Published in final edited form as:

Chem Commun (Camb). 2009 July 7; (25): 3693–3695. doi:10.1039/b902455g.

Antibacterial Cyclic D,L - α -Glycopeptides

Leila Motiei, Shai Rahimipour, Desiree A. Thayer, Chi-Huey Wong, and M. Reza Ghadiri

Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Abstract

We report the design, synthesis, membrane activity, biophysical characterization, and *in vitro* antibacterial activities of cationic cyclic D,L - α -glycopeptides bearing side chains derivatized with D -glucosamine (GlcNH₂), D -galactose (Gal), or D -mannose (Man).

The increasing prevalence of bacterial infections that are resistant to existing antibiotic therapeutics has fueled renewed interest in the design and discovery of novel antibacterial agents.¹ Consequently, a *Streptomyces* isolate known since the late 1950s to exhibit antibiotic activity against Gram-positive bacteria was recently reexamined, leading to the discovery and structure elucidation in 2002 of a new class of cationic glycopeptide antibiotics named mannopeptimycins.² Independently, we had described a new class of rationally designed supramolecular antimicrobial agents based on the self-assembling cyclic D,L - α -peptide architecture developed and studied in our laboratory since early 1990s.^{3–5} Following the disclosure of the mannopeptimycin structures, we noted that they possess a cyclic peptide architecture, backbone conformation, and potentially membrane-active antibacterial mode of action that are strikingly similar to that of the cyclic D,L - α -peptides. However, unlike mannopeptimycins, the cyclic D,L - α -peptides can be readily synthesized and subjected to multiple rounds of sequence-activity optimizations through either parallel or combinatorial library approaches.^{3,4,6} We therefore set out to expand the class of cyclic D,L - α -peptides to include glycosylated analogues and explore the utility of these derivatives as antimicrobial agents. Here we describe initial studies in the design, synthesis, membrane activity, and *in vitro* antibacterial activity of cationic cyclic D,L - α -glycopeptides bearing side chains derivatized with either D -glucosamine, D -galactose, or D -mannose.^{7,8}

As a progenitor peptide sequence for the present studies, we chose the membrane-active amphiphilic cyclic D,L - α -peptide [WLWKS α SK] (peptide **8**). The symmetrical disposition of hydrophilic amino acids in **8** seemed appropriate for probing rationally the effects of the position and type of *O*-glycosylserine residue substitutions on antibacterial activity and target membrane selectivity. Accordingly, glycopeptides **1–3** were designed to maintain a similar overall cationic character by individually substituting each of the three lysine residue with serine(β GlcNH₂). Similarly, both of the neutral serine residues were individually replaced with either serine(β Gal) or serine(α Man), providing glycopeptides **4** and **5** or **6** and **7**, respectively (Fig. 1, Table 1).

The requisite peracetylated *O*-linked glycosyl Fmoc-serine derivatives **9**, **10**, and **11** were synthesized according to published methods^{7,9} (Fig. 1). Cyclic peptides **1–8** were synthesized on the solid-support by first tethering Fmoc- D -Lys-OAll via its free ϵ -amino side chain moiety to a 2-chlorotrityl chloride polystyrene resin. Linear peptide synthesis was carried out according to standard Fmoc synthesis protocols under base-free HOBt/DIC coupling

conditions. Following the deprotection of *N*-terminal Fmoc and the *C*-terminal allyl ester, the linear peptides were cyclized on the solid support by exposure to activating agents. Following removal of the *O*-acetyl protecting groups with 16% hydrazine hydrate in MeOH, the cyclic peptides were cleaved from the resin and globally side chain deprotected under acidic conditions. The products were purified by RP-HPLC and characterized by mass spectrometry.

