Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 14 (2006) 77–82
Bioorganic & Medicinal Chemistry

Design of multifunctional peptides expressing both antimicrobial activity and shiga toxin neutralization activity

Yoshinao Yamada,[a,b] Yoshiko Miura,[a] Akio Sakaki,[a] Tetsuhiko Yoshida[b] and Kazukiyo Kobayashi[a,*]

[a]*Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Chikusa, Nagoya 464-8603, Japan*
[b]*Innovative Research Laboratory, Department of Research and Development, Toagosei Co., Ltd. 1-1, Funami-cho, Minato-ku, Nagoya 455-0027, Japan*

Received 13 May 2005; revised 27 July 2005; accepted 28 July 2005
Available online 19 September 2005

Abstract—We have designed novel short peptides expressing both antimicrobial and Shiga-toxin (Stx) neutralization activities by combining nuclear localization signal (NLS) peptides (*RIRKKLR*, *PKKKRKV*, and *PRRRK*) tandemly with globotriaoside (Gb3) mimic peptide (WHWTWL). These fusion peptides exhibited excellent antimicrobial activity against both gram-positive and gram-negative bacteria. A peptide WHWTWL*RIRKKLR* (Trp-His-Trp-Thr-Trp-Leu-*Arg-Ile-Arg-Lys-Lys-Leu-Arg*), especially, exhibited about 100 times higher activity than the original NLS peptide. SPR analysis demonstrated that the binding of this peptide to both Stxs was strong: $K_d = 6.6 \times 10^{-6}$ to Stx-1 and 6.8×10^{-6} to Stx-2. The in vitro assay against Stx-1 using HeLa cells showed that this peptide increased the survival rate of HeLa cells against the infection of Stx-1. The peptide has been found to maintain high antimicrobial activity, Stx neutralization activity, and no cytotoxicity at its concentration of 7.8–31.3 μg/mL (4.2–16.7 μM). The present peptide design has a prospect of developing potent multifunctional drugs to destroy proteinaceous toxin-producing bacteria and to simultaneously neutralize the toxins released by bacteriolysis.

1. Introduction

Antimicrobial peptides are a major component of innate self-defense system.[1] They provide an immediate response to invading microorganisms and display a broad spectrum of bactericidal or bacteriostatic actions. Various kinds of antimicrobial peptides have been identified from multicellular organisms, such as animals,[2] plants,[3] and insects,[4] during the past two decades. Development of resistance by sensitive microbial strains against these antimicrobial peptides is improbable because many antimicrobial peptides exert their effect by forming pores in microbial membranes or disrupting membrane structures.[5] Further efforts have been devoted to improve activity, specificity, and toxicity of antimicrobial peptides and to broaden clinical applications as next generation antimicrobials with high commercial potentials.

We have developed a new type of antimicrobial peptide based on NLS (nuclear localization signal) sequences.[6] NLS sequences consisting of several basic amino acids mediate the selective transport of proteins into the nucleus in eukaryotic cells.[7] NLS peptides are produced by eucaryotes and viruses as part of nuclear proteins.[8] We recently found that various types of NLS peptides displayed antimicrobial activity against gram-negative and gram-positive bacteria as a function different from nuclear localization signal, although their antimicrobial mechanism is still unknown.

We have also found that a peptide 'WHWTWLSEY' bound Shiga toxins (Stxs: Stx-1 and Stx-2) strongly and neutralized the toxins effectively.[9] Stxs produced by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 are known to cause hemolytic uremic syndrome (HUS).[10,11] Stxs consist of one toxic A-subunit and five carbohydrate-binding B-subunits.[12] Stxs infection is induced by binding the B-subunits to globotriaosyl (Gb3) ceramides in membrane surfaces and subsequently delivering the cytotoxic

Keywords: Peptides; Drug design; Antibacterial; Shiga toxins.
* Corresponding author. Fax: +81 52 789 2528; e-mail: kobayash@mol.nagoya-u.ac.jp

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.07.052

A-subunit into target cells.[13] Various types of Stx neutralization agents have been developed on the basis of Gb3 derivatives.[14] We have reported Stx-neutralizing glyco nanomaterials[15] and recently focused on Gb3 mimic peptides which were selected with biopanning from a phage display library against anti-Gb3 antibody.[9,16]

