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Supplementary Information

A β-Hairpin Peptide with pH-Controlled Affinity for Tumor Cells

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Experimental Procedures

Methods

Materials

Solvents of special grade were used unless otherwise specified. We obtained Fmoc-NH-SAL MBHA resin, NH_2-$(CH_2)_2$-NH-Trt resin, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asp(OAll), Fmoc-Glu(OtBu)-OH,Fmoc-Gly-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-D-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Trp(Boc)-OH, Boc-Trp(Boc)-OH, ethyl 2-cyano-2-((dimethylamino)(morpholino)methyloxyimino)acetate hexafluorophosphate (COMU), and 1*H*-benzo[*d*][1,2,3]triazol-1-ol anhydrous (HOBt) from Watanabe Chemical Industries, Ltd. (Japan). Acetic anhydride, acetic acid, ethyl 2-methylacetoatcetate, hydrazine monohydrate, *N*-bromosuccinimide (NBS), 2,2,2-trifluoroacetic acid (TFA), triisopropylsilane (TIS), tetrakis(triphenylphosphine)palladium(0), phenylsilane, 2,5-dihydroxybenzoic acid (DHBA), 5-hexynoic acid, *N*-(2-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-3',6'-dihydroxy-3-oxo-3*H*-spiro(isobenzofuran-1,9'-xanthene)-6-carboxamide (6-FAM-PEG_3-azide), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Ethanol, dichloromethane, acetonitrile, acetone, *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), diisopropylethylamine (DIPEA), deuterochloroform (chloroform-*d*), deuterodimethyl sulfoxide (dimethyl sulfoxide-d_6), and deuterium oxide (D_2O) were purchased from Kanto Chemicals Co., Inc. (Japan). Potassium carbonate, copper(II) sulfate pentahydrate, sodium acetate, sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate dihydrate, ascorbic acid, and *N*, *N*'-diisopropylcarbodiimide (DIPC) were purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Japan). 3-Mercaptopropanoic acid, 35 wt% deuterium chloride aqueous solution, and 40 wt% sodium deuteroxide aqueous solution were purchased from Merck/Sigma-Aldrich (Germany). NBS (8.0 g) was purified by recrystallization from water (200 mL) and lyophilized prior to use. Monobromobimane was prepared by referring to the previously reported method.[1,2]

Measurements

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired using an AVANCE III (Bulker Daltonics Inc., USA) spectrometer (400 MHz) ([sample] = 4 mg/mL) at 25 °C. The conformation of the peptide in water at

acidic and basic conditions was analyzed by nuclear overhauser effect spectroscopy using an AVANCE III (Bulker Daltonics Inc., USA) spectrometer (600 MHz) with a cryoprobe at 37 °C ([peptide] = 40 μM). The reversed-phase high performance liquid chromatography (HPLC) was performed by HPLC system (Prominence; Shimadzu Co., Japan) on an Inertsil WP300 C4 (10 × 250 mm, GL Science Inc., Japan) using a linear gradient of water-acetonitrile (containing 0.1% TFA). The matrix-assisted laser desorption ionization-time-of flight MS (MALDI-TOF MS) analyses were carried out on an Autoflex Speed II (Bulker Daltonics Inc., USA) using DHBA as a matrix. The circular dichroism (CD) spectra were recorded on a J-820 spectropolarimeter (JASCO Ltd., Japan) under nitrogen atmosphere. Experiments were performed in a quartz cell with a 1 mm path length over the range of 190-250 nm at 37 °C ([peptide] = 40 μM). The fluorescence spectra were measured using a JASCO FP-8300 spectrofluorometer (JASCO Ltd., Japan) at 37 °C ([peptide] = 40 μM).

Synthesis Procedure

Synthesis of 3,4-dimethyl-2-pyrazoline-5-one (1)

Ethyl 2-methylacetoacetate (20.0 g, 0.139 mol) was dissolved in 30 mL ethanol and slowly added hydrazine monohydrate (6.94 g, 0.139 mol) diluted by 10 mL of ethanol. The exothermic chemical reaction proceeded with fuming and white precipitation was generated. The mixture was additionally reacted for 1 h at ambient temperature and cooled to -20 °C forming white precipitation. The precipitation was collected by filtration and used for next reaction without further purification (Yield: 7.95 g, 50.0%). The chemical structure was determined by NMR spectroscopies.