In vitro antibacterial activities of glycopeptides **1-7** against the Gram-positive species tested were generally in the 5-10 μ M range (Table 1), indicating that glycosylation and the resulting increase in peptide hydrophilicity did not adversely influence cell membrane uptake and activity. Furthermore, glycosylation also did not diminish the bactericidal activity, as indicated by the rapid killing action (<2 h) observed with glycopeptides **2, 4**, and **6** against MRSA (Table 1, Fig. 2). However, depending on the position and the type of glycosyl residue employed, glycosylation significantly attenuated the *in vitro* toxicity toward erythrocytes (Fig. 3) and mammalian cells (mouse fibroblast) (Table 1). Glycopeptides **4** and **5** with the β -gal side chain modification were the most selective sequences, displaying low hemolytic activity against human and mouse red blood cells (Table 1, Fig. 3). However, incorporation of Ser(β GlcNH₂) or Ser(μ Man) residues either did not appreciably improve the bacteria vs. RBC membrane selectivity (glycopeptides **2, 3, 6**), or produced greater hemolytic activities (glycopeptides **1** and **7**). Of particular note is the significant hemolytic activity differences observed between human and mouse RBCs—in all cases, mouse red blood cells were more susceptible to hemolysis.

Although the hemolytic activity of membrane active antimicrobial peptides is typically used as an indicator of toxicity to mammalian cells, we have found that several non-hemolytic antimicrobial cyclic $\text{D,L-}\alpha$ -peptides were acutely toxic in mice.¹⁰ Therefore, in selecting promising cyclic peptide sequences for further studies, we also take into account their activity against mammalian tissue culture cells. Since *in vitro* cell culture cytotoxicity assays are performed in the presence of serum proteins in the culture media (typically fetal bovine serum, FBS), it is necessary to establish *a priori* that cyclic peptide binding to serum proteins does not significantly reduce its bioavailability and activity. We have established that measuring antibacterial activity in presence of 10-50% FBS (MIC in FBS) to be a facile functional surrogate assay for estimating the *in vitro* bioavailability of antimicrobial peptides under tissue culture-type conditions (Table 1). The MIC in FBS data correlate well with the extent of serum binding measured independently by membrane ultrafiltration assay (10 kD NMWL cellulose membrane). As shown in Table 1, the antibacterial activities of cyclic $\text{D,L-}\alpha$ -glycopeptides were not appreciably perturbed even in the presence of large amounts (up to 50% v/v) of fetal bovine serum (FBS). Furthermore, comparisons of the antibacterial activity against MRSA in FBS with observed levels of *in vitro* toxicity to mouse fibroblast cells (NIH 3T3) indicate that glycosylation considerably reduced mammalian cell toxicity (up to approximately five-fold in the cases of glycopeptides **2** and **4**) even after prolonged (48 h) exposure of tissue culture cells to cyclic $\text{D,L-}\alpha$ -glycopeptides (Table 1). Furthermore, the relative position of glycosylated serine side chain the in cyclic peptide sequence had up to a three-fold effect on mammalian cell membrane selectivity and toxicity.

Support for the self-assembling propensity of the cyclic glycopeptides and the membrane permeation mode action are provided by liposome-based fluorescence dye release assays (Fig. 4) and attenuated total reflectance (ATR) FT-IR spectroscopy in synthetic membranes (Table S1).¹¹ Peptide-induced membrane permeation is evidenced by the facile release of entrapped fluorescent dye (sulforhodamine B) from large unilamellar lipid vesicles (Fig. 4). Each cyclic glycopeptides studied (**2, 4, 5**, and **6**) showed rapid membrane activity even at concentrations >20-fold below MIC (data not shown). The ATR FT-IR study of cyclic glycopeptides **2, 4**, and **6** in dimyristoyl phosphatidylcholine (DMPC) multibilayers indicated that, like

nonglycosylated cyclic D,L - α -peptide nanotubes,^{4,5,11} the amphiphilic cyclic glycopeptide sequences studied also display typical characteristics of β -sheet-like hydrogen bonded tubular assemblies (amide-A = $\sim 3276\text{ cm}^{-1}$, amide-I = $\sim 1627\text{ cm}^{-1}$) oriented roughly parallel to the plane of the lipid membrane (Table S1).

