Polypyridine Ligands as Potential Metallo-β-lactamase Inhibitors

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Abstract

Bacteria have developed multiple resistance mechanisms against the most used antibiotics. In particular, zinc-dependent metallo-β-lactamase producing bacteria are a growing threat, and therapeutic options are limited. Zinc chelators have recently been investigated as metallo-β-lactamase inhibitors, as they are often able to restore carbapenem susceptibility. We synthesized polypyridyl ligands, N,N'-bis(2-pyridylmethyl)-ethylenediamine, N,N,N'-tris(2-pyridylmethyl)-ethylenediamine, N,N'-bis(2-pyridylmethyl)-ethylenediamine-N-acetic acid (N,N,N'-tris(2-pyridylmethyl)-ethylenediamine-N'-acetic acid, which can form zinc(II) complexes. We tested their ability to restore the antibiotic activity of meropenem against three clinical strains isolated from blood and metallo-β-lactamase producers (*Klebsiella pneumoniae, Enterobacter cloacae, and*

Stenotrophomonas maltophilia). We functionalized N,N,N'-tris(2-pyridylmethyl)-ethylenediamine with D-alanyl-D-alanyl-D-alanine methyl ester with the aim to increase bacterial uptake. We observed synergistic activity of four polypyridyl ligands with meropenem against all tested isolates, while the combination N,N'-bis(2-pyridylmethyl)-ethylenediamine and meropenem was synergistic only against New Delhi and Verona integron-encoded metallo-β-lactamase-producing bacteria. All synergistic interactions restored the antimicrobial activity of meropenem, providing a significant decrease of minimal inhibitory concentration value (by 8- to 128-fold). We also studied toxicity of the ligands in two normal peripheral blood lymphocytes.

Keywords: antibiotic; bacteria; meropenem; pyridine; synergy; zinc.

1.0 Introduction

Despite continuing progress in chemotherapeutics, antimicrobial resistance is an increasingly serious threat to public health. It has been estimated that yearly deaths attributable to antimicrobial resistance will rise from the current 700,000 to 10 million in 2050 [1]. The most used antibiotics are β-lactams: penicillins, cephalosporins, carbapenems, and monobactams [2].

Bacteria have developed multiple resistance mechanisms against β-lactams, mainly producing β-lactamase enzymes [3]. Metallo-β-lactamases (MBLs)-producing *Enterobacterales* have increasingly been reported worldwide with high mortality rates (> 30% for bacteremia) [4]. MBLs are zinc-dependent enzymes that catalyze the hydrolysis of almost all β-lactams, including the carbapenems, which are commonly used as the first choice treatment against multidrug-resistant (MDR) bacteria [5]. The active-sites of MBLs contain one or two Zn^{2+} ions. According to the primary structure, MBLs are classified into three subclasses (B1, B2, and B3) [6] with different metal scaffolds [7,8]. The B1 subclass enzymes have emerged as the most clinically significant [9]. The main B1 MBLs are Verona integron-encoded metallo-β-lactamases (VIM), imipenemase (IMP), and New Delhi MBL (NDM) types [5,10,11]. The Indian subcontinent, the Balkan region, and the Middle East are the main endemic areas for NDM enzymes [12]. However, this determinant is also emerging in Africa, raising enormous concern due to considerable migration flows [13]. Acquired MBLs are commonly encountered in *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* [14]. The mobile nature of acquired MBLs is of critical concern for public health and the horizontal transfer of MBL genes among different bacterial species through mobile genetic elements (mostly transposons and plasmids) promotes the global spread of these resistance determinants. Notably, other bacterial species can intrinsically produce MBLs.

A strategy for combatting the increasing resistance mediated by MBLs is the identification of MBL resistant β-lactams or MBL inhibitors, thus protecting the β-lactam drugs from hydrolysis [3,15,16]. MBL inhibitors could act as an antibiotic adjuvant, restoring β-lactam antimicrobial activity.

The number of MBL inhibitors have hugely increased over the past five years, with more than 900 inhibitors studied [17]. Recently an interactive website on MBL inhibitors has also been developed with the aim to facilitate the discovery of new, improved inhibitors [17]. The search for MBL inhibitors is now crucial for public health [5,18], and no MBL inhibitor has thus far been clinically approved.

Zinc chelators have been studied as one type of MBL inhibitor [3,14,15,18]. Although chelators have been used for diagnostic purposes and chelation therapy [19–21], they have never been included in any anti-MBL commercially available pharmacologic formulations. Chelators have not been used thus far in the clinical setting for two reasons: 1) infections due to MBL-producing bacteria are a relatively recent clinical problem, and 2) the development of a single inhibitor to neutralize MBLs has been deemed too technically challenging in part due to the difficulty in overcoming *in vivo* toxicity associated with cross-reactivity with human metalloenzymes [22]. Given this, provided their activity, efficacy, and tolerability are confirmed in preclinical and clinical studies, therapies including MBL inhibitors may become a reasonable alternative to classical therapeutic approaches for infections of MBL producers, especially when robust safety data become available.

Zn ligands can act as zinc "strippers" from MBL enzymes, or they can form a ternary complex, usually displacing a hydroxide ion shared by two Zn^{2+} ions at the MBL catalytic site [11,23–26]. Many zinc ligands such as ethylenediaminetetraacetic acid (EDTA), 1,4,7-triazacyclononane-1,4,7-triacetic acid, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, dithiocarbamates, N,N,N′,N'-tetrakis (2-pyridymethyl)ethylenediamine (TPEN), trispyridyl-amine, picolinic acid, dithiolopyrrolones, peptide aspergillomarasmine A, disulfiram, hydroxyquinolines, and other ligands have been proven to inhibit MBLs [24–33]. More often, the ligands have been studied on B1 MBLs. Recently some chelators have also been studied *in vivo* [34,35]. Zincophore ethylenediamine-N, N'-disuccinic acid improved survival of *Klebsiella pneumoniae* infected *Galleria mellonella* larvae when administrated in combination with imipenem, compared to imipenem monotherapy [36]. 1,10-phenanthroline and TPEN improved survival of *Aspergillus fumigatus* infected mice alone and in

combination with caspofungin [34]. Recently, we tested TPEN and nitroxoline *in vivo* and found that TPEN can restore meropenem activity in infected *G. mellonella* larvae [28].

Recently, even if the role of zinc complexation has not been highlighted, a combination of EDTA as a metal chelator and sulbactam (an MBL inhibitor) has been approved as antibiotic adjuvants of β-lactam ceftriaxone for the treatment of multiresistant septicemia [37,38]. Furthermore, some authors have suggested that endogenous zinc chelators in combination with conventional antibiotics could be used to treat infections caused by MBL-expressing pathogens [39].

Based on the recent interest in zinc chelators, in this paper, we synthesized polypyridyl multidentate ligands based on the ethylenediamine backbone. Specifically, we synthesized N,N'-bis(2-pyridylmethyl)-ethylenediamine (Bispicen), N,N,N'-tris(2-pyridylmethyl)-ethylenediamine (Trispicen), N,N,N'-tris(2-pyridylmethyl)-ethylenediamine-N'-acetic acid (TrispicenA), N,N'-bis(2-Pyridylmethyl)-ethylenediamine-N-acetic acid (BispicenA) to study their ability to restore meropenem activity (Fig. 1) against three clinical MBL (VIM-1, NDM-1 and L1) -producing strains isolated from blood. They were compared to TPEN. The ligands studied can form Zn complexes with the stability constants ranging from $\log K_{ZnL}$ = 11.4 (L=Bispicen) to $\log K_{ML}$ = 15.6 (L =TPEN) [40–43]. The modulation of stability constants is important for the selection of the ligand, as very strong ligands may indiscriminately bind to the metal of essential metalloproteins in the host cells [44].

