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Synthesis and biological evaluation of zinc chelating compounds as metallo- β -lactamase inhibitors†

Geir Kildahl-Andersen,^a Christian Schnaars,^a Anthony Prandina,^a Sylvie Radix, ^b Marc Le Borgne, ^b Lars Petter Jordheim,^c Tor Gjen, ^d Adriana Magalhes Santos Andresen,^d Silje Lauksund,^{ef} Christopher Frhlich,^{eg} rjan Samuelson, ^{ef} Pl Rongved^a and Ove Alexander Hgmoen strand ^{*a}

The syntheses of metallo- β -lactamase inhibitors comprising chelating moieties, with varying zinc affinities, and peptides partly inspired from bacterial peptide sequences, have been undertaken. The zinc chelator strength was varied using the following chelators, arranged in order of ascending binding affinity: dipicolylamine (DPA, tridentate), dipicolyl-1,2,3-triazolylmethylamine (DPTA, tetradentate) dipicolyl ethylenediamine (DPED, tetradentate) and trispicolyl ethylenediamine (TPED, pentadentate). The chosen peptides were mainly based on the known sequence of the C-terminus of the bacterial peptidoglycan precursors. Biological evaluation on clinical bacterial isolates, harbouring either the NDM-1 or VIM-2 metallo- β -lactamase, showed a clear relationship between the zinc chelator strength and restoration of meropenem activity. However, evaluation of toxicity on different cancer cell lines demonstrated a similar trend, and thus inclusion of the bacterial peptides did possess rather high toxicity towards eukaryotic cells.

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Introduction

Infectious diseases are a leading cause of death worldwide, including more than half of all child mortality under the age of five.¹ Currently, there is a serious concern regarding new and re-emerging infectious diseases, for which effective therapies are lacking.^{2–4} The emergence of multidrug-resistant (MDR) strains of common Gram-negative pathogens, such as

Escherichia coli, are of increased concern. Extensively drug-resistant or pandrug-resistant are now commonly used terms to describe clinically important isolates of *Pseudomonas aeruginosa* and *Enterobacteriales* that are resistant to virtually all clinically available antibiotic drugs.⁵

Metallo- β -lactamases (MBLs) are a group of hydrolytic enzymes that break down antibiotics of the β -lactam class, including penicillins, cephalosporins and carbapenems. Carbapenems is a class of last-resort antibiotics, and resistance towards these drugs are threatening our ability to treat serious infections. Worryingly, the MBLs are already widespread, and their occurrence is increasing.⁶ There are currently no MBL inhibitors on the market, and a successful inhibitor could extend the therapeutic lifetime of carbapenems. Even newly approved β -lactamase inhibitors, such as avibactam and vaborbactam, do not target MBLs.

MBLs are zinc dependent enzymes, carrying one or two zinc atoms within their active site. Several zinc-chelating compounds have inhibitory activity towards MBLs and some of these inhibitors have even reached clinical trials.^{7–11} By using chelators with moderately soft donor atoms, nitrogen in our case, selectivity towards rather soft metal ions, such as Zn^{2+} , can be achieved. Harder metal ions of biological relevance, such as Ca^{2+} and Mg^{2+} , are better chelated by oxygen donor atoms.¹² However, many zinc-chelating compounds suffer from low selectivity, low potency and/or high eukaryotic toxicity. We have earlier studied the effect of tris-

^a Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, PO Box 1068 Blindern, 0316 Oslo, Norway.