In summary, we have shown that specific glycosylation of membrane active self-assembling cyclic D,L - α -peptides can significantly reduce mammalian cell toxicity while maintaining potent bactericidal activities against multidrug-resistant Gram-positive species. The cyclic D,L - α -glycopeptides reported here are considerably simpler in amino acid side chain complexity than mannopeptimycins and can be readily subjected to combinatorial synthesis and structure-activity optimizations. We also note the potential utility of self-assembling cyclic D,L - α -glycopeptides in the design of other bioactive agents targeted to cells that express appropriate carbohydrate binding receptors.¹²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (GM 52190). We thank the Human Frontier Science Program and Fulbright for Postdoctoral Fellowships (S.R.), and NSF for a Predoctoral Fellowship (D.A.T.).

Notes and references

1. (a) Swartz MN. *Antimicrob. Agents Chemother* 2000;44:2009–16. [PubMed: 10898668] (b) Bonomo RA. *Clin. Infect. Dis* 2000;31:1414–22. [PubMed: 11096012] (c) Levy SB. *Clin. Microbiol. Infect* 2000;6:101–106. [PubMed: 11449641] (d) Pfaller MA, Jones RN, Marshall SA, Edmond MB, Wenzel RP. *Diagn. Microbiol. Infect. Dis* 1997;29:259–63. [PubMed: 9458983]
2. (a) Koehn FE. *J. Med. Chem* 2008;51:2613–2617. [PubMed: 18393404] (b) He H. *Appl. Microbiol. Biotechnol* 2005;67:444–452. [PubMed: 15702316] (c) Petersen PJ, Wang TZ, Dushin RG, Bradford PA. *Antimicrob. Agents Chemother* 2004;48:739–746. [PubMed: 14982758] (d) Weiss WJ, Murphy T, Lenoy E, Young M. *Antimicrob. Agents Chemother* 2004;48:1708–1712. [PubMed: 15105124] (e) Singh MP, Petersen PJ, Weiss WJ, Janso JE, Luckman SW, Lenoy EB, Bradford PA, Testa RT, Greenstein M. *Antimicrob. Agents Chemother* 2003;47:62–69. [PubMed: 12499170] (f) He H, Williamson RT, Shen B, Graziani EI, Yang HY, Sakya SM, Petersen PJ, Carter GT. *J. Am. Chem. Soc* 2002;124:9729–9736. [PubMed: 12175230]
3. Wilcoxon, KM. Ph. D. Thesis. The Scripps Research Institute; La Jolla, CA: 2002.
4. Fernandez-Lopez S, Kim H-S, Choi EC, Delgado M, Granja JR, Khasanov A, Kraehenbuehl K, Long G, Weinberger DA, Wilcoxon KM, Ghadiri MR. *Nature* 2001;412:452–456. [PubMed: 11473322]
5. (a) Hartgerink JD, Granja JR, Milligan RA, Ghadiri MR. *J. Am. Chem. Soc* 1996;118:43–50. (b) Ghadiri MR, Granja JR, Buehler LK. *Nature* 1994;369:301–4. [PubMed: 7514275] (c) Ghadiri MR, Granja JR, Milligan RA, McRee DE, Khazanovich N. *Nature* 1993;366:324–7. [PubMed: 8247126]
6. Redman JE, Wilcoxon KM, Ghadiri MR. *J. Comb. Chem* 2003;5:33–40. [PubMed: 12523832]
7. Lin H, Thayer DA, Wong C-H, Walsh CT. *Chem. Biol* 2004;11:1635–1642. [PubMed: 15610847]
8. For recent examples of glycopeptide antibiotic designs, see: (a) Lin H, Walsh CT. *J. Am. Chem. Soc* 2004;126:13998–14003.14003 [PubMed: 15506762] (b) Fu X, Albermann C, Jiang J, Liao J, Zhang C, Thorson JS. *Nat. Biotechnol* 2003;21:1467–1469.1469 [PubMed: 14608364] (c) Gobbo M, Biondi L, Filira F, Gennaro R, Benincasa M, Scolaro B, Rocchi R. *J. Med. Chem* 2002;45:4494–4504.4504 [PubMed: 12238928] (d) Ritter TK, Wong C-H. *Angew. Chem., Int. Ed* 2001;40:3508–3533.3533 (e) Ge M, Chen Z, Onishi HR, Kohler J, Silver LL, Kerns R, Fukuzawa S, Thompson C, Kahne D. *Science* 1999;284:507–11.11 [PubMed: 10205063]
9. Salvador LA, Elofsson M, Kihlberg J. *Tetrahedron* 1995;51:5643–56.