Moreover, COOH groups could improve the interaction with the active site of MBL, as found for other ligands containing carboxylate groups [18,45]. TrispicenA was also functionalized with the D-Ala-D-Ala-D-AlaOCH3 peptide with the aim to improve selectivity for bacteria. In bacteria, D-amino acids are found within peptidoglycan. Recently, it has been found that D-Ala derivatives can be incorporated into bacteria, one reason D-amino acid-derivatives have been tested as a high-value target for antibiotics [46,47].

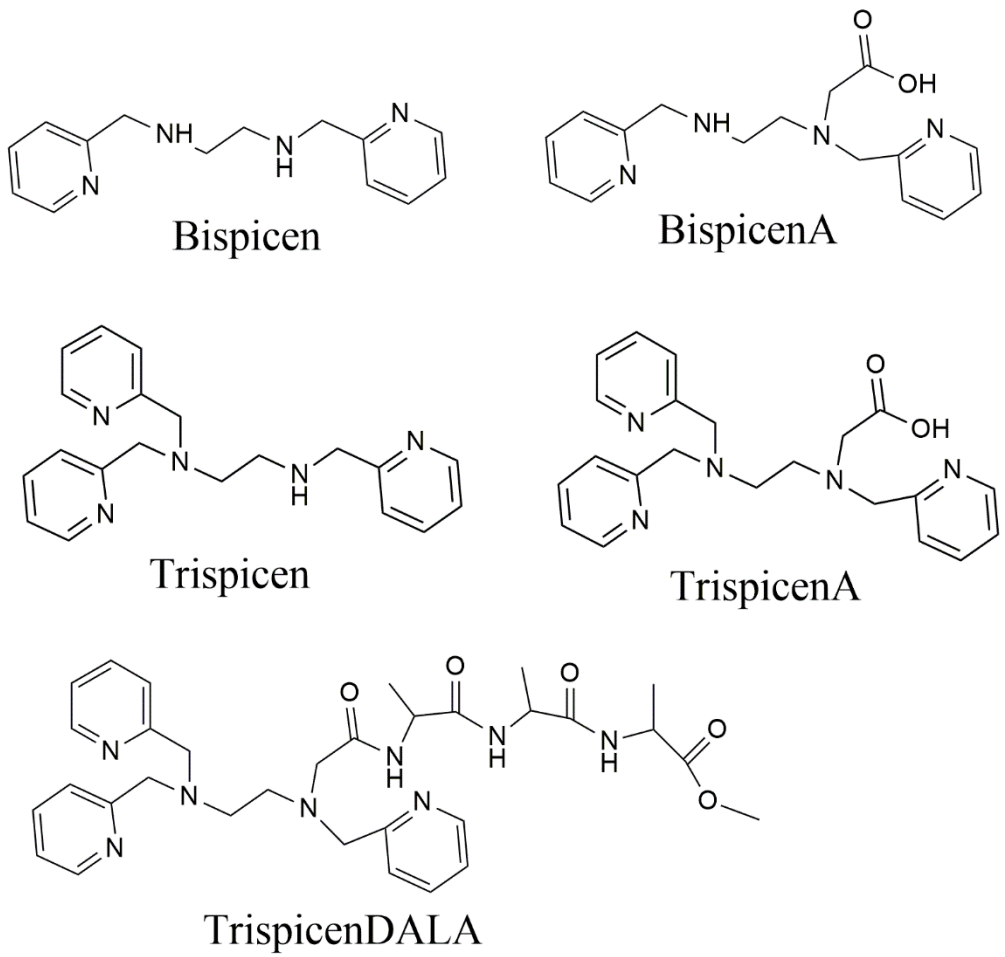

Figure 1. Polypyridine ligands investigated

2.0 Experimental Section

2.1 Materials

1-Hydroxybenzotriazole (HOBt), 1-ethyl-3 (3-dimethylamino propyl) carbodiimide (EDC), pyridine-2-carboxaldehyde and TPEN were purchased from TCI (TOKYO CHEMICAL INDUSTRY CO., Tokyo), D-Ala-D-Ala-D-Ala was purchased from Bachem (Tokyo, Japan) and modified in methyl-ester (D-Ala-D-Ala-D-AlaOCH_3). Bispicen, Trispicen, BispicenA, and TrispicenA have been synthesized as reported elsewhere [48–52]

2.1.1 Synthesis of N,N'-Bis(2-pyridylmethyl)-ethylenediamine (Bispicen)

Ethylenediamine (0.60 g, 10 mmol) was added to pyridine-2-carboxaldehyde (2.14 g, 20 mmol) in 20 ml of methanol. The solution was refluxed for 5 h, cooled at r.t. and $NaBH_4$ (1.52 g, 40 mmol, 10 ml of methanol) was added. The solution was refluxed overnight. The solvent was evaporated, and the product was extracted from the residue with CH_2Cl_2 (150 ml x 3). The extract was dried over $MgSO_4$. After evaporation of the solvent, Bispicen (1.99 g) was obtained as an oil and was freeze-dried. Yield 82%.

TLC: Rf= 0.72 (PrOH/AcOEt/H_20/NH_3 5:2:3:1).

^{1}H NMR (500 MHz, D_2O) δ: 8.19 (d, J = 4.6 Hz, 2H, H-6 Py), 7.58 (t, J = 7.7 Hz, 2H, H-4 Py), 7.15 (d, J = 7.9 Hz, 2H, H-3 Py), 7.09 (dd, J = 5.3 Hz, J = 7.5 Hz 2H, H-5 Py), 3.57 (s, 4H, CH_2Py), 2.49 (s, 4H, CH_2NH). ESI-MS: m/z = 243.1 $[M+H]^+$, 265.2 $[M+Na]^+$

2.1.2 Synthesis of N,N,N'-Tris(2-pyridylmethyl)-ethylenediamine (Trispicen)

Pyridine-2-carboxaldehyde (0.66 g, 6.15 mmol) was added to Bispicen (1.49 g, 6.15 mmol) in diethyl ether (20 ml) and the solution was stirred at r.t. overnight with $CaCl_2$ protection. The white precipitate formed was filtered and washed with diethyl ether. It was dissolved in 10 ml of CH_3OH and $NaBH_3CN$ (0.096 g, 1.54 mmol) and 1 ml of CF_3COOH were added. The solution was stirred at room temperature with $CaCl_2$ protection. After 8h, NaOH (water solution 15%, 65 ml) was added and the solution stirred overnight. The solvent was evaporated and the solid was extracted with CH_2Cl_2 (150 ml x 3). Trispicen (0.44 g) was obtained as a yellow oil and was freeze-dried. Yield 85%.

TLC: Rf= 0.63 (PrOH/AcOEt/H_20/NH_3 5:2:3:1)

^{1}H NMR (500 MHz, D_2O) δ: 8.19 (d, *J* = 5.0 Hz, 1H, H-6' Py), 8.15 (d, *J* = 5.0 Hz, 2H, H-6 Py), 7.58 (t, *J* = 7.7 Hz, 1H, H-4' Py), 7.52 (t, *J* = 7.7 Hz, 2H, H-4 Py), 7.16 (d, *J* = 7.8 Hz, 2H, H-3 Py), 7.09 (m, 4H, H-3', H-5 and H-5' of Py), 3.44 (s, 6H, CH_2Py), 2.44 (t, *J* = 6.1 Hz, 2H, CH_2N), 2.33 (t, *J* = 6.1 Hz, 2H, CH_2N). ESI-MS: m/z = 334.2 $[M+H]^+$, 356.2 $[M+Na]^+$

2.1.3 Synthesis of N,N'-bis([2-Pyridylmethyl)-ethylenediamine-N-acetic acid (BispicenA)

Tert-Butyl bromoacetate (0.42 g, 2.15 mmol) was added to Bispicen (0.52 g, 2.15 mmol) and K_2CO_3 (0.50 mg, 3.6 mmol) in 5 ml of CH_3CN. The solution was refluxed overnight under stirring. K_2CO_3 was filtered and washed with CH_3CN. The solvent was evaporated under vacuum and the residue obtained was purified by flash-chromatography (Silica column); Ethyl acetate/methanol 0→60% was used as the eluent.