E-mail: o.a.h.astrand@farmasi.uio.no; Tel: +47 22854478

^b EA 4446 Bioactive Molecules and Medicinal Chemistry, Facult de Pharmacie – ISPB, Universit de Lyon, Universit Lyon 1, SFR Sant Lyon-Est CNRS UMS3453 – INSERM US7, F-69373, Lyon cedex 08, France^c Universit de Lyon, Universit Claude Bernard Lyon 1, INSERM 1052, CNRS UMR5286, Centre Lon Brard, Centre de Recherche en Cancrologie de Lyon, 69008 Lyon, France^d Centre for Integrative Microbial Evolution (CIME), Faculty of Mathematics and Natural Sciences, University of Oslo, Blindern, Oslo, Norway^e Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, 9038 Troms, Norway^f Department of Pharmacy, UiT – The Arctic University of Norway, 9037 Troms, Norway^g The Norwegian Structural Biology Centre (NorStruct), Department of Chemistry, UiT The Arctic University of Norway, 9037 Troms, Norway

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picolylamine (TPA) derivatives as MBL inhibitors.¹³ In this work, we chose to vary the zinc binding strength by using *N,N*-dipicolylamine (DPA),¹⁴ *N,N*-dipicolyl-*N*-triazolylmethylamine (DPTA), *N,N*-dipicolyl ethylenediamine (DPED) and trispicolyl ethylenediamine (TPED) as starting points. The chelators were attached to various peptides, using peptide coupling reagents, to produce chelator-peptide constructs as putative MBL inhibitors.

We hypothesized that chelators attached to the bacterial peptide fragment D-Ala-D-Ala, could achieve affinity for bacterial penicillin-binding proteins (PBP). In addition, we also explored several other small peptides that could have an affinity to bacterial proteins such as *meso*-diaminopimelic acid (*m*-DAP)-D-Ala-D-Ala, as well as considering other attachment points (Fig. 1a). For compounds of this type, we chose to use L-Lys as a substitute for *m*-DAP, eliminating one carboxylic acid in *m*-DAP for ease of synthesis, but preserving the attachment points A and B (Fig. 1a). One can also imagine attaching the chelator at point D (Fig. 1a), as evidence from the literature suggests that compounds with larger substituents than methyl on the ultimate D-amino acid are acceptable substrates for the PBPs.^{15–17} We also created boronic acid-containing transition state intermediates (BATSI), knowing that the last D-Ala unit is cleaved by serine catalysis in the PBPs.¹⁸ For this reason, we prepared chelating compounds with L-Lys-boroglycine as peptides. Compounds of this type

could potentially target either PBPs or even serine β -lactamases, being aware that these proteins share an evolutionary past.¹⁹ Also, peptidomimetic boron-containing compounds binding to or inhibiting PBPs are well-known.^{20,21} As it would later turn out, current evidence suggests this reasoning to be too simplistic and such dual activity cannot be expected,²² but we have included such compounds in this study for completeness. In a recent report, we studied the linkers between the chelators and the peptides,²³ but found that the biological effect was mostly due to the strength of the zinc chelator. We, therefore, have not performed a comprehensive investigation of all possible chelator-linker-peptide combinations for the chelators and peptides in the present work.

Results and discussion

Synthesis of compounds

The full details of the syntheses are provided in the ESI.† The final assembly of the majority of the peptide-chelator compounds followed a common strategy. The part containing the chelating group was prepared either as a free acid (1–4), or as a free amine (5–7). The acids 1–4 were attached, using standard peptide coupling methodology, to methyl esters of dipeptides with free amine groups, available as either HCl or TFA salts, whereas the amines 5–7 were coupled to *N*-Boc-protected dipeptides containing a free acid. Then, coupling products were deprotected in the final step, to yield the desired products 8–20 (Scheme 1). We included the previously reported compound 15 here for completeness.²³ Note that methyl esters have been preserved in 8–14, as the corresponding free acids have performed poorly in biological assays (results not shown). All products 8–20 show an attachment of chelator moiety either at point C or point E (Fig. 1a).