^{1}H NMR (400 MHz, chloroform-*d*, tetramethylsilane (TMS)): δ = 1.80 (s, 3H), 2.11 (s, 3H), 2.51 (q, 1H), 11.49 (br, 1H).

^{13}C NMR (75 MHz, chloroform-*d*, TMS): 5.9, 10.4, 99.2, 143.3, 164.4 ppm.

Synthesis of 3,4-dimethyl-4-chloro-2-pyrazolin-5-one (2)

1 (3.00 g, 26.8 mmol) was dispersed to dichloromethane and cooled to 0 °C. Trichloroisocyanuric acid (2.04 g, 8.61 mmol) was added to the dispersion and reacted for 14 h at ambient temperature. The reaction mixture was filtered to remove precipitation. The filtrate was collected and concentrated to give the objective substance as a pale-yellow liquid (Yield: 3.74 g, 95.4%). The chemical structure was determined by NMR spectroscopies.

^{1}H NMR (400 MHz, chloroform-*d*, TMS): δ = 1.68 (s, 3H), 2.12 (s, 3H), 11.49 (br, 1H) ppm.

^{13}C NMR (75 MHz, chloroform-*d*, TMS): δ = 12.6, 21.7, 60.0, 159.9, 173.8 ppm.

Synthesis of 9,10-dioxa-syn(methyl, methyl)bimane (3)

Potassium carbonate (10.5 g, 76.5 mmol) was dispersed to 15 mL dichloromethane and cooled to 0 °C. Then, **2** (3.74 g, 25.5 mmol) diluted by 25 mL of dichloromethane was added to the dispersion. The mixture was stirred for 1h keeping at 0 °C, and additionally reacted for 15 h at ambient temperature. An excess of potassium carbonate was removed by filtration. The filtrate was concentrated and purified by recrystallization from 15 mL

of acetonitrile to give the pure substance as a yellow solid (1.87 g, 38.2 %). The chemical structure was determined by NMR spectroscopies.

^{1}H NMR (400 MHz, chloroform-*d*, TMS): δ = 1.80 (s, 6H), 2.29 (s, 6H) ppm.

^{13}C NMR (75 MHz, chloroform-*d*, TMS): δ = 6.7, 11.9, 112.0, 146.1, 160.5 ppm.

Synthesis of monobromobimane (4)

3 (1.87 g, 9.73 mmol) was dissolved in 200 mL of acetonitrile and cooled to 0 °C. NBS (1.73 g, 9.73 mmol) was added portionwise to the solution and reacted for 24 h at ambient temperature. The mixture was concentrated and passed through silica gel column using chloroform/acetone (v/v =1/1) mixture solution as an eluent. Yellow spot was collected, and the solvents were removed to give the objective substance as a yellow solid (1.40 g, 53.1%). The chemical structure was determined by NMR spectroscopies.

^{1}H NMR (400 MHz, chloroform-*d*, TMS): δ = 1.84 (s, 3H), 1.89 (s, 3H), 2.45 (s, 3H), 4.32 (s, 2H) ppm.

^{13}C NMR (75 MHz, chloroform-*d*, TMS): δ = 6.9, 11.5, 17.8, 113.2, 115.4, 144.2, 146.0 ppm.

Synthesis of 3-(((2, 5, 6-trimethyl-1, 7-dioxo-1*H*, 7*H*-pyrazolo[1, 2-*a*]pyrazol-3-yl)methyl)thio)propanoic acid (5)

4 (1.40 g, 5.16 mmol) and 3-mercaptopropanoic acid (0.60 g, 5.68 mmol) were reacted in 10 mL of acetonitrile in the presence of DIPEA (0.87 g, 6.19 mmol) for 24 h at ambient temperature. The mixture was concentrated and passed through silica gel column using chloroform/acetone (v/v = 1/1) mixture solution as an eluent. Fluorescent spot was collected, and the solvent were removed to give the objective substance as a yellow solid (0.42 g, 27.4%). The chemical structure was determined by NMR spectroscopies.