This paper reports that tandem fusion peptides between NLS peptides and Gb3 mimic peptides expressed a multifunction of both antimicrobial and Stx neutralization activities. Conventional antibiotics possess antimicrobial activity but no neutralization activity against bacterial toxins. The enterotoxins released by bacteriolysis cause serious HUS in colon and consequently damage human bodies seriously. It is expected that the proposed novel peptides can destroy bacteria and neutralize the released toxins simultaneously. Moreover, another effect on antimicrobial activity is expected by conjugating the NLS peptides rich in basic amino acids and the Gb3 mimic peptides rich in hydrophobic amino acids. Many antimicrobial peptides are known to possess two common features: overall positive charges, and distinct hydrophobic and hydrophilic faces.[17] In these respects, we have designed several fusion peptides and investigated their antimicrobial efficiency and interactions with Stxs.

2. Results

2.1. Design and synthesis of peptides

Peptides **1**–**8**, listed in Table 1, were synthesized by the standard solid-phase Fmoc chemistry on Wang resin, purified by reversed phase HPLC, and characterized by MALDI-TOF-mass spectroscopy. Peptides **1**–**4** consist of NLS peptide sequences and Stxs-binding sequences. These fusion peptides have been designed to be as short as possible to lower antigenicity to human bodies and to facilitate chemical synthesis. C-terminal italic '*RIRKKLR*' of peptides **1** and **2**, '*PKKKRKV*' of peptide **3**, and '*PRRRK*' of peptide **4** are NLS peptide sequences composed of several positively charged basic amino acids. These NLS peptides were of the low antimicrobial activity but the shortest peptides among the antimicrobial NLS library.[6] N-terminal underlined 'WHW' of peptide **1** and 'WHWTWL' of peptides **2**–**4** are the segments of Stxs-binding sequence 'WHWTWLSEY.' This peptide was recently selected as a segment of the most active Gb3 mimic peptide from phage display library and its N-terminal was reported to bind Stxs.[9] Peptides **5**–**7** are the original NLS peptide sequences and peptide **8** is a segment of Stxs-binding sequence.

Table 1. Antimicrobial activity of synthetic peptides

Peptide[a]	Medium[b]	MIC (μM)			
		24 h		48 h	
		E. coli IFO 3972	*S. aureus* FDA 209P	*E. coli* IFO 3972	*S. aureus* FDA 209P
1 WHW*RIRKKLR*	NB	3.1	3.1	3.1	6.3
	MHB	50	25	50	25
	MHB-ca	100	100	>100	100
2 WHWTWL*RIRKKLR*	NB	3.1	1.6	3.1	1.6
	MHB	6.3	3.1	6.3	3.1
	MHB-ca	12.5	6.3	12.5	6.3
3 WHWTWL*PKKKRKV*	NB	15.6	7.8	15.6	15.6
	MHB	125	62.5	125	125
	MHB-ca	250	250	500	250
4 WHWTWL*PRRRK*	NB	7.8	3.9	7.8	3.9
	MHB	15.6	7.8	15.6	15.6
	MHB-ca	62.5	15.6	62.5	31.3
5 *RIRKKLR*	NB	500	125	>500	125
	MHB	>500	>500	>500	>500
	MHB-ca	>500	>500	>500	>500
6 *PKKKRKV*	NB	>500	500	>500	>500
	MHB	>500	>500	>500	>500
	MHB-ca	>500	>500	>500	>500
7 *PRRRK*	NB	500	250	500	500
	MHB	>500	>500	>500	>500
	MHB-ca	>500	>500	>500	>500
8 WHWTWL	NB	500	500	>500	>500
	MHB	>500	>500	>500	>500
	MHB-ca	>500	>500	>500	>500

[a] The segments of Gb3 mimic peptides are underlined and the NLS peptides are italicized.
[b] NB, nutrient broth; MHB, Mueller–Hinton broth, and MHB-ca, cation adjusted Mueller–Hinton broth.

2.2. Antimicrobial activity

The minimum inhibitory concentrations (MICs) of synthetic peptides were determined against gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus aureus*) bacteria (Table 1). The antimicrobial activities of peptides **5**–**7** (NLS) and peptide **8** (Stxs-binding sequence) were weak, but those of fusion peptides **1**–**4** were increased. The activity was enhanced with the length of Stxs-binding sequences in the order of peptide **5** < peptide **1** < peptide **2**: peptide **2** was 80- to 150-fold higher than peptide **5**. Among the fusion peptides carrying various NLS sequences, the *RIRKKLR* sequence in **2** exhibited the highest activity. It is worth noting that peptide **2** maintained the high activity, even in a cation-adjusted MHB medium.