Assembly of the chelating groups (1–7), from commercially available precursors, was achieved as outlined in Scheme 2. The DPA amine 5 was prepared from *N*-Boc-protected 4-(2-aminoethyl)aniline as previously described.²³ Quantitative conversion to the acid 1 was achieved by reaction with succinic anhydride. For the dipicolyl 1,2,3-triazolylmethylamine chelators (DPTA), the amine 6 was obtained by copper(i)-mediated azide-alkyne cycloaddition (CuAAC) between *tert*-butyl 4-azidophenethylcarbamate (21),²⁴ and *N*-propargyl-di(2-picolyl)amine (22),²⁵ followed by deprotection with HCl. Preparation of the acid 2 from ethyl (4-bromophenyl)acetate (23) required conversion to the corresponding azide 24,²⁶ and subsequent CuAAC reaction with propargyl alcohol to afford the 1,2,3-triazole containing alcohol 25. Assembly of the chelator was then accomplished by converting the alcohol to the corresponding mesylate 26, followed by nucleophilic displacement with DPA to form the ethyl ester 27. *In situ* hydrolysis of 27, using lithium hydroxide, afforded 2, which was directly used in peptide coupling reactions. The TPED chelators 3 and 7 were synthesized in two steps, from *N,N,N'*-tris-pyridin-2-ylmethylethane-1,2-diamine (28), available following literature procedures.²⁷

Fig. 1 a) Potential chelator attachment points A–E based on tripeptide *m*-DAP-D-Ala-D-Ala. See main text for details. b) Chelators included in this study: dipicolylamine (DPA), dipicolyl 1,2,3-triazolylmethylamine (DPTA), dipicolyl ethylenediamine (DPED) and trispicolyl ethylenediamine (TPED).

Scheme 1 Preparation of products 8–20. Reagents: a. D-alanyl-D-alanine methyl ester hydrochloride (for 8, 10, 12, 14) or glycylglycine methyl ester hydrochloride (for 9), HATU, *N*-methylmorpholine, DMF. Compound 11: glycylglycine methyl ester hydrochloride, EDC·HCl, HOAt, *N*-methylmorpholine, ethanol/DMF; compound 13: L-alanyl-L-alanine methyl ester hydrochloride, HATU, *N*-methylmorpholine, DMF; b. 1) *N*-Boc-D-alanyl-D-alanoic acid, HATU, *N*-methylmorpholine, DCM, 2) TFA, DCM; c. 1) *N*-Boc-glycyl-glycine (for 16) or *N*-Boc-D-alanyl-D-alanoic acid (for 17), HATU, *N*-methylmorpholine, DMF, 2) TFA, DCM; d. 1) *N*-Boc-glycyl-glycine (for 18), *N*-Boc-D-alanyl-D-alanoic acid (for 19) or *N*-Boc-alanyl-alanoic acid (for 20), HATU, *N*-methylmorpholine or DIPEA (20 only), DMF or ACN (18 only), 2) TFA, DCM.

First, the amine was alkylated with either 29 or 31, in order to form the protected intermediates 30 and 32, which were subsequently deprotected to yield free acid (3) or free amine (7). All chelators 1–3 and 5–7 contain a phenyl ring in the link to the peptide. For the DPED series, we decided to circumvent the linker and directly couple the peptide to the acid 4, which was synthesized with only minor deviations from previously published methods.²⁸

In addition to the syntheses outlined in Schemes 1 and 2, some compounds were prepared using other routes. The DPA analogue 33, in which the linker was omitted, was obtained in two steps *via* the chloride 34, prepared directly from the H-D-Ala-D-Ala-OMe dipeptide (Scheme 3).