^{1}H NMR (500 MHz, D2O) δ 8.29 (d, *J* = 4.9 Hz, 1H, H-6 py), 8.20 (d, *J* = 4.8 Hz, 1H, H-6 py B), 7.67 (t, *J* = 7.7 Hz, 1H, H-4), 7.62 (t, *J* = 7.7 Hz, 1H, H-4 py B), 7.27 (d, *J* = 7.8 Hz, 1H, H-3 Py B), 7.22 (d, *J* = 8.0 Hz, 1H, H-3 Py), 7.19 (m, 1H, H-5), 7.15 (m, 1H, H-5 py B), 3.72 (s, 2H, CH2Py), 3.63 (s, 2H, CH2Py B), 3.15 (s, 2H, CH2CO), 2.68 (t, J = 5.5 Hz, 2H, CH2N), 2.55 (t, J = 5.5 Hz, 2H, CH_2NH), 1.23 (s, 9H, t-but). B ring is pyridine bound to the alkylated amino group.

^{13}C NMR (125 MHz, D_2O) δ 172.8 (COObut); 157.4 (C-2 of Py B), 156.0 (C-2 of Py), 146.5 (C-6 of Py), 146.1 (C-6 of Py B), δ 135.8 (C-4 of Py), δ 135.7 (C-4 Py B), δ 121.3 (C-3 py B) , 120.38 (C-3, C-5, C-5 B of Py), 81.3 (C(CH3)), 57.6 (CH_2Py), 54.2 (CH_2COOBut), 50.6 (CH_2N), 50.2 (CH_2 of Py), 42.5 (CH_2NH), 25.6 (CH_3).

The butyl ester was hydrolyzed with CF_3COOH (2 ml), under stirring. After 12 h, the solvent was evaporated, and the product was purified by Sephadex DEAE A-25 (HCO_3^- form) column using water as the eluent. The final product was freeze-dried. Yield: 0.13 g, 20%.

TLC: Rf = 0.85 (PrOH/AcOEt/H20/NH3 5:2:3:1)

^{1}H NMR (500 MHz, D2O) δ 8.48 (d, J = 5.7 Hz, 1H, H-6 Py), 8.36 (d, J = 5.9 Hz, 1H, H-6 Py B), 8.27 (t, J = 7.9 Hz, 1H, H-4 Py), 8.22 (t, J = 8.0 Hz, 1H, H-4 Py B), 7.81 (d, J = 8.0 Hz, 1H, H-3 Py B), 7.72 (t, J = 8.0 Hz, 1H, H-5 of Py), 7.63 (dd, J = 15.0, 7.6 Hz, 2H, H-5 py and H-3 Py B), 4.65 (s, 2H, CH2Py), 4.23 (s, 2H, CH2Py), 3.53 (s, 2H, CH2CO), 3.43 (t, J = 5.5 Hz, 2H, CH2N), 3.13 (t, J = 5.5 Hz, 2H, CH_2N). ESI-MS: m/z = 301.2 $[M+H]^+$, 323.2 $[M+Na]^+$

2.1.4 Synthesis of N,N,N'-tris([2-Pyridylmethyl)-ethylenediamine-N'-acetic acid (TrispicenA)

Trispicen (1.96 g, 5.88 mmol) was alkylated with tert-Butylbromoacetate (1.15 g, 5.88 mmol) and K_2CO_3 in 5 ml of acetonitrile. The reaction mixture was purified with flash-chromatography (Silica Column, eluent ethyl acetate/methanol 0→60%). The butyl ester was hydrolyzed with CF_3COOH (2 ml), under stirring. After 12 h, the solvent was evaporated, and the product was purified by Sephadex DEAE A-25 (HCO_3^- form) column using water as the eluent. The final product was freeze-dried.

Yield: 0.450 g, 20%.

TLC: Rf = 0.62 (PrOH/AcOEt/H_20/NH_3 5:2:3:1)

^{1}H NMR (500 MHz, D_2O) δ 8.10 (d, *J* = 5.0 Hz, 2H, H-6 of Py), 7.83 (d, *J* = 4.9 Hz, 1H, H-6' of Py), 7.50 – 7.40 (m, 3H, H-4 and H-4 Py), 7.15 (d, *J* = 7.9 Hz, 2H, H-3 of Py), 7.05 (d, *J* = 7.8 Hz, 1H, H-3' of Py), 7.02 (dd, *J* = 6.9, 5.6 Hz, 2H, H-5 of Py), 6.99 (dd, *J* = 7.5, 5.1 Hz, 1H, H-5' of Py), 3.95 (s, 2H, CH_2Py B), 3.24 (d, J = 11.1 Hz, 4H, CH_2Py), 3.12 (s, 2H, CH_2COOH), 3.08 (t, *J* = 5.7 Hz, 2H, CH_2CH_2N), 2.62 (t, *J* = 5.7 Hz, 2H, NCH_2N).

^{13}C NMR (125 MHz, D_2O) δ 170.0 (COOH); 156.4 (C-2 of Py), δ 149.2 (C-2' of Py), 148.6 (C-6 Py B), 147.9 (C-6 Py), 138.1 (C-4' of Py), 138.0 (C-4 of Py), 124.2 (C-3' and C-5' of Py), δ 123.7 (C-3 of Py), 123.1 (C-5 of Py), δ 58.5 (CH_2Py), 57.5 (CH_2 PyB), 55.0 (CH_2COOH), 49.9 (NCH2CO), 46.8 (CH_2N). ESI-MS: m/z = 392.2 $[M+H]^+$, 414.2 $[M+Na]^+$

2.1.5 Synthesis of D-Ala-D-Ala-D-AlaOCH_3

D-Ala-D-Ala-D-AlaOCH_3 was synthesized from tripeptide D-Ala-D-Ala-D-Ala (100 mg, 0.43 mmol) in 2 ml of CH_3OH at 0°C. 1 ml of acetyl chloride was added under stirring. After 4h, the solvent was evaporated and the product was dissolved in water and purified with chromatography (DEAE Sephadex A-25). ^{1}H NMR spectroscopy confirmed the presence of the OCH_3 group on the newly-formed ester. ^{1}H NMR (500 MHz, D_2O) δ: 4.25 (q, J = 7.3 Hz, 1H, CH); 4.14 (q, J = 7.2 Hz, 1H, CH); 3.59 (s, 3H, OCH_3); 3.42 (q, *J* = 6.9 Hz, 1H, CH); 1.26 (d, J = 7.5 Hz, 3H, CH_3); 1.24 (d, *J* = 7.4 Hz, 3H, CH_3); 1.14 (dd, *J* = 13.9, 6.7 Hz, 3H, CH_3).

2.1.6 Synthesis of N,N,N'-tris([2-Pyridylmethyl)-ethylenediamine-N'-acetyl-D-alanyl-D-alanyl-D-alanine methyl ester (TrispicenDAla)

HOBt (93 mg, 6.2 mmol) and EDC (117mg, 6.2 mmol) was added to TrispicenA (159 mg, 4.1 mmol) in DMF under stirring. After 15 min, D-Ala-D-Ala-D-AlaOCH$_3$ (100 mg, 4.1 mmol, in DMF) and Triethylamine (0,057 ml, 4.1 mmol) were added. The solution was stirred at r.t.. After 12 h, the reaction mixture was purified by flash-chromatography using an R$_P$ C18 column and a linear gradient of water/acetonitrile. The solid obtained was purified again in the same condition. The final product (20 mg) was freeze-dried.