^{1}H NMR (400 MHz, dimethyl sulfoxide-d_6, TMS): δ = 1.84 (s, 3H), 1.89 (s, 3H), 2.08 (t, 2H), 2.18 (t, 2H), 2.45 (s, 3H), 3.52 (s, 2H), 11.2 (br, 1H) ppm.

^{13}C NMR (75 MHz, dimethyl sulfoxide-d_6, TMS): δ = 6.9, 11.5, 27.5, 31.3, 38.6, 113.2, 115.4, 144.2, 146.0 ppm

Synthesis of β-hairpin formable RGD and RGE peptides containing alkyne group at *N*-terminus (βh-RGD and βh-RGE)

βh-RGD and βh-RGE peptides were prepared by solid phase peptide synthesis (SPPS) using Fmoc chemistry. The target sequences (Alkyne-SSRFEWEFESS-^{D}PRGDP-SSRFETEFESS and Alkyne-SSRFEWEFESS-^{D}PRGEP-SSRFETEFESS) were constructed on a Fmoc-NH-SAL MBHA resin by using Fmoc-amino acid derivatives (3 equiv.), 5-hexynoic acid (3 equiv.), COMU (3 equiv.), and DIPEA (6 equiv.) in DMF for coupling (1 h), and piperidine (20 vol%)/DMF for Fmoc removal (30 min). These peptides were cleaved from the resin by treating with TFA/dichloromethane/TIS (v/v/v = 8.5/1/0.5) for 4 h. The obtained peptides (white-color solid) were purified by repeated precipitation from a methanol/diethyl ether system and HPLC, and subsequently identify by MALDI-TOF MS analysis.

MALDI-TOF MS (DHBA): βh-RGD; $[M+H]^+$ = 3377.59 ($[M+H]^+_{theo.}$ = 3377.47), βh-RGE; $[M+H]^+$ = 3391.43 ($[M+H]^+_{theo.}$ = 3377.48).

Synthesis of fluorescein-labeled βh-RGD and βh-RGE (FITC-βh-RGD and FITC-β-RGE)

The peptides (βh-RGD: 29.3 mg, βh-RGE: 29.3 mg, 0.867 μmol), 6-FAM-PEG_3-N_3 (5.0 mg, 8.67 μmol), copper(II) sulfate pentahydrate (2.2 mg, 8.67 μmol), and ascorbic acid (15.3 mg, 86.7 μmol) were dissolved in 2 mL of methanol. The mixtures were deoxygenated by three freeze-pump-thaw cycles and reacted for 24 h at ambient temperature. After reaction, these solutions were poured to a membrane (MWCO: 1000 Da) and dialyzed with methanol for 3 days. The solutions were concentrated to give the objective peptides as a yellow solid.
MALDI-TOF MS (DHBA): FITC-βh-RGD; $[M+H]^+$ = 3953.43 ($[M+H]^+_{theo.}$ = 3953.60), FITC-βh-RGE; $[M+H]^+$ = 3967.56 ($[M+H]^+_{theo.}$ = 3967.62).

Synthesis of bimane and tryptophan-labeled βh-RGD (Bimane/Trp-βh-RGD) for NOESY and TrIQ experiments