Finally, a majority of the DPTA compounds was prepared from azido peptides, by the CuAAC reaction with *N*-propargyl-di(2-picolyl)amine (22) as either the final step in the case of 35–43 (38 excepted, Scheme 4), or the penultimate step in the case of the potential BATSI compounds 44 and 45

(Scheme 5). In this manner, we were thus able to provide compounds corresponding to attachment of chelator in position A (40, 41, 45), position B (42–44) and position D (38–39), (*cf.* Fig. 1a). The rather elaborate syntheses of the azides are described in detail in the ESI.†

Because the CuAAC reaction in these reactions created a powerful chelator, we attempted different work-up schemes in order to remove the copper from the reaction. No clear conclusion was reached whether one work-flow performed better than the other with regard to yield. Work-up with extraction from basic aqueous phases containing EDTA, in order to chelate Cu⁺/Cu²⁺,²⁹ gave, for instance, 62% yield in the preparation of Fmoc-protected 38, whereas only 27% yield of 48 was obtained. Solubility of the product in the aqueous phase was detrimental, and the subsequent purification on alumina column often yielded poorer separation than desired. An alternative work-flow, in which Chelex 100 was used for complexing Cu⁺/Cu²⁺, followed by purification of product

Scheme 2 Synthesis of precursors 1–3, 6 and 7.

on reversed-phase SPE material, was employed in the synthesis of 37, yielded also 62% of the expected product. Both methods were attempted for compounds 35 and 36, giving comparative yields in both cases, thus it seems that the choice can be a matter of convenience. More details can be found in the experimental description.

MIC determination in combination with meropenem

All the prepared compounds, as well as some of the intermediates, were tested in combination with the carbapenem meropenem against two different clinical isolates of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* expressing the

VIM-2 (Verona Integron-Mediated metallo- β -lactamase 2) and NDM-1 (New Delhi metallo- β -lactamase 1) metallo- β -lactamases, respectively (Table 1). The compounds were screened at a fixed concentration of either 50 or 125 μM , and MIC for meropenem was determined. In addition, a selection of the most active compounds was tested at reduced concentrations, interspersed with some of the less active compounds comprising the DPTA chelator (Table 2).

We have previously reported results on how variations in the linker between peptide fragment and zinc chelator influenced meropenem MIC restoration and eukaryotic toxicity.^{13,23} In this present paper, we have explored how variations in the peptide, the corresponding attachment point, as

Scheme 3 Preparation of compound 33, omitting the linker.

Scheme 4 Preparation of compounds 35–43 by way of CuAAC triazole formation.

well as differences in chelator strength, influenced the effect on meropenem MIC and toxicity of these compounds. As

Scheme 5 Preparation of potential BATSI compounds 44 and 45. Reagents: a. copper(II)sulfate, sodium-(+)-ascorbate, 22, water, *tert*-butanol; b. 1) LiOH hydrate, water, THF, 2) neutralize with 1 M HCl, 3) bis-trimethylsilyl-aminoethaneboronate pinacol ester, HATU, *N*-methylmorpholine, water, DMF.

shown in Tables 1 and 2, data suggest a strong dependence upon the strength of the chelator for reducing the MIC of meropenem. It should also be noted that none of the reported compounds displayed any antibiotic effect on their own (data shown in ESI,[†] Table S1). This includes the boronic acid containing compounds 44 and 45, despite being modelled from the bacterial peptide in the peptidoglycan cell wall. Overall, compound activities seems to follow the chelating strength. For our compounds, the order of chelating strength would be DPA @ DPTA @ DPED $\approx$ TPED @ TPEN. The DPA chelator performs weaker than those containing DPTA, whereas compounds with DPED and TPED both seem to perform equally well based on our data, and much better than DPTA. This also clearly illustrates the rather low chelating strength of N^3 in 1*H*-1,2,3-triazoles, compared to the aliphatic amine nitrogen present in DPED. The influence of the chelator strength was tested at low inhibitor concentration (Table 2). For the DPTA compounds, the inhibitory effect at 31 μ M is almost completely obliterated. Data also suggest no

Table 1 MIC values of meropenem (MEM) in combination with the prepared chelators against *P. aeruginosa* and *K. pneumoniae* harbouring VIM-2 and NDM-1, respectively. Assays were performed in duplicate, with at least three separate experiments. All prepared chelators were tested at a fixed concentration of 125 μM , except for compounds marked with an asterisk (*), which were tested at 50 μM . The MIC values of meropenem against both strains were determined to be 32–64 mg L^{-1} . TPEN = *N,N,N',N'*-tetrapicolyl ethylenediamine