TLC: Rf = 0.70 (PrOH/AcOEt/H$_2$0/NH$_3$ 5:2:3:1)

^{1}H NMR (500 MHz, D$_2$O) δ 8.28 (d, J = 4.8 Hz, 2H, H-6 of Py), δ 7.66 (q, J = 6.6 Hz, 3H, H-4 and H-6' of Py), δ 7.30 (d, J = 7.8 Hz, 2H, H-3 of Py), δ 7.22 (m, 5H, H-4', H-3', H-5 and H-5' of Py), δ 4.24 (q, J = 7.2 Hz, 1H, CH Ala), δ 4.13 (q, 7.1 Hz, 1H, CH Ala); 4.11 (q, J = 7.2 Hz, 1H, CH Ala), δ 3.74 (s, 2H, CH$_2$CO), δ 3.66 (s, 4H, CH$_2$Py), δ 3.63 (s, 3H, OCH$_3$), δ 3.60 (s, 2H, CH$_2$Py), δ 2.55 (m, 2H, CH$_2$CH$_2$N), δ 2.52 (m, 2H, CH$_2$CH$_2$N), δ 1.28 (d, J = 7.3 Hz, 3H, CH$_3$ of D-Ala), δ 1.24 (d, *J* = 7.2 Hz, 3H, CH$_3$ of D-Ala), δ 1.18 (d, *J* = 7.1 Hz, 3H, CH$_3$ of D-Ala).

^{13}C NMR (125 MHz, D$_2$O) δ 174.4 (COOH), 157.0 (C-2 of Py), 148.2 (C-2' of Py), 137.9 (C-4 and C-6' of py), δ 138.1 (C-6 of Py), δ 124.4 (C-3- of Py), δ 124.1 (C-5 of Py), δ 123.1 (C-4',C-3', and C-5'), δ 60.4 (CH$_2$CO), δ 59.9 (CH$_2$Py), δ 52.8 (OCH$_3$), δ 51.9 (CH$_2$CH$_2$N), δ 51.1(NCH$_2$N), δ 48.6 (CH Ala), δ 48.8 (CH Ala), δ 49.2 (CH Ala), δ 17.1 (CH$_3$ Ala), δ 16.3 (CH$_3$ Ala), δ 15.8 (CH$_3$ Ala).

ESI-MS: m/z = 619.4 $[M+H]^+$, 641.3 $[M+Na]^+$

Elemental Analysis for $C_{32}H_{42}N_8O_5$: calc C, 62.12; H, 6.84; N, 18.11; found C 61.92; H, 6.86; N, 18.05.

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. The 2D experiments (COSY, TOCSY, HSQCAD, HMBC, NOESY) were acquired by using 1000 data points, 256 increments, and a relaxation delay of 1.2 s. The spectra were referred to the solvent signal.

ESI mass spectra were acquired with an API 2000– ABSciex spectrometer.

Flash chromatography was carried out with a CombiFlash Automated Flash Chromatography System (Teledyne ISCO).

2.3 In vitro microbiological experiments

2.3.1 Bacterial isolates

We studied three MBL-producing clinical isolates (with acquired or chromosomally-encoded MBLs) responsible for invasive infections in patients hospitalized at the ‘‘Alessandro Manzoni’’ Hospital (Lecco, Italy). Bacterial isolates were selected on the basis of their origin (invasive, Intensive care unit (ICU) patients), resistance to carbapenems, typology of carbapenemases (chromosomally encoded or acquired MBLs), and availability of very few therapeutic alternatives. Bacterial isolates were as follows NDM-producing *Klebsiella pneumoniae* (LC954/14), L1-producing *Stenotrophomonas maltophilia* (LC669/17) and VIM-producing *Enterobacter cloacae (*LC1341/19*)* isolated from blood cultures. *K. pneumoniae* isolate (LC954/14) was isolated from blood in 2014 and has been previously described [13]. It expressed an acquired NDM-1 determinant, belonged to ST11, and represented the first described NDM-producing *K. pneumoniae* imported from Africa to Italy. *S. maltophilia* (LC669/17) was isolated from blood in 2017 and expressed the chromosomally encoded L1 carbapenemase. Both isolates have previously been used to evaluate the *in vitro* activity of the chelators, including TPEN in restoring carbapenem activity against MBL producers [28]. *E. cloacae* (this work) was isolated from blood in 2019 and expressed an acquired VIM carbapenemase. VIM carbapenemase was identified at group level (VIM-type), by the GeneXpert® System using the Xpert Carba-R test (Cepheid,Sunnyvale, CA), and then detected as VIM-1 by conventional PCR and sequencing In brief, the *bla*$_{VIM}$ gene was amplified by PCR using primers and conditions already

described [53]. Sequencing was performed using the Dye Terminator DNA sequencing kit V1.1 (Applied BiosystemsTM) followed by purification using the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany). The sequences obtained were corrected and analyzed using the 4Peaks program and then exported in FASTA format. Sequence alignments were performed by using BLAST (Basic Local Alignment Search Tool) and UniProt programs. The bioinformatic program Swiss-Model ExPASy was used to obtain the Protein Data Bank (PDB) format of VIM protein from the FASTA format.

Bacterial identifications have been performed by MALDI-TOF (Vitek MS, bioMerieux, Marcy l'Etoile, France), while antimicrobial susceptibility tests have been carried out by broth microdilution method using a dedicated TREK panel (DKMGN, Thermo Fisher Diagnostics, Milan, Italy). Antibiotic susceptibility profiles were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) v.7.1 clinical breakpoints. Minimum inhibitory concentration (MIC) values of meropenem (MEM) were further confirmed by standard broth microdilution (CLSI), using Cation-Adjusted Mueller-Hinton (CAMH, Thermo Fisher Diagnostics) broth.

2.3.2 Antimicrobial activity related to zinc chelators

Antimicrobial activity of six zinc chelators (TPEN, TrispicenDAla, TrispicenA, BispicenA, Trispicen and Bispicen) against the bacterial isolates have been investigated by standard broth microdilution (Clinical and Laboratory Standards Institute, CLSI) using Cation-Adjusted Mueller-Hinton (CAMH) broth to obtain MIC values.

2.3.3 Synergy between meropenem and zinc ligands

Interactions were determined by a preliminary qualitative double-disk diffusion test, and by subsequent quantitative checkerboard.

The double-disk diffusion method was performed with disks placed at 15 and 20 mm center-to-center for each combination chelator-meropenem, as previously described [28]. Standard 0.5 McF

(McFarland) inoculums for each isolate were plated on Mueller Hinton agar (MHA, bioMerieux). Ten µl of a concentrated solution of each chelator and MEM (1024 mg/L) were placed on blank disks on MHA. Synergy was defined as the presence of alterations in disk's inhibition rings. Positive antibiotic/chelator interactions in preliminary screening were then investigated by the checkerboard assay, as previously described [28]. The range of drug concentration used in the checkerboard analysis was such that the dilution range encompassed the MIC for each drug used in the analysis. Broth microdilution plates were inoculated with each bacterial isolate to yield ~ 10^6 CFU (Colony-Forming Unit)/ml in a 100-µl final volume and incubated for 18 h at 37°C. Synergy has been defined as requiring a fourfold reduction in the MIC of both antibiotics in combination, compared with each used alone, measuring the fractional inhibitory concentration index (FICI). The FICI was calculated for each combination using the following formula: $FICI = FIC_A + FIC_B$, where FIC_A = MIC of drug A in combination/MIC of drug A alone, and FIC_B = MIC of drug B in combination/MIC of drug B alone. The FICI was interpreted as follows: synergy, $FICI \leq 0.5$; indifference, $0.5 < FICI \leq 4$; antagonism, $FICI > 4$ [54]. All measurements were performed in triplicate assays for each method.

2.3.4 Antiproliferative activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The antiproliferative effect of compounds TPEN Trispicen, TrispicenA, Bispicen, BispicenA e TrispicenDala was examined on two normal peripheral blood lymphocytes (PBL A and B) that were stimulated with 1% phytohemoagglutinin (PHA) plus 100 U/ml Interleukin-2 (IL-2) IL-2 for a week. Stimulated PBLs were then exposed to compounds for 72 h. Once plated in 96-well round-bottomed microtiter plates at 10000 cells/well PBLs were centrifuged at 1100 rpm for 2 min. and compounds added after 6-8 h. All compounds were administered in five concentrations (1:10 dilution ratio starting from 100 uM concentration). Each well finally contained 200 µl. The MTT test was performed after 72 h as described elsewhere [55].