The peptide was prepared by SPPS using Fmoc chemistry. The target sequence (SSRFEWEFESS^{D}PRGDPSSRFETEFESS) was constructed on a NH_2-$(CH_2)_2$-NH-Trt resin by using Fmoc-amino acid derivatives (3 equiv.), COMU (3 equiv.), and DIPEA (6 equiv.) in DMF for coupling (1 h), and piperidine (20 vol%)/DMF for Fmoc removal (30 min). After removal Fmoc group on *N*-terminus, the resultant resin was treated with **5** (3 equiv.), COMU (3 equiv.) and DIPEA (6 equiv.) in DMF for 3 h at ambient temperature. The peptide was cleaved from the resin by treating with HFIP/dichloromethane (v/v = 3/7) for 6 h. The obtained peptide (white-color solid) was purified by repeated precipitation from a methanol/diethyl ether system. The peptide (50 mg, 0.014 mmol) and Boc-Trp(Boc)-OH (60 mg, 0.14 mmol), HOBt (22 mg, 0.14 mmol), and DIPC (36 mg, 0.28 mmol) were dissolved in 0.5 mL of DMSO and stirred for 24 h at ambient temperature. Subsequently, 5 mL of TFA/dichloromethane/TIS (v/v/v = 8.5/1/0.5) was added to the peptide solution and stirred for 3 h ambient temperature. After reaction, the solution was concentrated. The residue was dissolved in 10 mL methanol, poured to a membrane (MWCO: 1000 Da) and dialyzed with methanol for 3 days. The solutions were concentrated to give the objective peptides as a pale-yellow solid.
MALDI-TOF MS (DHBA): Bimane/Trp-βh-RGD; $[M+H]^+$ = 3846.63 ($[M+H]^+_{theo.}$ = 3846.65).

Tryptophan-induced quenching (TrIQ) measurement

The Bimane/Trp-βh-RGD peptide was dissolved in PBS at pH 5.0 to pH 9.0 (40 μM), and incubated for 30 min at 37 °C. Then, the fluorescence spectra were recorded on an FP-8000 spectrofuluorometer (Jasco, Tokyo, Japan) at the excitation wavelength of 381 nm.

Cell culture

HT-1080 cells derived from human sarcoma and MDA-MB-231 cells derived from human breast tumor were obtained from the American Type Culture Collection (ATCC, Virginia, USA). Normal human dermal fibroblasts

(NHDFs) were obtained from the Lonza (Warsaw, Poland). These cells were grown in Dulbecco's modified Eagle medium (DMEM) containing F12 (DMEM/F12) (Wako, Tokyo, Japan), supplemented with 10% (v/v) fetal bovine serum (FBS) (gibco), 100 U/mL penicillin (gibco), and 100 μg/mL streptomycin (gibco) in 5% CO_2 at 37 ºC.

Cellular uptake analysis by confocal laser scanning microscopy (CLSM) observation

The cells were plated on 35-mm glass-bottomed dishes (Iwaki Glass, Tokyo, Japan) at a density of 1.0×10^4 cells/dish and incubated overnight. Then, the fluorescein-labeled βh-peptides dissolved in pH-adjusted Opti-MEM (40 μM) (Wako) were applied to the dish. The pH of Opiti-MEM was adjusted to pH 6.2 and 7.4 with HCl. After the incubation for 6 h, the cells were washed twice with PBS and stained with LysoTracker Red DND-99 (Thermo Fisher Scientific, Waltham, MA, USA) (150 nM) for 1 h at 37 °C, followed by staining with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) (3 μg/ml) for 10 min at 37 °C. Confocal laser scanning microscopy (CLSM) images were acquired on a FluoView FV-3000 (Olympus, Tokyo, Japan) equipped with a 100× oil-immersion objective lens.

Cellular uptake analysis by flow cytometry

The cells were plated in 24-well plates at a density of 1×10^4 cells/well and incubated overnight. The fluorescein-labeled βh-peptides (40 μM) solutions at pH 6.2 and 7.4 were applied to the cells at 37 °C. After 6 h incubation, the cells were washed twice with PBS and collected by trypsin treatment. The collected cells were dispersed in PBS containing 1% BSA. Fluorescence intensities of collected cells were measured using a EC-800 flow cytometer (Sony Corp., Tokyo, Japan).

References

1 E. M. Kosower and B. Pazhenchevsky, *J. Am. Chem. Soc.*, 1980, **102**, 4983.

2 I. Neogi, P. J. Das and F. Grynszpan, *Synlett,* 2018, **29**, 1043.

Author Contributions

S. N. conceived the research. S. N. and K. N. designed and performed the experiments. M. T., S. N., and K. N. obtained funding for the project and oversaw the research. S. N., K. N., and M. T. co-wrote the manuscript. All authors discussed the results and commented on the manuscript. S. N. and K. N. contributed equally to this work.

