 (R)	>128 (R)
AZT + AVI	4 (R)	0.5 (S)	128 (R)	8 (R)

MEM, meropenem; AZT, aztreonam; AVI, avibactam; R, resistant; I, Intermediate; S, susceptible.

comparable with the aztreonam-avibactam (AZT-AVI) combination. Cyclam restored the susceptibility of meropenem in VIM-1-producing *E. coli* AQ/5 clinical strain. The IMP-1 producing *E. coli* AQ/41 was resistant to meropenem and all meropenem-ligand combinations tested in the present study. Even if the TrisIM and TetraIM showed good activity against the IMP-1 enzyme, these compounds were unable to restore the susceptibility to meropenem in *E. coli* AQ/41 clinical strain. Other mechanisms of resistance (i.e. porins modifications, PBP mutations) contributed to the resistance of carbapenems in E*nterobacterales*.

3.5. Cytotoxic activity

The effects of six zinc chelators (BisIM, TrisIM, TetraIM, Cyclam, Penta-AZA and DOTA) on human cell viability was also studied in PHA-stimulated PBMCs (Fig. 4). Cells were treated with increasing concentrations of compounds, ranging from 12.5 to 100 μM, for 48 h. The cell survival was assessed by the MTT assay. No significant cytotoxic effect was observed in cells exposed to TrisIM, Cyclam and DOTA. Treatments with TetraIM and Penta-AZA showed antiproliferative activity only at the highest concentration of 100 μM, with a 77.3% and 50.8% reduction of viability, respectively, while at lower concentrations, they did not show any effect. Instead, BisIM decreased viability slightly at 12.5 μM but was less cytotoxic than TetraIM and Penta-AZA at 100 μM, reaching a reduction of proliferation of 38.2% at the highest concentration.

4. Conclusion

In the 2000s, the wide use of carbapenems to fight the spread of ESBLs-producing Enterobacterales led to the emergence of antimicrobial resistance. The production of carbapenem-hydrolyzing enzymes, "carbapenemases", is the most important resistance mechanism. Among carbapenemases, the MBLs generally exhibit very broad activity spectra rendering the producing bacteria resistant to all β-lactams except monobactams. The introduction of clavulanic acid, sulbactam and tazobactam changed the *scenario* of antimicrobial therapy for β-lactamase-producing bacteria, except for MBLs that are completely resistant to these classical β-lactamase inhibitors. Numerous scientific efforts have been made in the last two decades, which have led to the discovery and synthesis of new molecules able to inhibit MBLs [18,34,38–43]. In this panorama, we have designed and synthesized a new family of zinc chelators based on imidazole (BisIM, TrisIM, TetraIM). Imidazole ring is widely present in biological systems, and many imidazole-containing compounds show pharmaceutical activities. New imidazole ligands showed efficient activity against NDM-1 and VIM-1, the most common MBLs in clinical strains. TetraIM ligand showed the best inhibitory activity against IMP-1, for which, to date, there are not many effective molecules. Furthermore, TetraIM did not show significant antiproliferative activity at a concentration ≤ 50 μM. Although animal

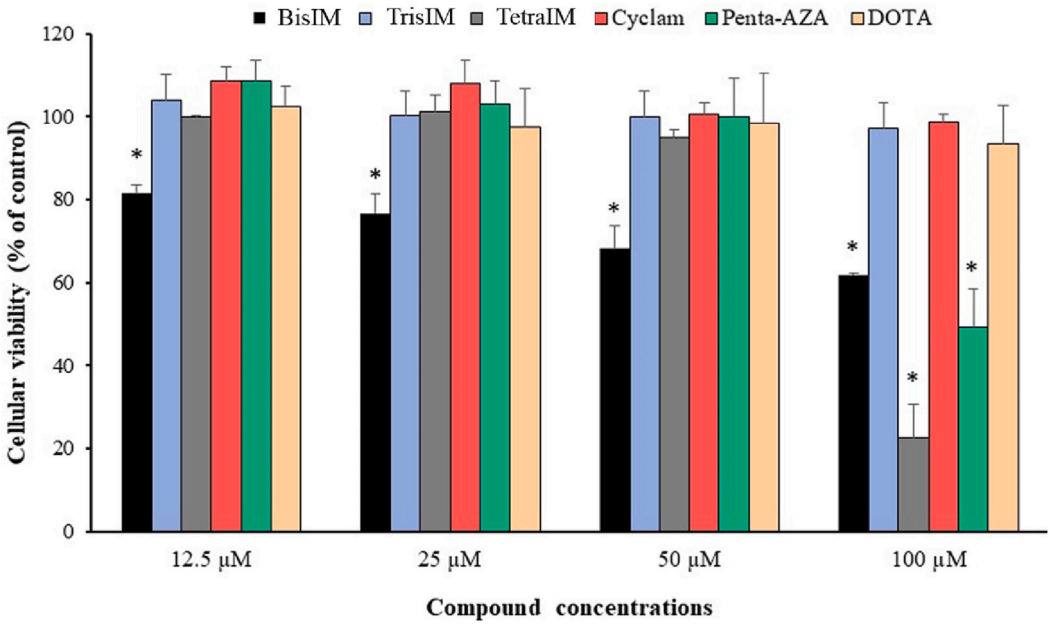

Fig. 4. Effects of compounds on PBMC viability. Cell survival was determined by the MTT assay. The viability of the control (untreated cells) was taken as 100%. The results represent the mean ± SD of three independent experiments. *Statistical differences were calculated using the Student's *t*-test. Data are significantly different from untreated cells ($p < 0.05$).

studies are needed to evaluate in vivo efficacy and safety of the new polyimidazole compounds, TetraIM may be a promising coadjuvant against MBL-producing Enterobacterales.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2023.112163.

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