3.0. **Results and discussion**

3.1 Synthesis and characterization

Bispicen and Trispicen have been synthesized through a reductive amination reaction, as reported elsewhere [48–50]. They were alkylated using tert-butyl bromoacetate [48,51].

NMR spectra confirmed the identity of Bispicen, Trispicen, and their alkylated derivatives (Fig. S1-S9). In the ^{1}H NMR spectrum of BispicenA, the protons of the two pyridine rings show different chemical shifts for the presence of the COOH group. The typical pattern of pyridine derivatives can be seen for each pyridine. Furthermore, the protons of the ethylene chain appeared as two triplets at 3.43 ppm and 3.13 ppm. The three singlets at 3.53 ppm, 4.23 ppm, and 4.65 ppm are due to the methylene protons.

In the ^{1}H NMR spectrum of TrispicenA, in addition to the pyridine protons in the aromatic region, the characteristic signals of the ethylene chain appear as two triplets. Three different singlets appeared due to methylene protons linked to the pyridine rings. In the ^{13}C NMR spectrum of TrispicenA at 170 ppm, the CO signal appeared (Fig. S8).

We functionalized TrispicenA with the tripeptide D-Ala-D-Ala-D-AlaOCH_3, to improve its permeability in the bacterial cell. The functionalization of TrispicenA was achieved through a condensation reaction in DMF using HOBt and EDC as condensing agents. The identity of the product was confirmed by NMR spectra (Fig. S10-S13).

In the ^{1}H NMR spectra (Fig. S10), the signals in the aromatic region can be assigned to the pyridine protons. The CH protons of the three D-Ala residues resonate at 4.24 ppm and 4.13 ppm while protons of CH_3 resonate at 1.28 ppm, 1.24 ppm, and 1.18 ppm as doublets. The protons of the ethylene chain resonate as multiplets at 2.55 ppm and 2.52 ppm. The signals of the methyl ester resonate at 3.63 ppm, while other methylene protons resonate at 3.74 ppm, 3.66 ppm, and 3.60 ppm. The ^{13}C NMR spectrum of the TrispicenDAla was also assigned by HSQC and HMBC spectra (Fig. S12, S13).

3.2 Zn complexes

The stability constants of the ligands with Zn^{2+} have been reported for TPEN and Bispicen [41,56]. We calculated the stability constants of Trispicen, BispicenA and TrispicenA (Table 1) as reported elsewhere [57]. The calculation of $logK_{ML}$ values was based on donor group additivity used successfully elsewhere [58]. In Table 1, we report the calculated $logK_{ML}$ for TPEN and Bispicen that are very similar to the experimental values. The calculated constant of Trispicen is the same as that found for the Trispicen methyl derivative (log K_{ZnL} = 13.3) [59].

Table 1. Stability constants (log K_{ZnL}) for complexes with TPEN, Bispicen, BispicenA, Trispicen and TrispicenA

	TPEN[56]	Bispicen[41]	BispicenA	Trispicen	TrispicenA
Log K_{ML} exp	15.7	11.4			
Log K_{ML} calc	15.5	10.9	13.3	13.3	15.6

We studied the complexation reaction of TrispicenA and TrispicenDAla with Zn^{2+} by ^{1}H NMR. Similar studies have already been reported for Bispicen [40], Trispicen [42], and TPEN [60]. Zn^{2+} coordination with pyridine and amino nitrogen atoms has been proposed [61–63].

A solution of TrispicenA in D_2O at pH = 7.4 was titrated with a $ZnCl_2$ solution (Fig 2). When zinc was added, new signals representing a Zn complex species appeared in the spectra together with the signals of the free ligand (Fig. 2). In the aromatic region, new signals appeared at 8.74, 8.08, 7.99, and 7.81 ppm. In the aliphatic region, new signals at 4.02, 3.9, 1.10, and 1.02 ppm appeared. Spectra changed up to a Zn/L ratio = 1. 2D experiments were conducted on the solution of $[Zn(TrispicenA)]^{+}$ species (Fig. S14-S18) and the full ^{1}H and ^{13}C NMR spectra assignments was obtained (Table S1). The complexation reduced conformational freedom of the ligand. In the spectrum, twelve signals due

to the protons of the pyridine rings appeared. The complexation produced a shift of the signals, and all methylene protons appeared at different chemical shifts because of the coordination of the metal. Similarly, in the ^{13}C NMR spectrum (Fig. S14), pyridine rings showed 15 signals, in addition to 6 signals due to methylene carbons and CO at 178 ppm.

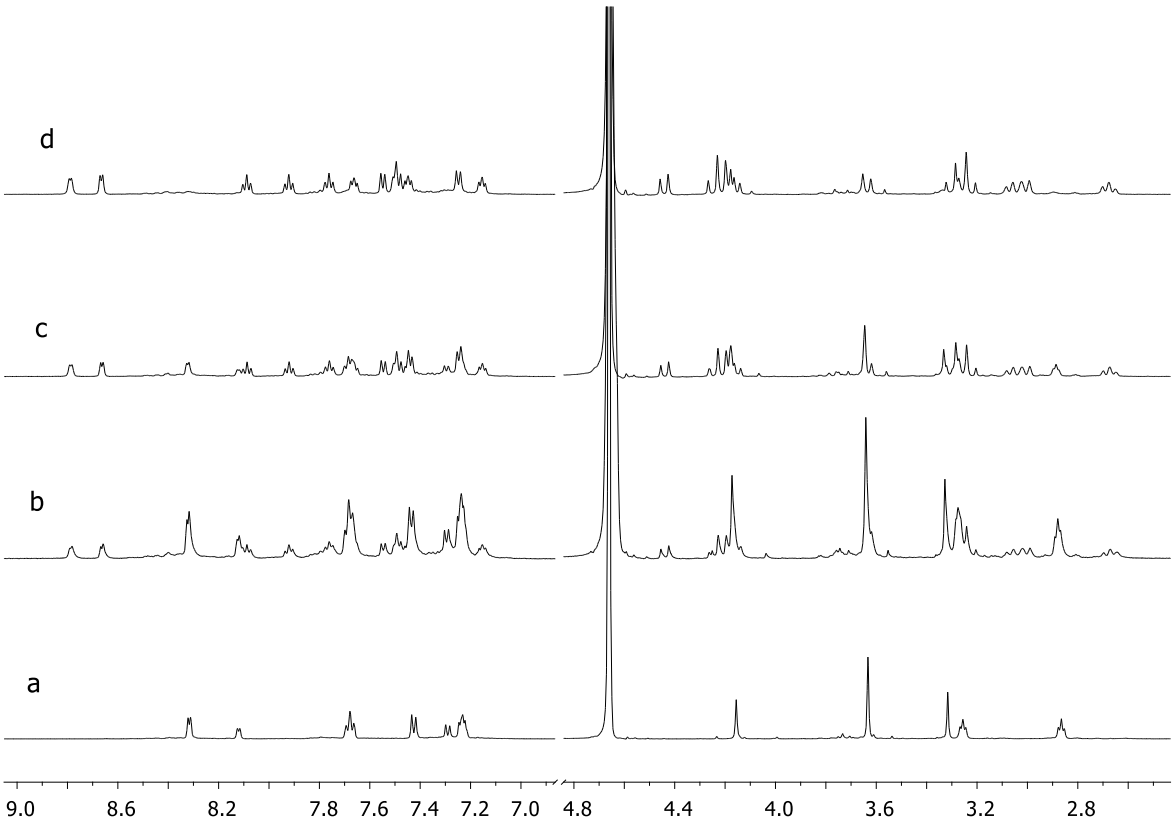

Figure 2. ^{1}H NMR spectra in *D_2O (pD 7.4): a) TrispicenA alone; b) TrispicenA/Zn 1:0.3; c) TrispicenA/Zn 1:0.5; d) TrispicenA/Zn 1:1.*

NOESY spectra of $[Zn(TrispicenA)]^{+}$ (Fig S18) showed the correlations among the protons of the different pyridine rings in the zinc complex.