Compound	MIC MEM (mg L^{-1})	
	<i>P. aeruginosa</i> VIM-2	<i>K. pneumoniae</i> NDM-1
DPA class		
8	32	8–16
15	16	8
33	16	8–16
DPTA class		
9	8*	4*
10	8*	4*
16	8*	4*
17	4*	4*
27	16*	4*
35	2	<0.5
36	8	2
37	4	2
38	Precipitated	Precipitated
39	4	1
40	16*	4*
41	16*	8*
42	32*	8*
43	32*	8*
44	2	0.5
45	2	0.5
DPED class		
14	1*	0.125*
TPED class		
11	1	0.125
12	1	0.125
13	1	0.125
18	Precipitated	Precipitated
19	2	0.125
20	2	0.125
28	0.5	<0.25
TPEN	1	<0.5

Table 2 MIC values of MEM (mg L^{-1}), in combination with lower concentrations of inhibitor (31 and 16 μM), against *P. aeruginosa* and *K. pneumoniae* harbouring VIM-2 and NDM-1, respectively. The MIC values of meropenem against both strains were determined to be 32–64 mg L^{-1}

Compound	<i>P. aeruginosa</i> VIM-2		<i>K. pneumoniae</i> NDM-1	
	31 μM	16 μM	31 μM	16 μM
35	64	32	16	32
36	16	32	8	16
44	16	32	8	16
45	32	32	8	16
14	1–2	32	0.125	16
11	2	32	2	16
12	4	32	0.125	16
13	1	32	0.125	8
19	2	32	0.125	8
20	4	32	0.125	16
28	1	32	0.125	8

active role of the peptide for the biological effect. Neither change of configuration from D-amino acids to L-amino acids, change of attachment points nor addition of boronic acids seem to be of consequence. It should also be noted that testing of compound 44 and 45 against strain with reduced susceptibility to meropenem due to class A and class D carbapenemases (KPC-2 and OXA-48, respectively, data shown in ESI,† Table S2) revealed no potentiation of meropenem in those assays, suggesting that they do not function as transition-state inhibitors of those enzymes. This is perhaps unsurprising when we keep in mind that the β -lactamases need to be inert against the endogenous peptides of the bacterium.

Based on these data, a zinc-depletion mechanism seems to be the most likely mode of action of our compounds. A similar mechanism was concluded for the fungal natural product aspergillomarasmine A, identified as a potent inhibitor of NDM-1 and other clinically relevant metallo- β -lactamases.^{8,30,31} There are also recent reports on a class of spiro-indoline-thiazole analogues working similarly.³²

Toxicity in human cell lines

Toxicity of a selection of the compounds was assessed in three different cancer cell lines, with IC_{50} -values as stated in Table 3. Also, included in the same table, are estimated values for the stability constants pK_D of the corresponding zinc complexes. The value for the DPA chelators are taken directly from the literature,³³ whereas the DPTA chelators have been given a value of 1.3 log units above DPA, based on data provided in a study where coordinating and non-coordinating triazole moieties were compared.²⁵ The DPED chelator is given a value of 11.8, based on structural similarities with the previously reported chelator DPESA.²⁷ Finally, TPED is estimated to 12.3, also based on structural similarity with the known chelator TPESA.²⁷ We note that this value might be underestimated, as another source put TPEN itself at 15.6, with a corresponding statement that zinc is bound to TPEN in a pentadentate manner, suggesting that the contribution of the fourth pyridine ring in TPEN compared to TPED could be relatively minor.³³

