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The *in vitro* inhibitory activity of polypyridine ligands towards subclass B1 metallo-β-lactamases

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ABSTRACT

Antimicrobial resistance leads to the ineffectiveness of antimicrobials, hampering the ability to cure infections. Zinc-dependent metallo ß-lactamases (MBLs) producing bacteria represent a threat to public health due to limited therapeutic options. Zinc chelators are able to inhibit MBLs and have the potential to restore carbapenem susceptibility. Recently, polypyridine ligands named Bispicen (N,N'-Bis(2-pyridymethyl)-ethylenediamine), Trispicen (N,N,N'-Tris(2-pyridylmethyl)-ethylenediamine), TrispicenA (N,N,N'-tris([2-pyridylmethyl)-ethyl-enediamine-N'-acetic acid) and TrispicenDALA (N,N,N'-tris([2-pyridylmethyl)-ethylenediamine-N'-acetyl-D-alanyl-D-alanyl-D-alanine methyl ester) were evaluated for their ability to interfere with the Zn-ion network by interactions in the binding site of MBLs. We report on the inhibitory activity of these ligands towards three subclass B1 MBLs (NDM-1, VIM-1 and IMP-1) by kinetic analysis, demonstrating that all compounds, except TrispicenDALA, acted as competitive inhibitors towards NDM-1 and VIM-1. Bispicen was more active against VIM-1 with K_i value of 0.13 μM while NDM-1 was highly inhibited by Trispicen with K_i value of 0.08 μM. The IMP-1 enzyme was resistant to these compounds showing K_i and IC_{50} values higher than 500 μM. Docking study reveals that all compounds interact with residues of the MBL active site mainly by the formation of pi-interactions and chelation of both Zn ions. Polypyridine ligands deserve attention for their inhibitory potential towards NDM-1 and VIM-1.

Introduction

β-lactams are the most prescribed antibiotics in clinical practice and β-lactamases represent the major defense mechanism, especially in Gram-negative bacteria, against this class of antibiotics [1,2]. According to their amino acid sequence, β-lactamases are classified in four molecular classes A, B, C and D [3]. On the basis of their mechanism of action, β-lactamases are categorized into two big groups, serine-β-lactamases (SBLs) (classes A, C and D) and metallo-β-lactamases (MBLs) (class B). To date more than 7000 different β-lactamases have been identified and additional classification schemes, based on substrate profile and susceptibility to β-lactamase inhibitors, have been performed [4,5]. β-lactamases are bacterial hydrolases that, very efficiently, catalyze the irreversible hydrolysis of the amide bond of the β-lactam ring of β-lactams [6]. The SBLs hydrolyze β-lactams acting by a three-steps mechanism involving the transient formation of an acyl-enzyme in which the hydroxyl group of the active serine residue is esterified by the carbonyl group of the antibiotic moiety [7]. MBLs catalyze β-lactams hydrolysis through a non-covalent mechanism involving one or two zinc ions [8]. On the basis of their active site residues, zinc ligands, zinc stoichiometry, structure and substrate profiles, MBLs are further divided into subclasses B1, B2 and B3 [9]. Subclasses B1 and B3 include di-zinc enzymes whereas subclass B2 includes mono-zinc enzymes (e.g. CphA). From a structural point of view, MBLs showed a typical αββα sandwich with a metal binding site including two Zn ions (Zn1 and Zn2) which coordinate six conserved amino acid residues [8,10–12]. The Zn1 is coordinated by 3 His (3H site: His116-His118-His196, BBL numbering) and Zn2 is coordinated by Asp120, Cys221 and His263 (BBL

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numbering). The active site is also surrounded by several loops playing a pivotal role in substrate binding, Zn coordination and enzyme stability [10]. MBLs showed broad substrate spectrum activity, as a result of the high flexibility and adaptability of the active site, but a low degree of activity against monobactams. From clinical point of view, the most common enzymes belonging to subclass B1 are NDM-1 (New Delhi Metallo-β-lactamase-1), VIM-1 (Verona ImipeneMase) and IMP-1 (IMipenemase). These variants are in constantly evolving and numerous NDM-, VIM- and IMP-types have been identified (https://www.bldb.eu/). To date, 54 NDM-, 86 VIM- and 99 IMP- variants have been found in clinical and environmental strains [13]. Concerning NDM-types the most widespread and clinically relevant variants showed V88L, M154L and A233V amino acid substitutions which contributed to increase carbapenemase activity [14,15]. It is relevant to note that, at present, we do not have effective inhibitors against MBLs to be used in human infections [16]. In fact, first generation β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam are ineffective against MBLs and the diazabicyclooctanones such as avibactam and relebactam (second-generation of β-lactamase inhibitors) and boronate compounds (i.e. vaborbactam, third-generation of β-lactamase inhibitor) are not markedly active against MBLs [17–21]. The promising novel boronates such as taniborbactam and QPX7728 seem to have a good activity against MBLs but they have not yet been approved by drug regulatory authorities [21–24]. Zinc chelators have been studied as potential MBL inhibitors [25–30]. Zinc ligands can strip zinc from MBL enzymes, or they can displace a hydroxide ion shared by two Zn^{2+} ions at the MBL catalytic site and form ternary complex with Zn. Some zinc chelators have also been tested in *in vivo* models [31,32].