Spectra suggested the complexation of Zn^{2+} with the pyridine and ethylenediamine nitrogens and COO^- with Zn ion in an octahedral environment, as found in the solid-state [63].

TripicenDAla was also titrated with $ZnCl_2$ in D_2O. The shift of the signals of the D-Ala moiety suggested the involvement of the chain in the coordination of Zn^{2+}. In this case, the spectra showed broad signals, probably due to the presence of an equilibrium between complex species (Fig S19). The spectra changed up to Zn/L = 1. In addition to the coordination of pyridine and amine groups, the coordination of amide bonds may be suggested as found for zinc with peptides [33]. A stability constant value of Zn^{2+} complex can be estimated higher than Trispicen and similar to TrispicenA.

Competition experiments with TPEN were performed by ^1H NMR spectroscopy (Fig. 3). TPEN is a commonly used Zn^{2+} chelator in cells [64]. Preliminarily, NMR spectra of $[Zn(TPEN)]^{2+}$ complexes were acquired at different Zn/TPEN molar ratios. When zinc was added, new signals of the zinc complex appeared in the spectra. Four signals appeared at 8.13, 7.87, 7.38 and 7.34 ppm, while the signals of the ligand protons disappeared. The two doublets at 4.26 ppm and 4.01 ppm due to the zinc complex protons also appeared (Fig. 3e).

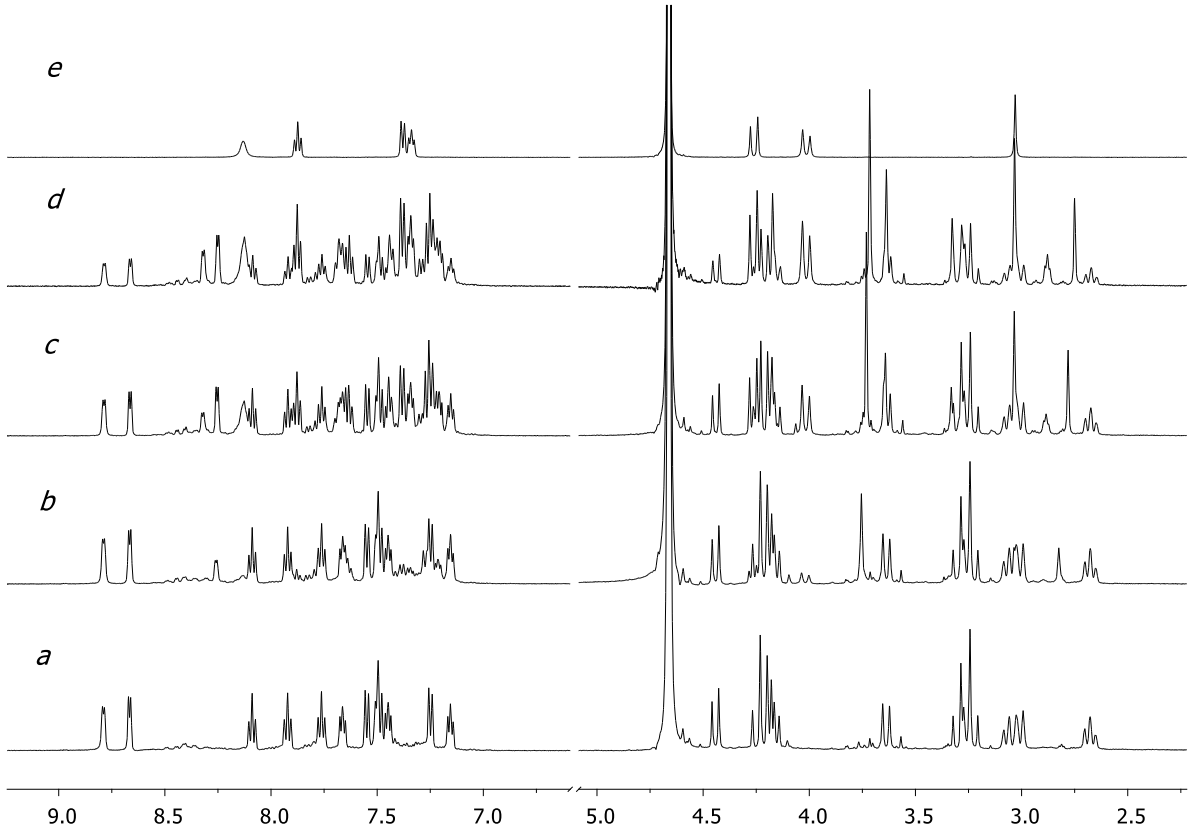

Figure 3. ^{1}H *NMR spectra* in D_2O *(pD 7.4*): *a)* $[Zn(TrispicenA)]^{+}$ *alone; b)* $[Zn(TrispicenA)]^{+}$*/TPEN 3:1; c)* $[Zn(TrispicenA)]^{+}$*/TPEN 2:1; d)* $[Zn(TrispicenA)]^{+}$ */TPEN 1:1. e)* $[Zn(TPEN)]^{2+}$

TPEN was added to the $[Zn(TrispicenA)]^{+}$ solution. NMR signals due to $[Zn(TrispicenA)]^{+}$ did not change, suggesting that $[Zn(TrispicenA)]^{+}$ did not lose Zn^{2+} when small amounts of TPEN were added. We found that NMR spectra showed the formation of the $Zn(TPEN)^{2+}$ complex when the Zn/TPEN/TrispicenA ratio was 2/1/2 two hours after TPEN addition. The signals of $[Zn(TrispicenA)]^{+}$ decreased but did not disappear in the spectra when Zn/TPEN/TrispicenA were 1/1/1 (Fig. 3). NMR data indicated that TrispicenA bonded zinc ion with a stability constant similar to that of TPEN. NMR data were used to compare the stability of the two complexes.

Considering the reaction

$[Zn(TrispicenA)]^{+} + TPEN = [Zn(TPEN)]^{2+} + TrispicenA^{-}$

$K = [ZnTPEN][TrispicenA]/[ZnTrispicenA][TPEN] = K_{ZnTPEN}/K_{ZnTrispicenA}$

[ZnTPEN]/[TPEN] and [TrispicenA]/[ZnTrispicen] can be determined from the integration of the signals in the NMR spectra. From the spectra $K_{ZnTPEN}/K_{ZnTrispicenA} \approx 1.3$. This data is consistent with the calculated stability constant value for Zn-TrispicenA.

3.3 Toxicity in human cells

All the ligands were tested for their cytotoxicity in two normal peripheral blood lymphocytes. IC_{50} values are reported in Table 2. Four of 6 compounds showed a pharmacologically significant antiproliferative activity with IC_{50} ranging from 4.91±0.12 μM to 17.8±4.1 μM. TPEN toxicity values has been reported in cancer cells and the zinc chelation was proposed as the prevailing cytotoxicity mechanism [65–67]. Bispicen showed lower toxicity than Trispicen and TrispicenA.

BispicenA and TrispicenDala showed no activity on stimulated PBLs (the value chosen to arbitrarily define an efficient *in vitro* antiproliferative activity was 30 μM). Interestingly the functionalization with the peptide D-Ala-D-Ala-D-AlaOCH_3 reduced the antiproliferative activity of the ligand compared to TPEN or TrispicenA. The peptide residue may modify the lipophilicity of the ligand and reduce its toxicity as recently reported for similar derivatives [68].

Table 2. Concentrations (μM) of TPEN, Trispicen, TrispicenA, Bispicen, BispicenA, and TrispicenDala inhibiting 50% proliferation (IC_{50}) of normal PBL stimulated with PHA and IL-2.

Ligand	PBL A	PBL B
TPEN	5.08±0.23	5.54±0.41
TrispicenDAla	>85	>100
Trispicen	4.91±0.12	5.39±0.21
TrispicenA	5.17±0.41	5.39±0.20
Bispicen	13.9±1.7	17.8±4.1
BispicenA	>100	>100

The values express the mean±SD of 4 data.