10. Dartois V, Sanchez-Quesada J, Cabezas E, Chi E, Dubbelde C, Dunn C, Granja J, Gritzen C, Weinberger D, Ghadiri MR, Parr TR Jr. *Antimicrob. Agents Chemother* 2005;49:3302–3310. [PubMed: 16048940]
11. Kim HS, Hartgerink JD, Ghadiri MR. *J. Am. Chem. Soc* 1998;120:4417–4424.
12. For selected recent examples, see:(a)Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. *Science* 2001;291:2370–6.6 [PubMed: 11269318](b)Bertozzi CR, Kiessling LL. *Science* 2001;291:2357–2364.2364 [PubMed: 11269316](c)Koeller KM, Wong C-H. *Nat. Biotechnol* 2000;18:835–841.841 [PubMed: 10932151](d)Dwek RA. *Chem. Rev* 1996;96:683–720.720 [PubMed: 11848770](e)Varki A. *Glycobiology* 1993;3:97–130.130 [PubMed: 8490246]

Fig. 1. Glycosyl serine derivatives and the solid-phase approach employed in the synthesis of cyclic $\text{D,L-}\alpha$ -glycopeptides (see Supporting Information for experimental details).

Fig. 2. Time-kill curves of cyclic D,L - α -peptides at concentrations of 2xMIC against MRSA (ATCC33591), signifying their rapid bactericidal mode of action (CFU = bacterial colony forming units).

Fig. 3. Dose-dependant hemolytic activity of cyclic peptides against human (left panel) and mouse (right panel) red blood cells.

Fig. 4. Cyclic D,L - α -peptides cause rapid release of dye (sulforhodamine B) from DPPC vesicles (LUVs). Data shown are for 5 μ M peptide concentrations with a peptide:lipid ratio of 1:23. Dye-release activities were also observed at low peptide concentrations (0.125 μ M) at a peptide:lipid ratios of 1:92 (data not shown).

Table 1
In vitro antibacterial, hemolytic, and cytotoxic activities of cyclic $\text{D,L-}\alpha$ -glycopeptides.^a

Glycopeptide	MIC ^b (μM)			HD ₅₀ ^g (μM)			LD ₅₀ (μM)
	MRSA ^c (MBC) ^d	MRSA ^c in FBS	B. cereus ^e	m-RBC ^h	h-RBC ⁱ	NIH3T3 ^j	
1 [WLWKSXS]	5	15	10	65	85	20	
2 [WLWKSXS]	5 (10)	10	10	90	140	50	
3 [WLWXSXS]	5	5	15	80	150	20	
4 [WLWKSZK]	5 (10)	10	10	150	260	50	
5 [WLWKZKS]	5	5	5	105	190	25	
6 [WLWKSUK]	2.5 (5)	5	10	70	120	25	
7 [WLWKUKS]	2.5	10	5	50	105	15	
8 [WLWKSXS]	5	5	5	65	120	10	

^aPeptide sequences are shown in single-letter amino acid codes with the underlining representing D-residues. The bold letters depict O-glycosylserine residues with X = Ser(βGlcNH_2), Z = Ser(βGal), and U = Ser(αMan). All measurements were done at least in duplicate. We estimate errors in MIC to be 1 μM and errors in LD₅₀ and HD₅₀ to be 5 μM .

^bMinimum inhibitory concentration.

^cMethicillin-resistant *Staphylococcus aureus* (ATCC33591).

^dMinimum bactericidal concentration.

^eATCC11778.

^fVancomycin-resistant *Enterococcus faecalis* (ATCC51575).

^gHemolytic dose.

^hMouse (Balb-C)

ⁱhuman red blood cells (erythrocytes).

^j*In vitro* toxicity against mouse fibroblast cells (NIH 3T3) measured after 48 h of continued exposure to tested agents.