In a previous *in vitro* study, we have explored the activity of the polypyridine ligands N,N'-bis(2-pyridymethyl)-ethylenediamine (Bispicen), N,N,N'-tris(2-pyridylmethyl)-ethylenediamine ethylenediamine (Trispicen), N,N,N'-tris([2-pyridylmethyl)-ethylenediamine-N'-acetic acid (TrispicenA) and N,N,N'-tris([2-pyridylmethyl)-ethylenediamine-N'-acetyl-D-alanyl-D-alanyl-D-alanine methyl ester (TrispicenDALA) which were able to restore the antimicrobial activity of meropenem against MBLs-producing Gram-negative bacteria [30]. Based on these recent data on the polypyridine ligands, the aim of the present study was to evaluate the inhibitory activity of these ligands towards subclass B1 MBLs by kinetic experiments and docking study. Specifically, we studied ligands Bispicen, Trispicen, Trispicen A and TrispicenDALA against NDM-1, VIM-1 and IMP-1..

Materials and methods

Compounds and β-lactamases

Bispicen, Trispicen, TrispicenA and TrispicenDALA (Fig. 1) have been synthesized as previously reported [30]. The NDM-1 (accession ID, E9NWK5_KLEPN), VIM-1 (accession ID, Q5GN09_KLEPN) and IMP-1 (accession ID, Q8G9Q0_PSEAI) metallo-β-lactamases were purified from overnight cultures of recombinant *E. coli* DE3/pET24-NDM-1, *E. coli* DE3/pET-24-VIM-1 and *E. coli* DE3/pET-24-IMP-1 using the procedures reported in our previous papers [33,34]. Ligands reported in Fig. 1 can form stable complexes with Zn^{2+} from $\log K_{ZnL} = 11.4$ (L = Bispicen) to $\log K_{ZnL} = 15.6$ (L = TrispicenA) as reported elsewhere [30,35–38].

Kinetic parameters determination

Purified NDM-1, VIM-1 and IMP-1 variants were used to determine by kinetic assays K_i and IC_{50} values against Bispicen, Trispicen, Trispicen A, TrispicenDALA. Steady-state kinetic experiments were carried out under initial-rate conditions using Hanes plot linearization [39,40]. Competitive inhibition assays with all compounds were directly monitored using 100 μM meropenem as the reporter substrate and 30–80 nM of each enzyme. The limit of 500 μM was established as the highestvalue for all compounds. The kinetic K_i values were calculated using the following equation: $v_0/v_i = 1 + (K_m \times I)/(K_m + S) \times K_i$, 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 $K_m/(K_m + S) \times K_i$ [41–42].

Docking study

Flexible docking studies were performed using GOLD 2022 CCDC Software Ltd. (https://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 enzyme active site. 3D structure of NDM-1, VIM-1 and IMP-1, with PDB codes: 5ZGR, 5N5I and 1JJT, respectively, were retrieved from Protein Data Bank (PDB, https://www.rcsb.org). In NDM-1, VIM-1 and IMP-1, we used the BBL consensus numbering. Ligand structures were obtained from the Cambridge Structural Database (CSD) and modified by Mercury software of Cambridge Crystallographic Data Center CCDC (CSD code: GAGCEW for Bispicen, ABORUI for Trispicen, TrispicenA and TrispicenDALA). All compounds were docked at the substrate binding site by setting as flexibles the following residues: Ile31, Leu61, Met63, Val69, Tyr89 and Asn233 in NDM-1; Tyr69, Trp71, Phe64, Asp119 and Asn233 in VIM-1; Val64, Phe90, Trp67, Val70 and Lys224 in IMP-1. Low energy ligand structures were obtained by Conformer Generator module of Mercury. The solvent and substrate were deleted. Ten poses (docking output) were generated for each ligand into each enzyme and ranked by docking