2.3. Cytotoxicity

Figure 1 illustrates the cytotoxicity assay of peptides **1**, **2**, and **5**, and polymyxin B for HeLa cells. Polymyxin B, a branched-cyclic antibiotic peptide consisting of 11 amino acids produced by *Bacillus polymyxa*, was used as a reference. The cell survival rates were determined with a cell counting kit (WST-8) by measuring the absorbance at 490 nm at peptide concentrations from 2000 μg/mL in the four times serial dilutions. Almost 100% of cell survival rates were kept in the presence of peptides **1** and **2** at 125 μg/mL (84.5 and 66.5 μM, respectively) concentrations. Peptide **5** was not cytotoxic, even at much higher concentration, but polymyxin B was apparently cytotoxic at 2000 μg/mL. It is important to note that peptides **1** and **2** showed no cytotoxicity to HeLa cells at their MIC against bacteria listed in Table 1.

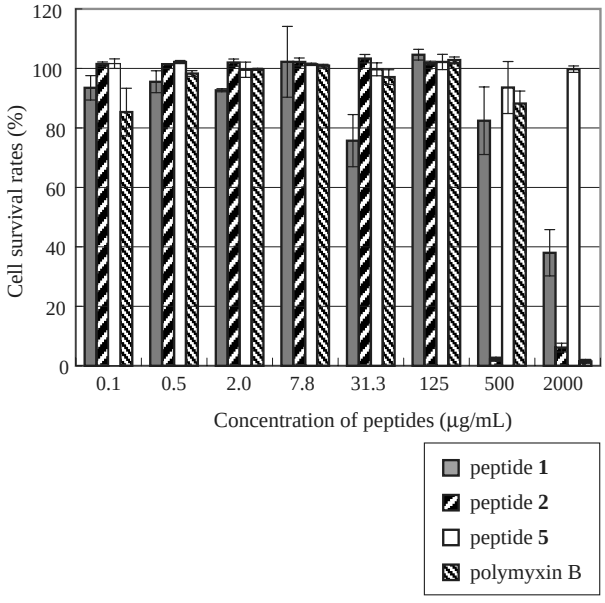

Figure 1. Cytotoxicity of peptides for HeLa cells. Survival rates of HeLa cells are indicated as an average of four experiments in the presence of various peptide concentrations. Weight concentration of each peptide (μg/mL) can be converted to molar concentration (μM) as follows: peptide **1**: 0.676 μM; peptide **2**: 0.532 μM; peptide **5**: 1.032 μM; polymyxin B: 0.721 μM.

2.4. Shiga-toxin-binding assay by SPR analysis

The binding affinities of the shortest fusion peptide **1** and the highest antimicrobial peptide **2** to Stx-1 and -2 were examined with a Biacore2000 biosensor (SPR). Table 2 lists the equilibrium (K_d) and rate (k_a and k_d) constants in association and dissociation of peptides. Peptide **2** exhibited high affinities to both Stxs: its dissociation equilibrium constants (K_d) to Stx-1 and -2 were 6.6 and 6.8×10^{-6}, respectively. Their affinities to both Stxs, in contrast to the reported result of Gb3Cer: Gb3Cer-incorporated membrane of 1-palmitoyl 2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), exhibited a lower affinity to Stx-2 ($K_d = 1.0 \times 10^{-6}$) than to Stx-1 ($K_d = 2.2 \times 10^{-7}$).[18] The affinity of peptide **5** to both Stxs was too weak to be determined by the data fitting to a 1:1 Langmuir-binding model. The affinity of peptides **1** and **2** to non-carbohydrate-binding proteins, such as BSA, trypsin, and fibrinogen, could also not be determined. We assume that these peptides recognized both Stxs specifically.