3.4 In vitro microbiological results

Evaluation of antibiotic MIC values and antimicrobial activity of zinc ligands

Bacterial isolates were resistant to several antibiotics, demonstrating a multidrug-resistant profile. They showed high MIC values for the majority of tested antimicrobials (Table S2). As expected, all isolates were resistant to carbapenems (ertapenem, imipenem, and meropenem). Low MIC values were also observed for trimethoprim/sulfamethoxazole *in S. maltophilia*, for colistin in NDM-producing *K. pneumoniae*, and amikacin in VIM-1 producing *E. cloacae* (Table S2). MIC values were high for MEM in NDM-producing *K. pneumoniae* (128 mg/L) and *S. maltophilia* (256 mg/L), while in VIM-1 producing *E. cloacae* the MIC value was 16 mg/L (Table 3).

Standard broth microdilution of polypyridine ligands showed high MIC values of TPEN and TrispicenDAla (256 mg/L and >256 mg/L, respectively) for all strains studied, confirming the absence of antimicrobial activity against these strains. High MIC values were also observed for TrispicenA (512 mg/L) and BispicenA (>512 mg/L) in all strains, Trispicen (256 mg/L) and Bispicen (64 mg/L) in NDM-producing *K. pneumoniae*, and VIM-producing *E. cloacae*. In *S. maltophilia*, Trispicen and Bispicen MIC values were 64 mg/L and 32 mg/L, respectively (Table 3). Notably, these compounds showed higher antimicrobial activity than MEM (256 mg/L) alone against *S. maltophilia*.

Table 3. *Antimicrobial activity of Meropenem (MEM), TPEN, Bispicen, BispicenA, TrispicenDAla, Trispicen and TrispicenA*

Microorganisms	**MIC values (mg/L)**						
	MEM	**TPEN**	**TrispicenDAla**	**TrispicenA**	**BispicenA**	**Trispicen**	**Bispicen**
K. pneumoniae NDM-1	128	256	>256	512	>512	256	64

S. maltophilia L1	256	256	>256	512	>512	64	32
E. cloacae VIM-1	16	256	>256	512	>512	256	64

Synergy tests

Double-disk diffusion tests showed alterations in disk inhibition rings for combinations of TPEN-MEM and TrispicenA-MEM against all tested isolates (Fig.4). No alteration was observed for combinations of BispicenA-MEM. For the combination of TrispicenDAla-MEM, synergy was observed only in VIM-producing *E. cloacae*. Alterations in disk inhibition rings were observed for the combination of Trispicen-MEM and Bispicen-MEM in NDM-producing *K. pneumoniae* and VIM-producing *E. cloacae*. Synergistic activities between chelators and MEM against VIM-producing *E. cloacae* are shown in Figure 4.

Table 4 – Synergistic interaction for TPEN, Bispicen, BispicenA, Trispicen, TrispicenDAla and TrispicenA with Meropenem (MEM) determined by FICI versus NDM-1 producing *K. pneumoniae*, L1-producing *S. maltophilia* and VIM-1 producing *E. cloacae* clinical strains.

Checkerboard microdilution assays and synergistic concentrations							
Compound: TPEN				**Compound: TrispicenDAla**			
Microorganisms	**MEM in combination (mg/L)**	**Compound concentration (mg/ L)**	**FICI**	**Microorganisms**	**MEM in combination (mg/L)**	**Compound concentration (mg/ L)**	**FICI**
K. pneumoniae (NDM-1*)*	32 16 16 8 4 2	16 16 32 32 32 32	0.31 0.18 0.25 0.18 0.15 0.14	*K. pneumoniae* (NDM-1*)*	32 16 8 4 2	32 32 32 32 32	0.31 0.18 0.12 0.09 0.07
S. maltophilia (L1)	64 32 16 8	16 32 32 32	0.31 0.25 0.18 0.15	*S. maltophilia* (L1)	64 32	64 128	0.37 0.37

	4 2	32 32	0.14 0.13				
E. cloacae (VIM-1)	4 2	16 16	0.31 0.18	*E. cloacae* (VIM-1)	4 2	32 32	0.31 0.18
	Compound: TrispicenA				**Compound: Trispicen**		
Microorganisms	**MEM in combination (mg/L)**	**Compound concentration (mg/ L)**	**FICI**	**Microorganisms**	**MEM in combination (mg/L)**	**Compound concentration (mg/ L)**	**FICI**
K. pneumoniae (NDM-1*)*	32 16 8 4 2	16 16 16 16 16	0.28 0.16 0.09 0.06 0.05	*K. pneumoniae* (NDM-1*)*	32 16 8 4 2	16 16 16 16 16	0.31 0.19 0.12 0.09 0.07
S. maltophilia (L1)	64 32 16 16 8 8 4 2	32 32 32 64 64 128 128 128	0.31 0.19 0.12 0.19 0.16 0.28 0.26 0.26	*S. maltophilia* (L1)	64 32	16 16	0.50 0.37
E. cloacae (VIM-1)	4 2 1 0.5 0.5 0.25	16 16 16 16 32 32	0.28 0.16 0.09 0.06 0.09 0.08	*E. cloacae* (VIM-1)	4 2 1 1 0.5 0.25	8 8 8 16 16 16	0.28 0.16 0.09 0.12 0.09 0.08
	Compound: Bispicen				**Compound: BispicenA**		
Microorganisms	**MEM in combination (mg/L)**	**Compound concentration (mg/ L)**	**FICI**	**Microorganisms**	**MEM in combination (mg/L)**	**Compound concentration (mg/ L)**	**FICI**
K. pneumoniae (NDM-1*)*	32 16 8 4 2	16 16 16 16 16	0.50 0.37 0.31 0.28 0.26	*K. pneumoniae* (NDM-1*)*	-	-	NI
S. maltophilia (L1)	-	-	NI	*S. maltophilia* (L1)	-	-	NI
E. cloacae (VIM-1)	4 2 1 0.5 0.25	16 16 16 16 16	0.5 0.37 0.31 0.28 0.26	*E. cloacae* (VIM-1)	-	-	NI

NI = no synergistic interaction, FICI = FIC_A + FIC_B, where FIC_A = MIC of drug A in combination/MIC of drug A alone, and FIC_B = MIC of drug B in combination/MIC of drug B alone

Checkerboard analysis (Table 4) confirmed the presence of synergistic interactions demonstrated by double-disk diffusion tests and confirmed the absence of interaction for BispicenA-MEM in all strains

and Bispicen-MEM in *S. maltophilia*. Moreover, the analysis also showed the presence of synergistic interactions for a combination of TrispicenDAla-MEM in *S. maltophilia* and NDM-producing *K. pneumoniae* and Trispicen-MEM in *S. maltophilia*.