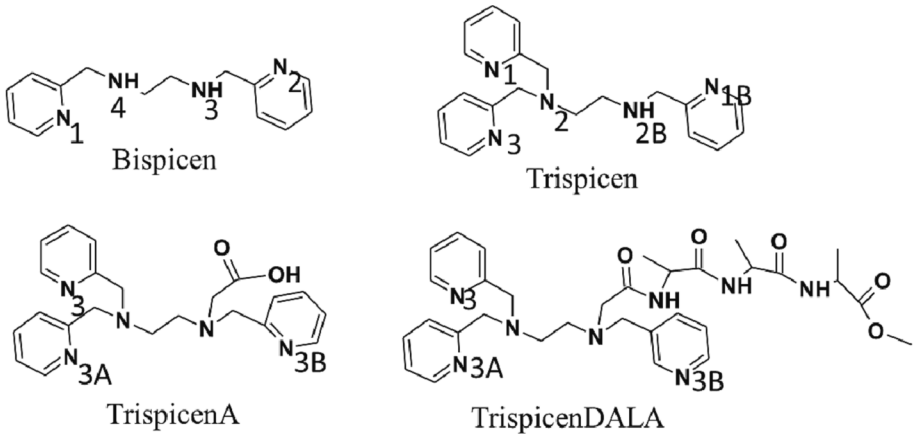

Fig. 1. Ligands investigated in the present work and atom numbering for molecular docking.

score.

Results and discussion

Kinetic inhibition assays

The K_m values of meropenem were determined for NDM-1, VIM-1 and IMP-1 (80 μM, 48 μM and 26 μM, respectively). The inhibition activity of Bispicen, Trispicen, TrispicenA and TrispicenDALA was evaluated against the three aforementioned subclass B1 MBLs. TrispicenDALA showed K_i and IC_{50} values for NDM-1, VIM-1 and IMP-1 higher than 500 μM, thus this compound cannot be considered a good inhibitor. Bispicen, Trispicen and TrispicenA compounds acted as competitive inhibitors towards NDM-1 and VIM-1 (Table 1). TrispicenA was capable to inhibit NDM-1 and VIM-1 with K_i values of 6 and 27 μM. Trispicen highly inhibited NDM-1 with K_i value of 0.08 μM and VIM-1 with K_i value of 3 μM. Bispicen was very active against VIM-1 (K_i = 0.13 μM) and it was less active towards NDM-1 (K_i = 117 μM) and IMP-1 (K_i = 412 μM) (Table 1). None of these compounds was able to inhibit IMP-1 enzyme.

Docking analysis

In order to clarify the mechanism of action of the assayed compounds against NDM-1, VIM-1 and IMP-1, molecular docking was performed. Docking scores are reported in Table 2. To gain insight into their interaction mode all compounds were docked on the binding site of NMD-1, VIM-1 and IMP-1 enzymes.

Considering the high structural homology along MBLs all designed compounds were able to bind with target molecules. The best poses of ligands into the binding site of the target molecules are shown in Fig. 2.

Overall, the presence of pyridine moieties allows the formation of pi-interactions like metal-pi interactions, polar-pi interactions, pi-stacking interactions along with H-bonding interactions with conserved residues of adjacent loops. The binding mode of Trispicen, the most active compound, into the active site of NDM-1 reveals that hydrogen bond interactions occur among the side chain of Asn233 and both pyridine atom N3 (d: 3.03 Å) and N2 atom (d: 3.16 Å) of the ligand (Fig. 3). The N2B atom of Trispicen coordinates Zn2 (d: 2.21 Å) and it is also involved in H bonding interaction with Asp120 (d: 3.34 Å). The ligand also establishes hydrophobic interactions with Ile31 and Val69 (residues of L3 loop) through the pyridine rings. Additionally, an electrostatic interaction (pi-anion interaction) involving the side chain of Asp236, along with a pi-sulfur interaction between Met63, and the pyridine rings of the ligand was also observed. The Asp236 residue interacts with the pyridine rings by a pi-donor H bond.

The carboxyl group of TrispicenA binds Zn1 ion with a distance of 2.49 Å and it is also H bonded to side chains of Asn233 (d: 2.94 Å). The Zn2 ion is coordinated by the pyridine N3 atom (d: 2.61 Å). Another H bonding interaction occurs between the N3B pyridine atom of Trispicen A and Gln119 (d: 2.80 Å). The pyridine ring of the ligand stacks His263 residue. Like Trispicen, TrispicenA is anchored to the enzyme active site by interactions with Ile31, Val69 (pi-Alkyl interactions) and Met63 (pi-sulfur interaction). Moreover, the ligand establishes carbon H bonding interactions and pi-donor hydrogen bonds with Asp120 and Asn233. The good inhibitory effect of Trispicen and TrispicenA is because these ligands interact with residues coordinated by zinc ions. In addition, L3 loop residues (Val69 and Met63) play an important role in anchoring these ligands to the active site. The interaction of Trispicen and TrispicenA with Ile31, situated at the tip of β1 strand, facilitated their binding and their inhibitory activity. In NDM-1, Ile31 plays an important role in substrate specificity and binding contributing to an increase of the hydrophobicity of the L3 loop [43,44]. Trispicen and TrispicenA also interact with *L*10 loop (residues 233 and 236) which plays an important role in substrate binding and zinc ions coordination [43]. Less active Bispicen was predicted to bind Zn2 ion through N3 (d: 2.21 Å) atom. Additionally, N3 and N4 are H bonded to the side chain of Asp120 (d: 3.01 Å and d: 3.08 Å, respectively). Ligand results anchored also by other two H bonding interactions with Asn233 (d: 3.01 Å) and Gln119 (d: 2.64 Å). Further, the ligand stacks His118 and His263 through its pyridine rings and Ile31 provides a pi-alkyl interaction with the ligand. For TrispicenDALA the binding affinity is considerably overestimated. This compound with an extra peptide group on the ethylenediamine chain is a much more sterically bulky compound, certainly affecting the existing coordination network and the resulting docking output. Despite it was predicted to bind the Zn atom, the distance between the pi-system and the ion is greater than the ligands considered so far (d greater than 4 Å).