2.5. Neutralization assay of Stx-1 with peptides 2 and 5

HeLa cells were incubated with a mixture of Stx-1 at 5.0 pg/100 μL and peptide **2** or peptide **5** in a range of concentrations (0–125 μg/mL) for 2 days. Figure 2 shows that the cell survival rate in the presence of Stx-1 was about 40%, which increased to over 50% by

Table 2. Kinetic constants[a] of peptides **1**, **2**, and **5** to Stxs estimated by SPR

Peptide	Stx	k_a (1/Ms)	k_d (1/s)	K_d (M)
1	Stx-1	1.7×10^3	1.9×10^{-1}	1.1×10^{-4}
1	Stx-2	5.3×10^4	1.5×10^{-1}	2.8×10^{-6}
2	Stx-1	2.7×10^4	1.8×10^{-1}	6.6×10^{-6}
2	Stx-2	2.7×10^4	1.8×10^{-1}	6.8×10^{-6}
5	Stxs	—[b]	—	—

[a] k_a, association rate constant; k_d, dissociation rate constant; K_d, dissociation equilibrium constant.
[b] The affinity was too weak to be determined.

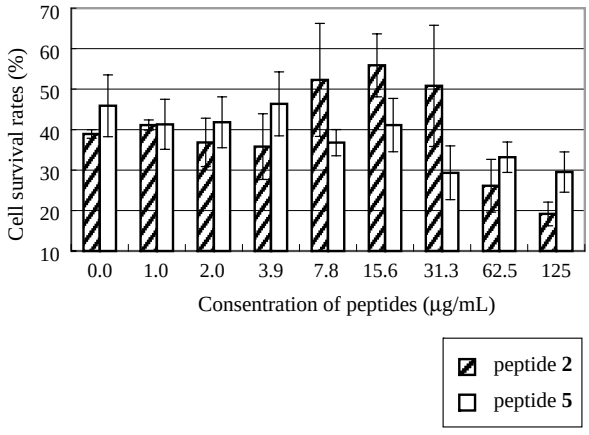

Figure 2. Stx-1 neutralization activity assay of synthetic peptides **2** (WHWTWL*RIRKKLR*) and **5** (*RIRKKLR*). Survival rates of HeLa cells against Stx-1 at 5.0 pg/100 μL are indicated as an average of two experiments in the presence of various concentrations of these peptides.

adding peptide **2** at a concentration of 7.8–31.3 μg/mL (4.2–16.7 μM). Peptide **2** in 7.8–31.3 μg/mL clearly improved the cell survival rate in comparison with peptide **5**. Hence, peptide **2** was suggested to neutralize Stx-1 and protect the HeLa cells against the infection of Stx-1. The cell survival rate was decreased at 62.5 μg/mL or higher concentration of **2**, suggesting that the cells were damaged on account of the synergic effect of both Stx-1 and the peptide.

2.6. CD spectroscopy

Conformational information of peptide **2** based on CD spectroscopy is essential for discussing its antibiotic activity mechanism. Figure 3 shows the CD spectra of peptide **2** in PBS containing 0–50% trifluoroethanol (TFE). While the spectrum in PBS had a negative cotton effect at 200 nm and positive effect around 225 nm, the spectra in 25% and 50% TFE solution had negative cotton effects around 208 nm and 220 nm. The negative cotton effect at 200 nm in PBS buffer indicated that the random coil conformation[19] and the peak around 225 nm were attributable to Trp.[20] The addition of TFE converted the negative peak at 200 nm to larger negative maximum at 208 nm and smaller shoulder around 220 nm, indicating the conformation change from random coil to a mixture of α-helix and 3_{10}-helix.[19] The NLS peptide **5** was found to take a random coil conformation not only in PBS but also in 25% and 50% TFE solution. Hence, the addition of the hydrophobic Gb3 sequence to the NLS sequence enhanced the tendency to form a helical conformation in a hydrophobic condition. The relation of the conformation to antimicrobial activity is described in Section 3.

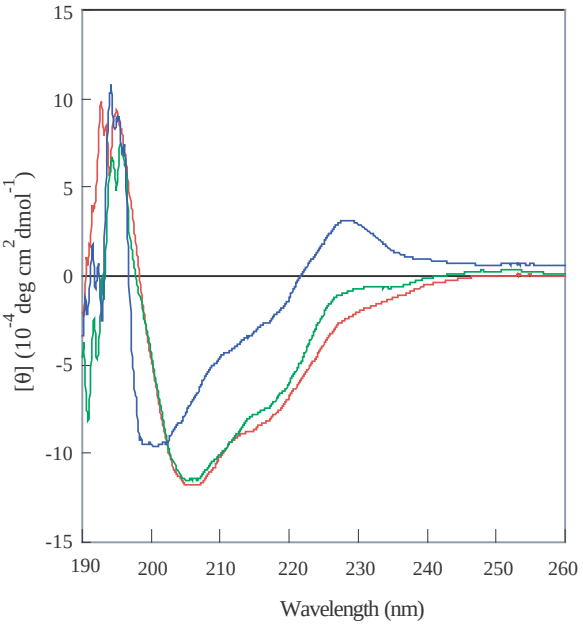

Figure 3. CD spectra of synthetic peptide **2** (WHWTWL*RIRKKLR*) in PBS (blue line), PBS containing 25% trifluoroethanol (green line), and PBS containing 50% trifluoroethanol (red line) at [peptide] = 200 μM at 25 °C.