The IC_{50} values shown in Table 3, corresponding to toxicity in three different human cancer cell lines, separates clearly the compounds in two different groups, with the weaker chelators DPA and DPTA exhibiting lower toxicity than DPED and TPED. Thus, it would seem that both ability to potentiate the activity of meropenem and intrinsic toxicity correlate with each other through chelator strength. Initially, we hoped for some visible detoxifying effect of the vector or linker for the strongest chelator, but this seems not to be the case. For instance, by comparison between 12, 19 and 20, we see that attachment to C- or N-terminus of the peptide (attachment points E and C, respectively, in Fig. 1a), or change from D-amino acids to L-amino acids, have no effect on the toxicity with the TPED chelator. If the obtained toxicity data are representative for normal human cells, the MIC values of

Table 3 Properties and intrinsic toxicity of selected zinc chelator constructs sorted by estimated pK_D

Compound	clog <i>P</i>	Peptide characteristics		Chelator characteristics		Intrinsic toxicity ^a (μM)		
		Sequence	Attachment point ^b	Type	Est. pK_D ^c	MDA-MB 231	Mia-PaCa	Colo-357
8	0.3	D-Ala-D-Ala	C	DPA	7.6	141 ± 42	45 ± 15	152 ± 8
15	0.4	D-Ala-D-Ala	E	DPA	7.6	127 ± 92	118 ± 32	21 ± 5
33	0.1	D-Ala-D-Ala	C	DPA	7.6	184 ± 46	98 ± 6	90 ± 12
10	2.1	D-Ala-D-Ala	C	DPTA	8.9	125 ± 27	119 ± 21	105 ± 23
35	0.2	D-Ala-D-Ala	C	DPTA	8.9	152 ± 17	116 ± 9	133 ± 18
36	0.2	L-Ala-L-Ala	C	DPTA	8.9	157 ± 10	105 ± 16	96 ± 11
37	-0.8	Gly-Gly	C	DPTA	8.9	168 ± 77	176 ± 11	137 ± 78
38	-0.1	D-Ala-D-Lys	D	DPTA	8.9	127 ± 55	106 ± 10	114 ± 3
39	1.0	D-Ala-D-Lys	D	DPTA	8.9	131 ± 15	128 ± 8	101 ± 15
44	-3.5	L-Lys-boroGly	B	DPTA	8.9	101 ± 21	66 ± 13	112 ± 33
45	-3.5	L-Lys-boroGly	A	DPTA	8.9	128 ± 44	119 ± 3	82 ± 6
14	0.1	D-Ala-D-Ala	C	DPED	11.8	4.5 ± 0.6	5.1 ± 0.4	4.3 ± 0.2
11	0.7	Gly-Gly	C	TPED	12.3	7.8 ± 0.4	12 ± 3	12 ± 4
12	1.7	D-Ala-D-Ala	C	TPED	12.3	4.1 ± 1.4	5.9 ± 2.3	5.4 ± 0.8
18	1.2	Gly-Gly	E	TPED	12.3	15 ± 3	21 ± 23	24 ± 6
19	2.2	D-Ala-D-Ala	E	TPED	12.3	4.9 ± 0.7	4.8 ± 1.2	6.8 ± 1.4
20	2.2	L-Ala-L-Ala	E	TPED	12.3	5.1 ± 0.2	5.5 ± 0.9	6.8 ± 1.6

^a IC₅₀ values reported in μM with standard deviation. ^b See Fig. 1a. ^c See main text for explanation.

meropenem at a lower chelator concentration illustrates the dilemma of the strongest chelators as they have little to no therapeutic window. Application of such compounds in the clinic may be viewed as balancing acts, wherein the chelator strength must be strong enough for significant potentiation, but not too strong, in order to limit side effects. Consequently, we have in parallel investigated the use of the TPA chelator in order to explore this region of intermediate chelator strength between DPTA and DPED.¹²