In VIM-1, the most active compound Bispicen is anchored to the protein by an H bonding interaction between the N2 pyridine atom and Asp119 (d: 2.98 Å). The N1 and N4 atoms of Bispicen coordinate the Zn2 ion with a distance of 2.89 Å and 2.61 Å, respectively (Fig. 4). Furthermore, in the ligand structure, the two pyridine rings provide for hydrophobic interaction (pi-pi T shaped interaction) with the side chain of His263 and electrostatic interaction (pi-anion interaction) with the side chain of Asp119. In addition, non-conventional H bond interactions with His196, Cys221 and Asp120 as well as pi-donor H bond and pi-sigma interactions with Asp119 were found (Fig. 3). Because of the presence of an additional pyridine ring in the Trispicen molecule, Bispicen and Trispicen don't share the same network of interaction. Hence, Trispicen interacts with both Zn ions: the N2B atom of the ligand coordinates the Zn1 (d: 2.57 Å) while the pyridine ring establishes an electrostatic interaction (pi-cation) with the Zn2 ion (d: 4.78 Å). N2B is also H bonded to the side chain of His118 (d: 2.98 Å). Other H bond interactions occur among N1 and N3 atoms with the side chain of Asn233 with a distance of 2.98 Å for both. The interaction of Trispicen with Ala67, Ala235 (pi-Alkyl interactions), Tyr69 (pi-pi stacking interaction) and Asp236 (pi-anion interaction) allows to well accommodate the ligand structure into the binding site. In TrispicenA the carboxyl group interacts with the Zn1 ion (d: 2.36 Å) and with His196 by a pi-donor H bond. Another pi-donor H bond was predicted between the ligand and Asp119. No conventional H bond interactions were found in the TripsicenA molecule. However, the ligand is anchored to the binding

Table 2
Best scores of ligands after docking with NMD-1, VIM-1 and IMP-1.

Ligand	CHEMPLP (KJ/mol) NDM-1	CHEMPLP (KJ/mol) VIM-1	CHEMPLP (KJ/mol) IMP-1
Bispicen	82.25	81.42	81.48
Trispicen	96.25	101.81	86.18
TrispicenA	102.88	112.83	108.63
TrispicenDALA	98.10	110	104.65

Table 1
Kinetic parameters for meropenem and inhibitory activity of four polypyridine ligands against NDM-1, VIM-1 and IMP-1.

MBLs subclass B1	Bispicen		Trispicen		TrispicenA		TrispicenDALA	
	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)
NDM-1	117 ± 2	300 ± 5	0.08 ± 0.01	0.16 ± 0.03	6 ± 0.5	7 ± 1	>500	>500
VIM-1	0.13 ± 0.05	0.48 ± 0.05	3 ± 0.5	13 ± 1	27 ± 1	12 ± 1	>500	>500
IMP-1	412 ± 3	>500	>500	>500	>500	>500	>500	>500