3. Discussion

We have demonstrated that the fusion peptide **2** WHWTWL*RIRKKLR* carries two distinct chemical and biological properties: the positively charged NLS peptide with antimicrobial activity and the hydrophobic Gb3 mimic peptide with Stx-binding activity.

The antimicrobial activity of the NLS peptide *RIRKKLR* has been strongly enhanced by combining the hydrophobic WHWTWL sequence to its N-terminal. The amphipathic feature is common to many naturally occurring antimicrobial peptides. The activity of many antimicrobial peptides is explained by the Shai–Matsuzaki–Huang (SMH) model.[21] When a peptide forms a certain secondary structure, the positively charged domain is proposed to initiate electrostatic interaction with the negatively charged components of the microbe membrane and the hydrophobic domain is supposed to insert the peptides into the membrane. Then, the peptides collapse the membrane into fragments and disrupt the target microbial membrane physically. Although the NLS peptide **5** itself was in a random coil conformation, peptide **2** has formed a helical conformation in a hydrophobic condition, as shown in Figure 3. The basic residues of Arg, His, and Lys, and the hydrophobic residues of Trp, Leu, and Ile are supposed to be assembled into the separated domain in the α-helix (Fig. 4). We assume that the separated domain structure of peptide **2** can enhance the electrostatic and hydrophobic interactions to microbe membrane and that the SMH model can rationalize the excellent antimicrobial activities of peptide **2**. It is also important that the antimicrobial activity was maintained even after 48 h incubation, suggesting that this peptide was bactericidal rather than bacteriostatic.

The affinities of peptide **2** to Stx-1 and -2 were strong enough to neutralize Stxs. In addition to its high antimicrobial activity and lack of cytotoxicity, peptide **2** expressed neutralization activity to Stx-1 at a concentration from 7.8 to 31.3 μg/mL. It is suggested that the

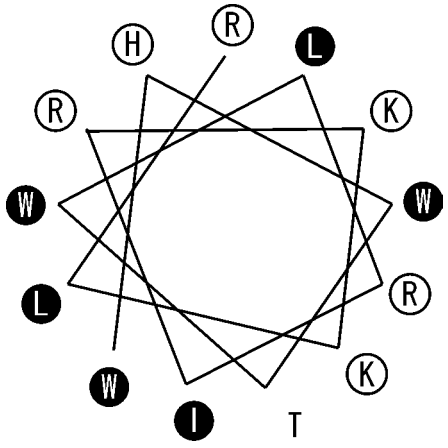

Figure 4. Helical wheel diagram of peptide **2**. The white filled circles are positively charged amino acids and the black filled circles are hydrophobic amino acids.

Gb3-mimic domain at the N-terminal bound and neutralized Stx-1, and rescued HeLa cells from the infection of Stx-1. Conventional antibiotics are often known to cause deterioration of the HUS because a large amount of Stxs (particularly Stx-1) is released from the destroyed EHEC. Then, the Stxs neutralization activity of peptide **2** becomes indispensable. The peptide **2** will be a potent multifunctional drug to treat patients seriously infected with Stxs-producing bacteria.

We expect that the antimicrobial activity may further be enhanced by modification of the terminal carboxylate group and that the Stxs neutralization activity of peptide **2** may further be enhanced by extension of the Stxs-binding sequence, as well as by expression of the multivalency effect. It is also suggested that the high antimicrobial activity of peptide **2**, even in cation-rich medium conditions, is effective as a drug for an intravenous or subcutaneous injection.

4. Conclusion

Fusion peptide **2** (WHWTWL*RIRKKLR*) between the NLS peptide *RIRKKLR* and the Gb3 mimic peptide WHWTWL has successfully expressed a multifunction of antibacterial activity against gram-positive and gram-negative bacteria, as well as neutralization activity against Stx. The present design of fusion peptides is a promising strategy to develop a new type of antimicrobial drugs that express another function, such as proteinaceous toxin neutralization activity, by appropriate combinations between various kinds of antimicrobial peptides and carbohydrate mimic peptides.