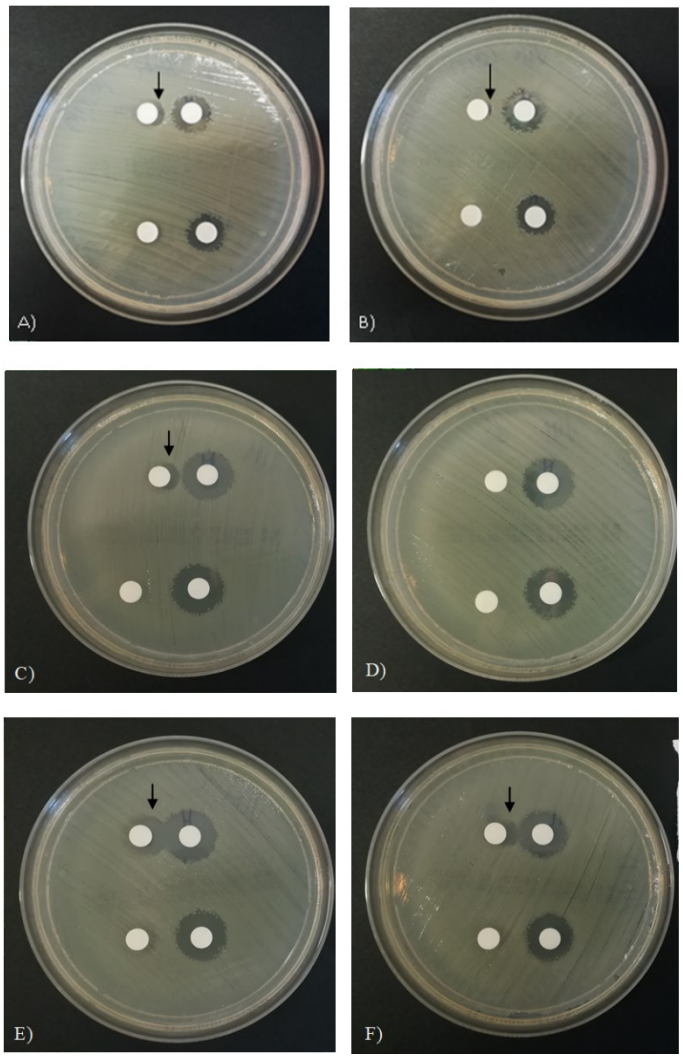

Figure 4. *Synergistic activity evaluated by double-disk diffusion test between MEM and A) TPEN, B) TrispicenDAla, C) TrispicenA, D) BispicenA, E) Trispicen, F) Bispicen, against VIM-producing E. cloacae (15 and 20 mm center-center). Arrows indicate alterations of the inhibition zone around the discs.*

Overall, we found a synergistic activity between MEM and TrispicenDAla, TrispicenA, Trispicen, and TPEN against all tested isolates. In contrast, the combination of Bispicen-MEM was synergistic only against NDM-producing *K. pneumoniae* and VIM-producing *E. cloacae*.

The synergistic activity suggests that the ligands can cross the Gram-negative cell wall, enter the periplasm where the MBLs are located [23], and inhibit MBL activity.

MIC values of MEM in the presence of the ligands (at approximately 30 μM) decreased compared to MEM alone. In particular, several synergistic interactions restored the antimicrobial activity of MEM, providing a decrease of MIC values 8- to 128-fold, meaningfully below the susceptibility breakpoint (2 mg/L). Particularly, Trispicen and TrispicenA were the most effective ligands, providing, at 16–32 mg/L, a MIC value for MEM of 0.25 mg/L compared to 32 mg/L of MEM alone in *E. cloacae*; Trispicen and TrispicenA also showed very low FICIs (<0.1).

Unlike some data reported elsewhere for other classes of ligands [45], we did not find any strong evidence of the advantage of the carboxylate groups.

Bispicen did not show any synergistic activity in L1-producing *S. maltophilia*, which has a relatively impermeable cell membrane [69], which potentially explains the lower effect of TrispicenA on *S. maltophilia* and the lack of synergistic activity of Bispicen. Furthermore, efflux mechanisms may not be excluded. The lack of synergy can also be related to the different MBL enzyme of *S. maltophilia*. Notably, L1 has a higher affinity for zinc of other MBLs, and it is also active as a Zn1 enzyme [8]. Indeed, Bispicen is the ligand with the lowest stability constant among the tested ligands.

In VIM-1 and NDM-1 producing bacteria, we did not observe a strong dependence of the synergistic effect of the ligand on the stability constant of the zinc complex. Bispicen with $\log K_{ZnL} = 11.4$ and Trispicen with $\log K_{ZnL} = 13.3$ are effective in restoring MEM activity in these bacteria.

The moiety D-Ala-D-Ala-D-AlaOCH_3 in TrispicenDAla did not improve the synergistic activity in the studied bacteria in comparison to trispicenA. TrispicenDAla showed a similar effect of TPEN in *K. pneumoniae* and a slightly less effect in other clinical strains. Interestingly, TrispicenDAla was not

toxic in human cells and this data can be important for the selection of the ligand to study *in vivo* experiments.

Microbiological data suggest that zinc polypyridine ligands are potential candidates as adjuvants in antibiotic therapy with meropenem, for infections caused by MBL producers. Nevertheless, cytotoxicity of the compounds suggest that a further investigation is needed on these class of ligands *in vivo* models for the selection of the best ligands. However, the functionalization of the ligands is a promising strategy to modulate the toxicity as reported for other ligands. Although the cytotoxic of TPEN and other similar ligands is known, they have been used in vivo as potential therapeutics. TPEN has been administrated as an antiasthma agent and an antifungal agent *in vivo* [34,70] and to protect against botulinum neurotoxin in mice [71]. Bispicen has also been evaluated *in vivo* for its ability to form zinc complexes as a potential inhibitor of p53-dependent apoptotic pathways [72]. Cytotoxicity of a drug may not necessarily be a limitation to the clinical application. However, risk/benefit should be carefully evaluated especially for life-threatening disease and *in vivo* studied are important to highlight the potential of polypyridyl ligands.

Conclusion

Few therapeutic options are currently available for the treatment of infections caused by metallo-beta-lactamase (MBL) producers. Recent research suggests that Zn^{2+} ligands appear to be promising as potential adjuvants of beta-lactam antibiotics. Metal chelation therapy was first proposed approximately 50 years ago for human pathologies produced by metal overload and has a long clinical history [73]. 8-hydroxyquinolines, penicillamine, deferiprone, and deferoxamine are also used clinically for chronic diseases [74–76]. The use of metal ligands to inhibit metalloenzymes has also

been studied and applied [77]. Furthermore, zinc chelators have been used in humans therapeutically, especially for neurological disorders [78,79].

In this *in vitro* study, we explored the effect of a family of polypyridine ligands in Gram-negative bacteria producing VIM-1, NDM-1 (B1 MBLs), and L1(B3 MBL). We found that TrispicenDAla, TrispicenA, Trispicen, Bispicen, and TPEN at μM concentrations were effective in restoring the antimicrobial activity of meropenem, providing an 8- to 128-fold decrease of the initial MIC values observed for tested isolates. In particular, Trispicen and TrispicenA were the most effective ligands in *E. cloacae*. Interestingly the functionalization of Trispicen functionalized with D-Ala-DAla-DAlaOCH3 peptide reduced the cytotoxicity of Trispicen moiety in human cells.

Provided they are sufficiently effective and safe in the different phases of clinical development, suitable functionalized zinc chelators based on pyridine rings combined with β-lactams may become an important last resort or perhaps a frontline therapeutic option for infections caused by MBL producers.

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Abbreviations

BLAST	Basic Local Alignment Search Tool
Bispicen	N,N'-bis(2-pyridylmethyl)-ethylenediamine
BispicenA	N,N'-bis(2-pyridylmethyl)-ethylenediamine-N-acetic acid
CAMH	Cation-Adjusted Mueller-Hinton
CFU	Colony-Forming Unit
CLSI	Clinical and Laboratory Standards Institute
EDC	1-ethyl-3 (3-dimethylamino propyl) carbodiimide
EDTA	EthyleneDiamineTetraAcetic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FICI	Fractional Inhibitory Concentration Index
HOBt	1-Hydroxybenzotriazole
IC50	Concentrations inhibiting 50% proliferation
ICU	Intensive Care Unit
IMP	Imipenemase
IL2	Interleukin-2
MBL	Metallo-β-Lactamases
McF	McFarland
MEM	Meropenem
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MTT	3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide
NDM	New Delhi Metallo-β-lactamase

PBL	Peripheral Blood Lymphocytes
PHA	Phytohemoagglutinin
TPEN	N,N,N′,N’-tetrakis (2- pyridymethyl)ethylenediamine
Trispicen	N,N,N’-tris(2-pyridylmethyl)-ethylenediamine
TrispicenA	N,N,N’-tris(2-pyridylmethyl)-ethylenediamine-N’-acetic acid
VIM	Verona integron-encoded metallo-β-lactamase