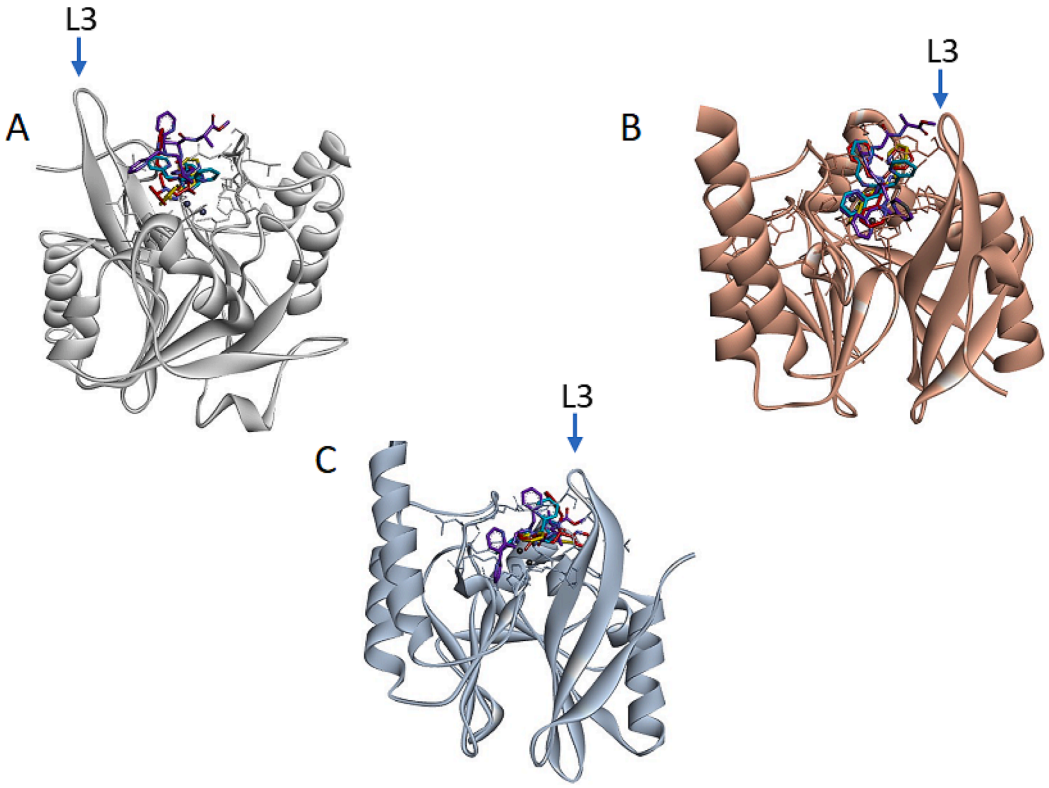

Fig. 2. Best pose of Bispicen (yellow), Trispicen (cyan), TrispicenA (red), TrispicenDALA (purple) into the binding site of NDM-1 (A), VIM-1 (B), IMP-1 (C).