5. Experimental

5.1. Solid-phase peptide synthesis

Peptides were synthesized, according to the standard solid-phase synthesis techniques with a Milligen 9050 synthesizer (PerSeptive Biosystems, Framingham, MA). Protected amino acids and chemicals were purchased from Novabiochem (San Diego, CA), Applied Biosystems (Foster, CA), and Peptide Institute, Inc (Osaka, Japan). The Wang resin (0.1 mmol scale) was treated with piperidine (20%) in dry DMF and then with a mixture of Fmoc-amino acid (4-fold excess), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), and *N*,*N*′-diisopropylethylamine. All the other Fmoc-amino acids were sequentially coupled to a growing peptide chain for 1.5 h. A solution of piperidine (20%) in dry DMF was used to remove the Fmoc group at all steps. After deprotection of the last Fmoc group, the peptide resin was washed with methanol and dried in vacuo to yield the protected peptide resin. Deprotection and cleavage from the resin were carried out with a mixture of 80% trifluoroacetic acid, 6% ethanedithiol, 12% thioanisole, and 2% *m*-cresol with stirring at room temperature for 4 h. After filtration of the exhausted resin, the residue was crystallized in diethyl ether to finish the product as a white powder. The product was purified by reverse-phase HPLC on a C18 column (X-Terra MS C18 4.6×15 mm: Waters, Milford, MA) for 30 min, using a linear gradient of 5–60% acetonitrile in water containing 0.1% trifluoroacetic acid (v/v), at a flow rate of 1.5 mL/min. The purified peptide was lyophilized and the molecular weight was determined by MALDI-TOF-mass spectroscopy. (Voyager-DE^{TM} PRO: PerSeptive Biosystems, Framingham, MA). Calcd for peptide **1** $[M+H]_+$: 1479.9. Found: 1479.2. Calcd for peptide **2** $[M+H]^+$: 1880.3. Found: 1879.8. Calcd for peptide **3** $[M+H]^+$: 1794.3. Found: 1794.1. Calcd for peptide **4** $[M+H]^+$: 1623.0. Found: 1622.4. Calcd for peptide **5** $[M+H]^+$: 970.3. Found: 969.7. Calcd for peptide **6** $[M+H]^+$: 884.2. Found: 883.9. Calcd for peptide **7** $[M+H]^+$: 712.9. Found: 712.4. Calcd for peptide **8** $[M+H]^+$: 929.1. Found: 928.4.

5.2. Bacteria culture

Escherichia coli (IFO 3972) and *S. aureus* (IFO 12732) were cultured on Mueller–Hinton agar (Difco Laboratories, Detroit, MI) at 37 °C for 18 h and the growth was measured (OD_{660}) just before the MIC examination.

5.3. Antibacterial activity

Minimal inhibitory concentrations (MICs) of peptides against bacteria were determined using a standard microtiter dilution method in nutrient broth, Mueller–Hinton broth, and cation-adjusted Mueller Hinton broth (Difco Laboratories, Detroit, MI). Polymyxin B sulfate was used as a positive control to evaluate the reliability of each test. The peptide solution was prepared by the two methods. Aqueous solutions (2 mM) of peptides **2** and **3** were diluted with each medium to yield 100 μM concentration. The other peptides were dissolved in each medium to yield 500 μM concentration. Twofold serial dilutions of peptides (100 μL) were added to 96-well plates. The bacteria adjusted to 2×10^7 colony-forming units/mL with physiological saline (5 μL) were added to each peptide solution. The final inoculum was 1.0×10^6 colony-forming units/mL. The plates were incubated at 37 °C for 24 and 48 h, and the lowest peptide concentration to inhibit growth was determined as MIC.