What seems to have an effect is the changing the peptide from Ala-Ala, in any configuration, to Gly-Gly, as shown for 11, and even more pronounced, for 18. The dominating effect of this switch to glycine might be to make the compounds more hydrophilic, which in turn points toward another strategy for tuning the toxicity of the strongest chelators. We have in fact prepared DPED and TPED compounds (50 and 51, Fig. 2, syntheses given in ESI†) taking this strategy to the extreme by the inclusion of carbohydrates as “hydrophilic vec-

tors”, and, compared their toxicity to their peptide counterparts 14 and 19 (Table 4). The human cells are not the same as used in the first and large screen (Table 3), but allow comparing the interesting compounds with each other. However, no clear conclusion as to the hydrophilic vector could be drawn, as compound 50 was 17-fold less toxic than 14, and compound 51 was 8-fold more toxic than 19. Further, *in vitro* and *in vivo* testing of 50, and maybe 51 also, must be undertaken to fully evaluate their clinical utility.

Conclusion

In this work, we have prepared zinc chelators bound to small peptide fragments, with theoretical dissociation constants for bound zinc (K_d) ranging from 10^{-8} to 10^{-14} . In co-administration with meropenem towards meropenem-resistant bacterial strains harbouring the MBLs VIM-2 or NDM-1, we observed potentiation of meropenem for all chelator types. However, for the weakest chelator variants, higher concentrations of chelator were needed to obtain any effect, while stronger chelators were effective down to 31 μM concentration.

Toxicity of the new compounds was evaluated *in vitro* in three human cancer cell lines. The data suggested a correlation between chelator strength and increased toxicity. Our initial hypothesis that inclusion of peptide fragments of bacterial origin might lead to decreased toxicity in human cells, did not hold up, as the corresponding inhibitors containing L-Ala-L-Ala showed exactly the same results as the inhibitor containing D-Ala-D-Ala. Instead, data for inhibitors containing Gly-Gly point towards decreased lipophilicity as a path towards lowering the toxicity for the strongest chelators. We postulate that by choosing zinc chelators of intermediate strength, toxicity might be limited, while preserving a

Fig. 2 Compounds containing strong chelators coupled with carbohydrates.

Table 4 Direct comparison of MIC values of meropenem (MEM) and toxicity of two different chelators (DPED and TPED) with similar side chains. The MIC values of meropenem against both strains were determined to be 32–64 mg L⁻¹

Compound	clog <i>P</i>	Chelator	MIC data towards MEM [mg L ⁻¹] at 31 and 16 μM				Intrinsic toxicity IC ₅₀ ^a (μM) HepG2
			<i>P. aeruginosa</i> VIM-2		<i>K. pneumoniae</i> NDM-1		
			31 μM	16 μM	31 μM	16 μM	
14	0.1	DPED	1–2	32	0.125	16	6.83 ± 1.05
50	−1.7	DPED	1	32	0.25	32	117.6 ± 1.11
19	2.2	TPED	2	32	0.125	8	13.56 ± 1.30
51	0.7	TPED	2	32	0.125	8	1.70 ± 6.17

^a IC₅₀ values reported in μM with standard deviation.

therapeutic utility. Compound 50, with its poly-alcohol side chain, looks promising and it might seem these hydrophilic side chains have more potential than peptides.

Experimental

Chemical synthesis

The detailed experimental procedures for all new compounds can be found in the ESI,[†] except for commercially available TPEN and the previously reported compound 15.²³