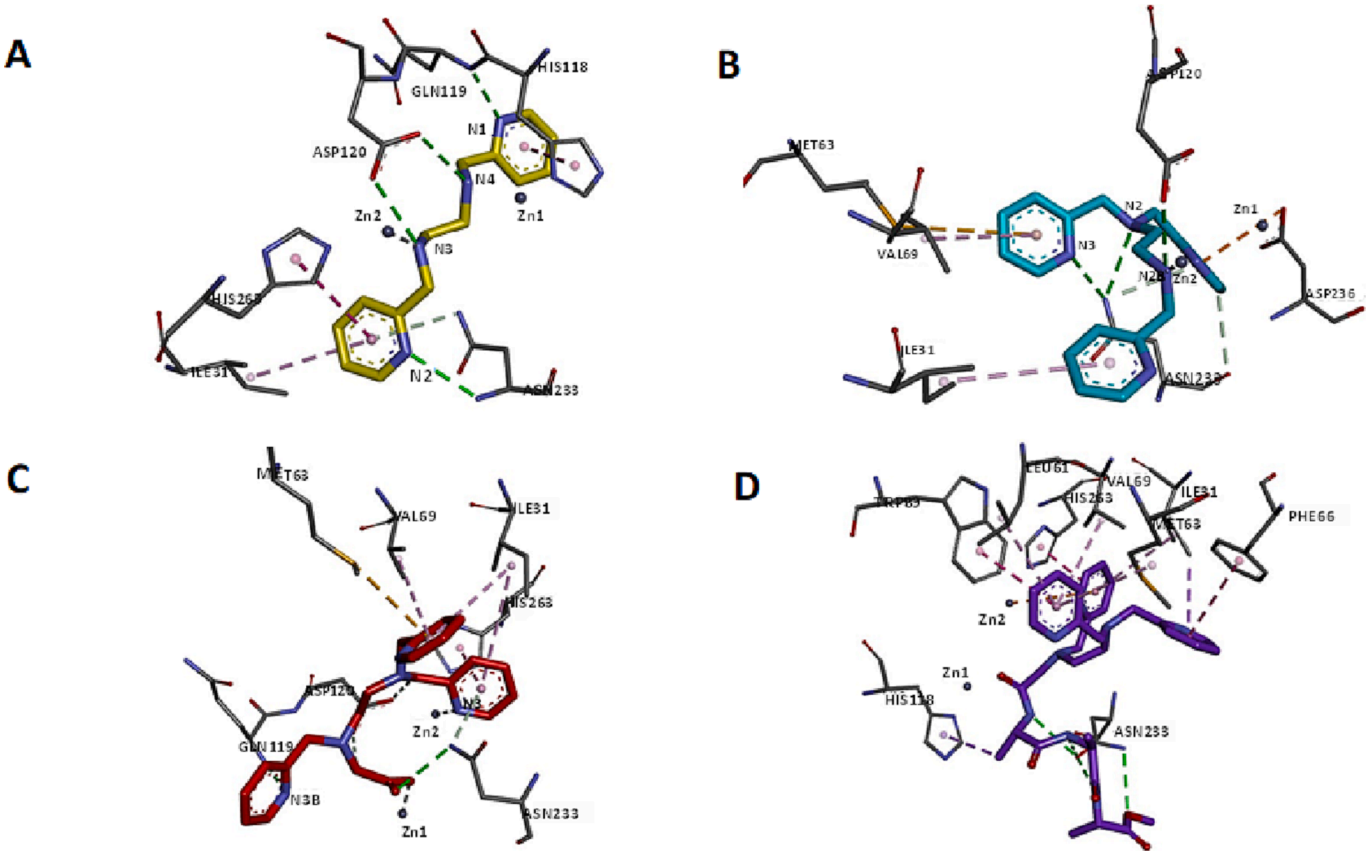

Fig. 3. Best docking poses of Bispicen (A), Trispicen (B), TrispicenA (C), TrispicenDALA (D) in the active site of NDM-1. H-bonding interactions are represented as green dotted lines. Hydrophobic interactions are shown as pink dotted lines. Grey dotted lines indicate metal coordination. Pi-anion/cation and pi-sulfur interactions are depicted as orange dotted lines. Pi-sigma interactions are in purple.

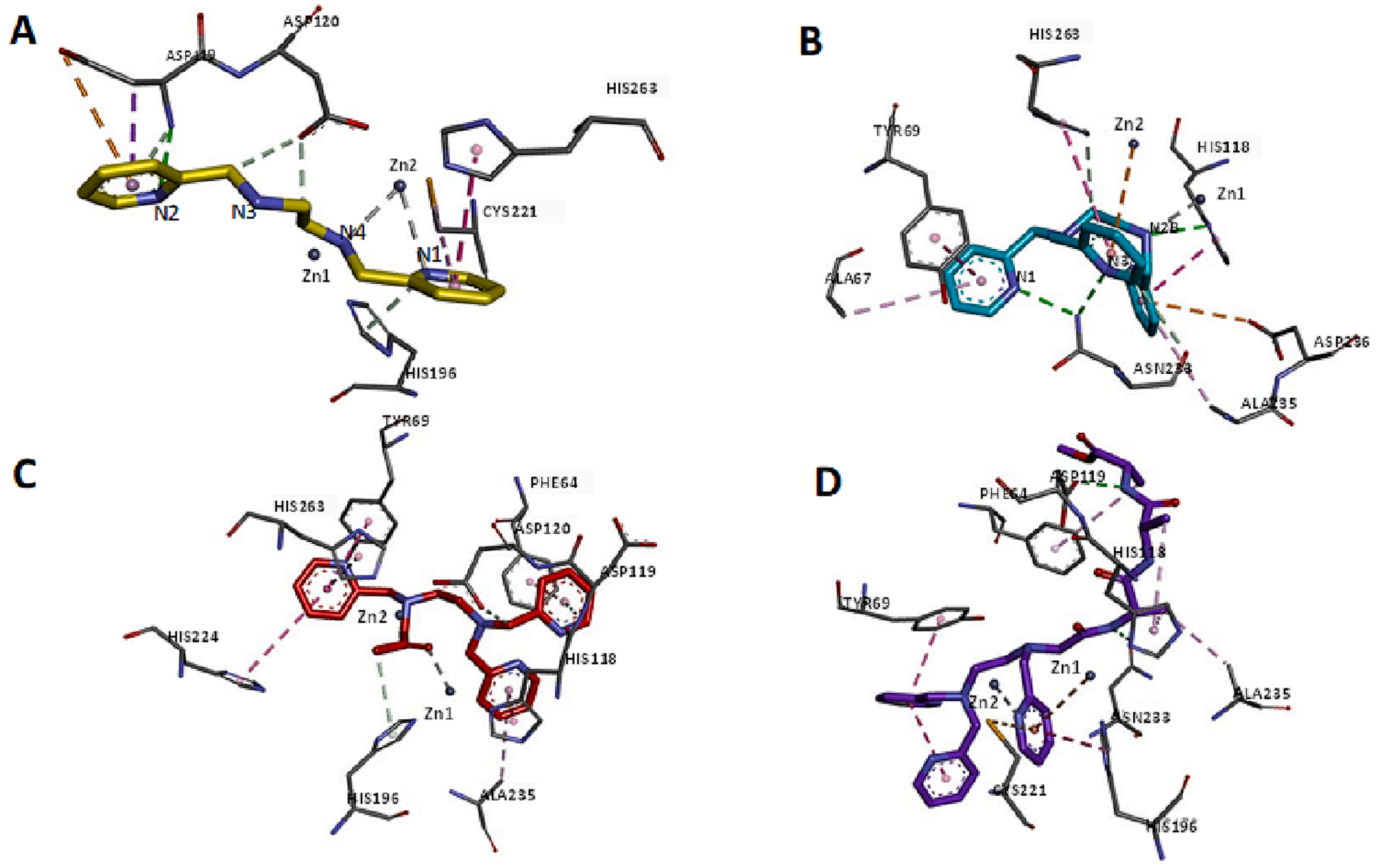

Fig. 4. Best docking poses of Bispicen (A), Trispicen (B), TrispicenA (C), TrispicenDALA (D) in the active site of VIM-1. H-bonding interactions are represented as green dotted lines. Hydrophobic interactions are shown as pink dotted lines. Grey dotted lines indicate metal coordination. Pi-anion, pi-cation and pi-sulfur interactions are depicted as orange dotted lines.