5.4. Surface plasmon resonance

Binding of peptides to Shiga toxins (Stx-1 and -2) was estimated by surface plasmon resonance (SPR) with a BIAcore 2000 Biosensor (BIAcore, Inc., Uppsala, Sweden). Peptides were coupled onto carboxymethyl dextran biosensor chips (CM-5) using the BIAapplications handbook protocol for ligand thiol immobilization. For kinetics measurements, serial dilutions of Stx-1 or 2 (3.6×10^{-1}, 1.8×10^{-1}, 8.9×10^{-2}, and 4.5×10^{-2} μM) were injected in running HBS-N buffer (10 mM Hepes, pH 7.4, 150 mM NaCl; BIAcore, Inc.) at a flow rate of 10 μL/min for 1 min, followed by a dissociation period of 0.5 min at 25 °C. The equilibrium (K_d) and rate (k_a and k_d) constants in association and dissociation were determined from a fit of the data to

a 1:1 Langmuir-binding model using the manufacturer's analysis software (BIAevaluation 3.1). The bindings of BSA, trypsin, and fibrinogen to peptides were also similarly estimated.

5.5. Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) under 5% CO_2 at 37 °C. The cells (5.0×10^3 cells/100 μL) in logarithmic growth phase in DMEM were plated on 96-well microplates (100 μL/well) and then incubated under 5% CO_2 at 37 °C for 1 day.

5.6. Cytotoxic assay of peptides

After cell adhesion on 96-well microplates, the medium was removed and replaced with DMEM (100 μL/well) containing a range of peptide concentrations (1.0×10^{-1}–2.0×10^3 μg/mL) and the plates were incubated under 5% CO_2 at 37 °C for 2 days. The Cell Counting Kit-8 (WST-8) solution (10 μL) (Dojindo Molecular Technologies, Gaithersburg, MD) was added to each well. After further incubation under 5% CO_2 at 37 °C for 3 h, the cell survival rate was determined by measuring the absorbance at 490 nm (655 nm as a reference) with a Microplate Reader Model 550 (Bio-Rad Laboratories, Richmond, CA).

5.7. Neutralization activity of peptides 2 and 5 against Stx-1[15]

After cell adhesion on 96-well microplates, the medium was removed and replaced with DMEM (100 μL/well) containing a mixture of a range of peptide concentrations (0–125 μg/mL) and Stx-1 at 5.0 pg/100 μL. The plates were incubated under 5% CO_2 at 37 °C for 2 days. The Cell Counting Kit-8 solution (10 μL) was added to each well. After further incubation under 5% CO_2 at 37 °C for 3 h, the cell survival rate against Stx-1 was determined by the same method as the cytotoxic assay mentioned above.

5.8. CD spectroscopy

The CD spectra were recorded on a JASCO (Tokyo, Japan) model J-727 with 190–260 nm wavelength, 0.1 nm step resolution, 100 nm/min speed, 2 s response time, 1 nm bandwidth, and integration frequency of 4 times at 25 °C. Sample solutions of peptide **2** (200 μM) were prepared in PBS (pH 7.4), PBS containing 25% TFE, and PBS containing 50% TFE.