Meropenem MIC determination

The MICs of meropenem alone or in combination with the compounds were determined using pre-made broth micro-dilution plates, containing meropenem in 2-fold dilution steps ranging from 0.03 to 64 mg L⁻¹ (TREK Diagnostic Systems/Thermo Fisher Scientific, East Grinstead, UK). The compounds were initially screened at a final concentration of 125 or 50 μM. The MIC assays were performed using two MBL-producing meropenem resistant strains, one NDM-1-producing *K. pneumoniae*³⁴ and one VIM-2-producing *P. aeruginosa*.³⁵ In brief, the compounds were diluted in MH-broth (Becton Dickinson, Franklin Lakes, NJ USA) and 50 μL of the suspension was added to each well of the meropenem plate. Bacterial colonies were suspended in 0.9% saline buffer to 0.5 McFarland and further diluted 1 : 100 in MH broth (Thermo Fisher Scientific, East Grinstead, UK). 50 μL of the diluted bacterial suspension was added to the meropenem plates to a final volume of 100 μL. The plates were incubated for 20 h at 37 °C. Each MIC was determined in duplicate. To investigate any intrinsic antibacterial activity of the compounds the MIC of each compound were determined in a 2-fold serial dilution series in MH broth (Becton Dickinson, Franklin Lakes, NJ USA) ranging from 2–1000 μM. 50 μL of each dilution step was then mixed with 50 μL of a bacterial inoculum, prepared as described above, in 96 well microtiter plates (Thermo Fisher Scientific, Roskilde, Denmark). The plates were incubated, and MIC determined as described above.

Cell viability assays

Human breast cancer cells (MDA-MB-231) and pancreatic cancer cells (MIA-PaCa2 and Colo-357), all obtained from

ATCC, were cultured at 37 °C with 5% CO₂ in DMEM media, containing 10% fetal bovine serum and antibiotics. For cell survival assays, cells were seeded in 96 well plates (3000 cells per well, 100 μL medium), adhered for 24 h and exposed to different concentrations (from 300 μM to 0.3 μM) of the Zn chelators (dissolved in DMSO) for an additional 72 h. Then, metabolically active cells were quantified by the MTT assay by adding 100 ng MTT per well, incubating cells for 2 h, replacing media with 100 μL isopropanol/HCl/H₂O (90/1/9, v/v/v) and determining optical density at 540 nm. IC₅₀ values were determined using the CompuSyn software. Results are expressed as mean values of IC₅₀ ± SD (μM) of three independent experiments performed in triplicate.

The zinc chelators were dissolved in DMSO at concentrations ranging from 1 to 10⁻³ mM, for compounds 14 and 50, and from 0.1 to 10⁻⁴ mM, for compounds 19 and 51. They were added to white 96-well plates (maximum DMSO concentration in wells lower than 1%) containing 20,000 HepG2 cells per well, obtained from ATCC. Plates were incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. After 24 h, AlamarBlue cell viability reagent (Thermo Fisher, Carlsbad, CA, USA) was added as a 10% solution, and plates were placed back in the incubator for 4 h. AlamarBlue is a red-ox indicator yielding a fluorescence signal proportional to the number of viable cells in each well. The fluorescence signal was measured in a microplate reader (Clariostar, BMG Labtech, Ortenberg, Germany) at 550 nm/603 nm (excitation/emission). Data from eight replicates were used to calculate the IC₅₀ and standard deviation using Sigmoidal, 4PL, where X is log(concentration) analysis, and a four-parameter logistic regression from GraphPad Prism 6 (GraphPad Software Inc., USA).

clog *P* calculations

The partition coefficient for all compounds were calculated using PerkinElmer Informatics ChemDraw Professional version 15.1.0.144.

Author contributions

G. K.-A. – Synthesis and characterization of compounds, project supervision, manuscript composition and writing.

C. S. – Synthesis and characterization of compounds, project supervision, manuscript writing.

A. P. – Synthesis and characterization of compounds, biological testing of compounds.

S. R., M. L. B. – Supervision of synthesis, manuscript writing.

L. P. J., C. F. – Biological testing of compounds, manuscript writing.

T. G., A. M. S. A., S. L. – Biological testing, data analysis.

Ø. S. – Biological testing and supervision, data analysis, manuscript writing.

P. R. – Synthesis supervision, project conception, data analysis and manuscript writing.

O. A. H. Å. – Synthesis and characterization of compounds, supervision of synthetic work, project conception, data analysis, manuscript composition and writing.

Conflicts of interest

OAHA, ØS, PR, GKA and CS have filed patents on this discovery.

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