site of the enzyme mainly by hydrophobic interactions. In detail, these hydrophobic interactions involve all pyridine rings of TrispicenA and His263, Phe64, His118 (pi-pi stacked), Ala235 (pi-alkyl interactions), Tyr69, His224 (pi-pi T shaped) residues. Yet, a carbon-hydrogen bond with Asp120 was also found.

The evaluation of *in silico* data of IMP-1 and Bispicen, Trispicen, TrispicenA and TrispicenDALA indicates different accommodations of these ligands in the active site of IMP-1. The IMP-1 enzyme interacts with i) Bispicen by Zn1, Val64, Trp67, Val70, His118, Asp120 and His263; ii) Trispicen by Val64, Trp67, Cys221, Asn230 and His118; iii) TrispicenA by Zn1, Zn2, Val64, Trp67, Val70, Asp120 and His263; iv) TrispicenDALA by Zn2, Val64, Trp67, Phe90, Lys224, Phe117, His118, Ser119, Asn230 and His263 (Fig. 5). The distances between Bispicen and Zn1 was 2.7 Å, between TrispicenA and Zn1 and Zn2 were 2.61 Å/2.54 Å and 1.85 Å, respectively, between TrispicenDALA and Zn1 was 2.60 Å. Trispicen was not able to interact with Zn ions. Even if the four ligands interact with catalytic residues of IMP-1, as well as in NDM-1 and VIM-1, they were unable to inhibit IMP-1. It seems that the distance between Zn1 and Zn2 is too long (higher than 4 Å). We speculate that the higher distance Zn1-Zn2 found in IMP-1 in the presence of ligands could affect their inhibitory activity. The new polyimidazole ligands, synthesized in our laboratories, inhibited NDM-1 and VIM-1 with good efficiency but did not work against IMP-1 [45]. In some NDM-1 laboratory mutants we have demonstrated that differences in Zn1-Zn2 distance affected the hydrolytic activity of the enzymes [34]. Recently, Zhang et al. stated how the distance between Zn1 and Zn2 could influence the enzymatic activity of NDM-1 [46]. Correlation with active site conformational changes and enzymatic activity was also demonstrated in BCII enzyme a subclass B1 metallo-β-lactamase [47].

Conclusions

Recently, many clinically relevant bacterial pathogens rapidly evolved towards multidrug- or extensively-drug resistance phenotypes limiting therapeutic options to life-saving antibiotics, such as carbapenems or colistin [48,49]. This determines the selection of carbapenem- and/or colistin-resistant isolates, and the emergence of pan drug-resistant isolates [2,48,49]. There is urgent to discover new antibiotics, especially active on pathogens already showing pan-drug resistance, including major Gram-negative pathogens such as *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* [48,49]. However, MBLs-producing bacteria showed resistance not only to the life-saving carbapenems but also to innovative non-β-lactam β-lactamase inhibitors, such as diazabicyclooctanones (e.g., avibactam, relebactam, nacubactam) and boronic acids (vaborbactam).

The sequence divergence and the disparity in zinc stoichiometry and coordination between subclasses B1, B2 and B3 made difficult to identify and develop an inhibitor effective for all MBLs.

In the present study we related on the activity of four polypyridine ligands against the most clinically relevant MBLs (NDM-1, VIM-1 and IMP-1). The *in vitro* studies confirmed the previous microbiological results proving that Bispicen, Trispicen, TrispicenA showed good inhibitory activity against NDM-1 and VIM-1 [30]. The IMP-1 enzyme showed high resistance to these compounds but, to date, no effective inhibitors have been found effective towards this enzyme and its natural variants [17,18,20]. Overall, *in silico* study indicates that these ligands lie across the binding site of the MBLs by intercalating between the two MBL subclass zinc ions. Differences in *in vitro* results could suggest alternative binding modes of ligands which appear located less deeply into the active site and farther to Zn ions when docked with IMP-1 notably in