References and notes

1. (a) Zasloff, M. *Curr. Opin. Immunol.* **1992**, *4*, 3–7; (b) Boman, H. G. *Cell* **1991**, *65*, 205.
2. (a) Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449; (b) Selsted, M. E.; Novotny, M. J.; Morris, W. L.; Tang, Y. Q.; Smith, W.; Cullor, J. S. *J. Biol. Chem.* **1992**, *267*, 4292; (c) Ganz, T.; Lehrer, R. I. *Curr. Opin. Hematol.* **1997**, *4*, 53; (d) Hancock, R. E. W.; Falla, T.; Brown, M. H. *Adv. Microb. Physiol.* **1995**, *37*, 135.
3. Broekaert, W. F.; Cammue, B. P. A.; De Bolle, M. F. C.; Thevissen, K.; De Samblanx, G. W.; Osborn, R. W. *Crit. Rev. Plant Sci.* **1997**, *16*, 297.
4. (a) Steiner, H.; Hultmark, D.; Engstrom, A.; Bennich, H.; Boman, H. G. *Nature* **1981**, *292*, 246; (b) Lamberty, M.; Zachary, D.; Lanot, R.; Bordereau, C.; Robert, A.; Hoffmann, J. A.; Bulet, P. *J. Biol. Chem.* **2001**, *276*, 4085; (c) Habermann, E. *Science* **1972**, *177*, 314; (d) Hoffman, J. A.; Reichart, J.-M. *Cell Biol.* **1997**, *7*, 309.
5. (a) Mor, A. *Drug Dev. Res.* **2000**, *50*, 440; (b) Zaslof, M. *Nature* **2002**, *415*, 389; (c) Hancock, R. E. W.; Chapple, D. S. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317.
6. Yoshida, T.; Kume, M.; Yamada, Y.; Matsuda, Y.; Kourai, H. Patent WO 03091429, **2003**.
7. (a) Dingwall, C.; Sharnick, S. V.; Laskey, R. A. *Cell* **1982**, *30*, 449; (b) Görlich, D.; Mattaj, I. W. *Science* **1996**, *271*, 1513; (c) Weis, K. *Trends Biochem. Sci.* **1998**, *23*, 185.
8. (a) Kalderon, D.; Roberts, B. L.; Richardson, W. D.; Smith, A. E. *Cell* **1984**, *39*, 499; (b) Mizuno, T.; Okamoto, T.; Yokoi, M.; Izumi, M.; Kobayashi, A.; Hachiya, T.; Tamai, K.; Inoue, T.; Hanaoka, F. *J. Cell Sci.* **1996**, *109*, 2627; (c) Sudbeck, P.; Scherer, G. *J. Biol. Chem.* **1997**, *272*, 27848; (d) Truant, R.; Cullen, B. R. *Mol. Cell. Biol.* **1999**, *19*, 1210.
9. (a) Miura, Y.; Sakaki, A.; Kamihira, M.; Tsuda, Y.; Iijima, S.; Kobayashi, K. *Biochim. Biophys. Acta*, to be submitted; (b) Miura,Y.; Sakaki, A.; Kobayashi, K. JP. Patent 350686(A), **2004**.
10. Griffin, P. M.; Tauxe, R. V. *Epidemiol. Rev.* **1991**, *113*, 60.
11. Cohen, A.; Hannigan, G. E.; Williams, B. R. G.; Lingwood, C. A. *J. Biol. Chem.* **1987**, *262*, 17088.
12. (a) Samdvig, K. *Toxicon* **2001**, *39*, 1629; (b) Kozlov, Y. V.; Chernaia, M. M.; Fraser, M. E.; James, M. M. *J. Mol. Biol.* **1993**, *232*, 704.
13. (a) Waddel, T.; Cohen, A.; Lingwood, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7898; (b) Arab, S.; Lingwood, C. A. *Glycoconjugate J.* **1996**, *13*, 159.
14. (a) Matsuoka, K.; Terabatake, M.; Esumi, Y.; Terunuma, D.; Kuzuhara, H. *Tetrahedron Lett.* **1999**, *40*, 7839; (b) Kitov, P. I.; Read, R. J.; Bundle, D. R. *Nature* **2000**, *403*, 669.
15. (a) Dohi, H.; Nishida, Y.; Mizuno, M.; Shinkai, M.; Kobayashi, T.; Takeda, T.; Uzawa, H.; Kobayashi, K. *Bioorg. Med. Chem.* **1999**, *7*, 2053; (b) Dohi, H.; Nishida, Y.; Takeda, T.; Kobayashi, K. *Carbohydr. Res.* **2002**, *337*, 983.
16. Miura, Y.; Sasao, Y.; Kamihira, M.; Sasaki, A.; Iijima, S.; Kobayashi, K. *Biochem. Biophys. Acta* **2004**, *1673*, 131.
17. Giangaspero, A.; Sandri, L.; Tossi, A. *Eur. J. Biochem.* **2001**, *268*, 5589.
18. Nakajima, H.; Kiyokawa, N.; Katagiri, Y. U.; Taguchi, T.; Suzuki, T.; Sekino, T.; Mimori, K.; Ebata, T.; Saito, M.; Nakao, H.; Takeda, T.; Fujimoto, J. *J. Biol. Chem.* **2001**, *276*, 42915.
19. Yoder, G.; Polese, A.; Silva, R. A. G. D.; Formaggio, F.; Crisma, M.; Broxterman, Q. B.; Kamphuis, J.; Toniolo, C.; Keiderling, T. A. *J. Am. Chem. Soc.* **1997**, *119*, 10278.
20. Woody, R. W. *Eur. Biophys. J.* **1994**, *23*, 253.
21. (a) Matsuzaki, K. *Biochim. Biophys. Acta* **1999**, *1462*, 1; (b) Yang, L.; Weiss, T. M.; Lehrer, R. I.; Huang, H. W. *Biophys. J.* **2000**, *79*, 2002; (c) Shai, Y. *Biochim. Biophys. Acta* **1999**, *1462*, 55.