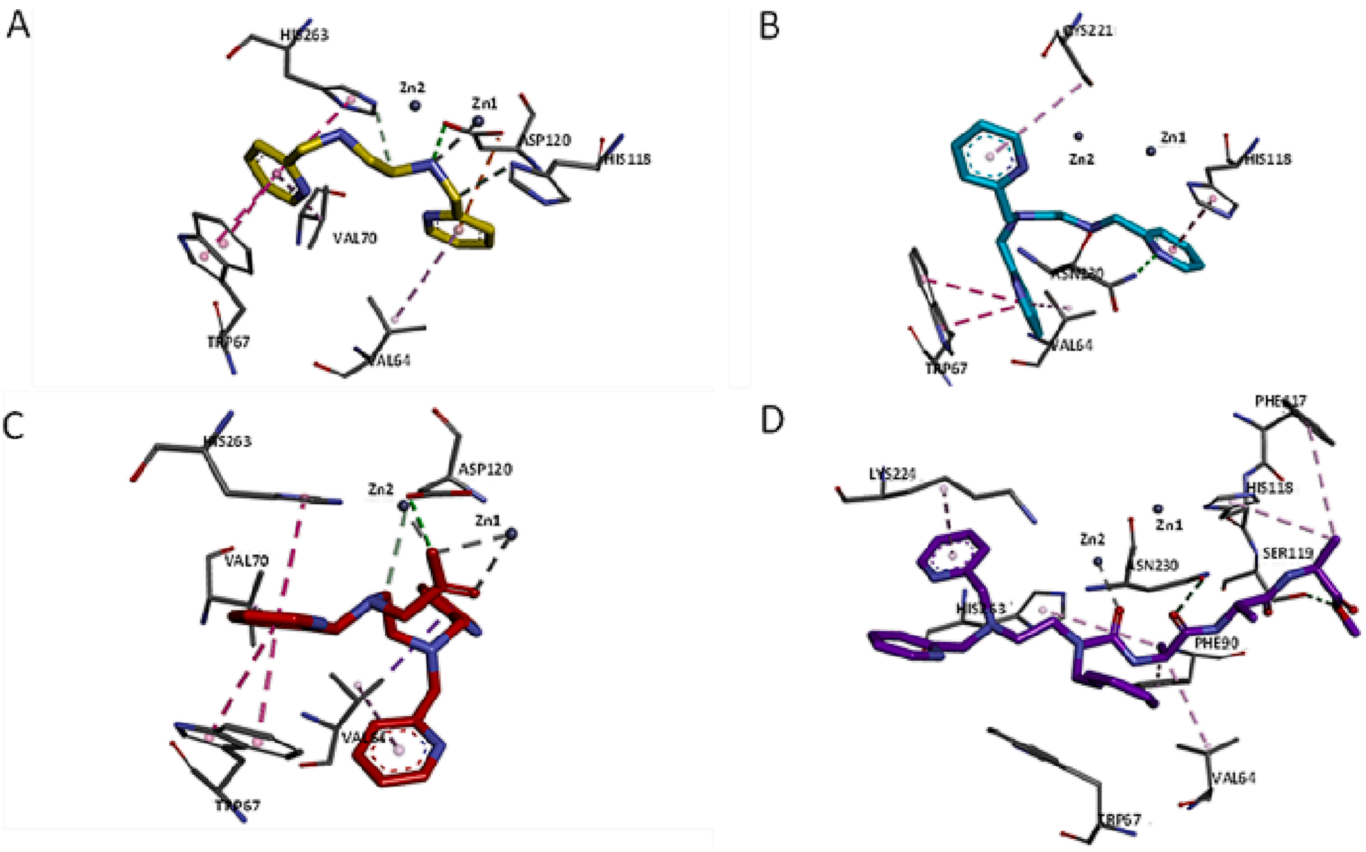

Fig. 5. Best docking poses of Bispicen (A), Trispicen (B), TrispicenA (C), TrispicenDALA (D) in the active site of IMP-1.

contrast to NDM-1 and VIM-1. Since subclass B1 contains the most clinically relevant β-lactamases, the effort of much research is to develop inhibitors with specific selectivity on subclass B1 enzymes. In this context, the design and synthesis of the zinc polypyridine ligands could be a promising approach for novel molecules to use in combination with β-lactams.

Accession CODES

- NDM-1: E9NWK5_KLEPN
- VIM-1: Q5GN09_KLEPN
- IMP-1: Q8G9Q0_PSEAI

CRediT authorship contribution statement

Livia Basile: Investigation, Methodology, Writing – original draft. **Alessandra Piccirilli:** Investigation, Methodology, Writing – original draft. **Fabrizia Brisdelli:** Investigation, Writing – original draft, Funding acquisition. **Mariagrazia Perilli:** Conceptualization, Data curation, Writing – original draft, Funding acquisition. **Noemi Bognanni:** Investigation, Methodology. **Luana La Piana:** Investigation, Methodology. **Luigi Principe:** Data curation, Visualization. **Stefano Di Bella:** Data curation, Visualization. **Graziella Vecchio:** Conceptualization, Data curation, Writing – original draft, Funding acquisition.

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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