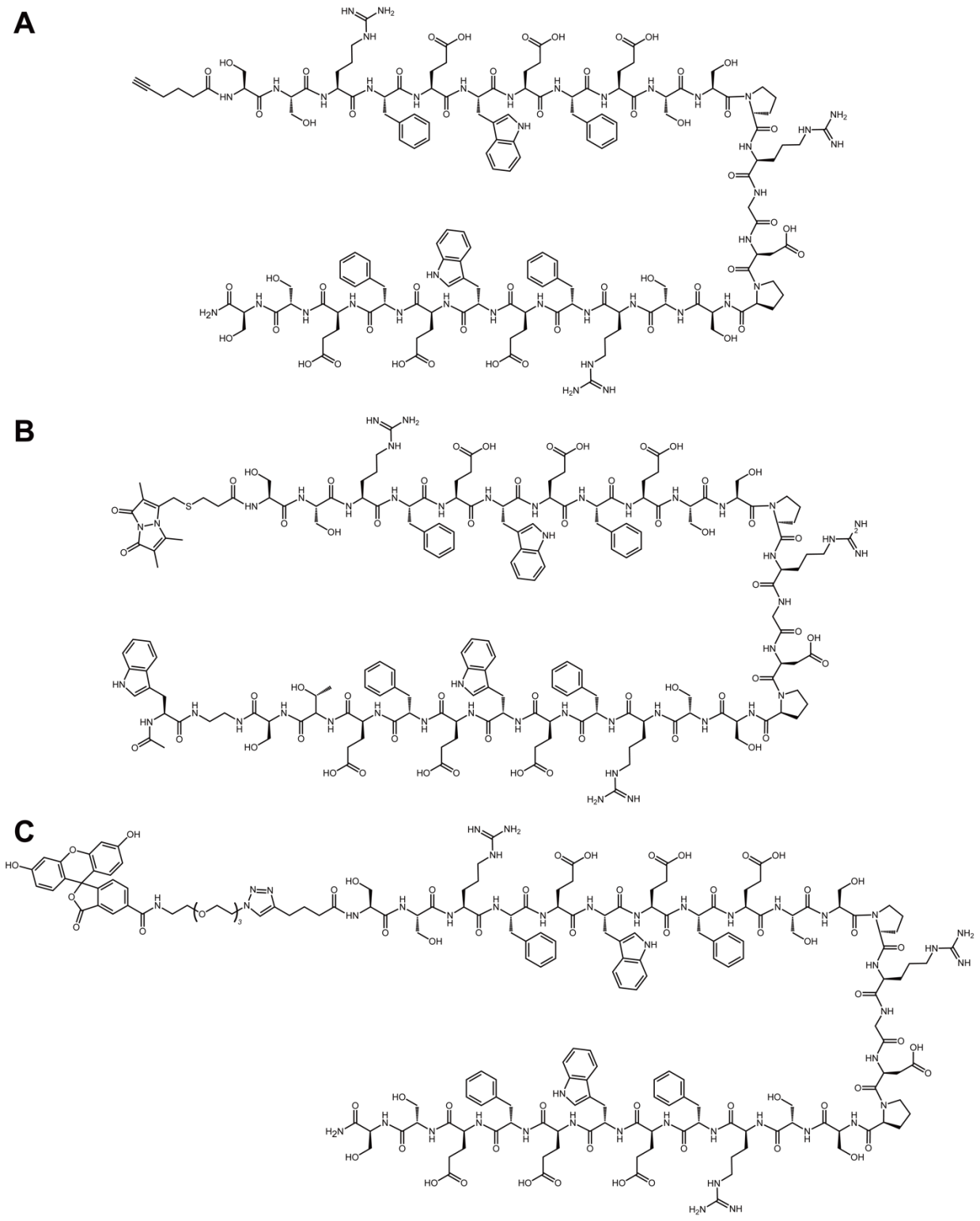

Figure S1. Chemical structures of various β-hairpin formable RGD peptides. (A) Alkyne (βh-RGD), (B) bimane-tryptophan (Bimane/Trp-βh-RGD), and (C) FITC-terminated (FITC-βh-RGD) peptides.

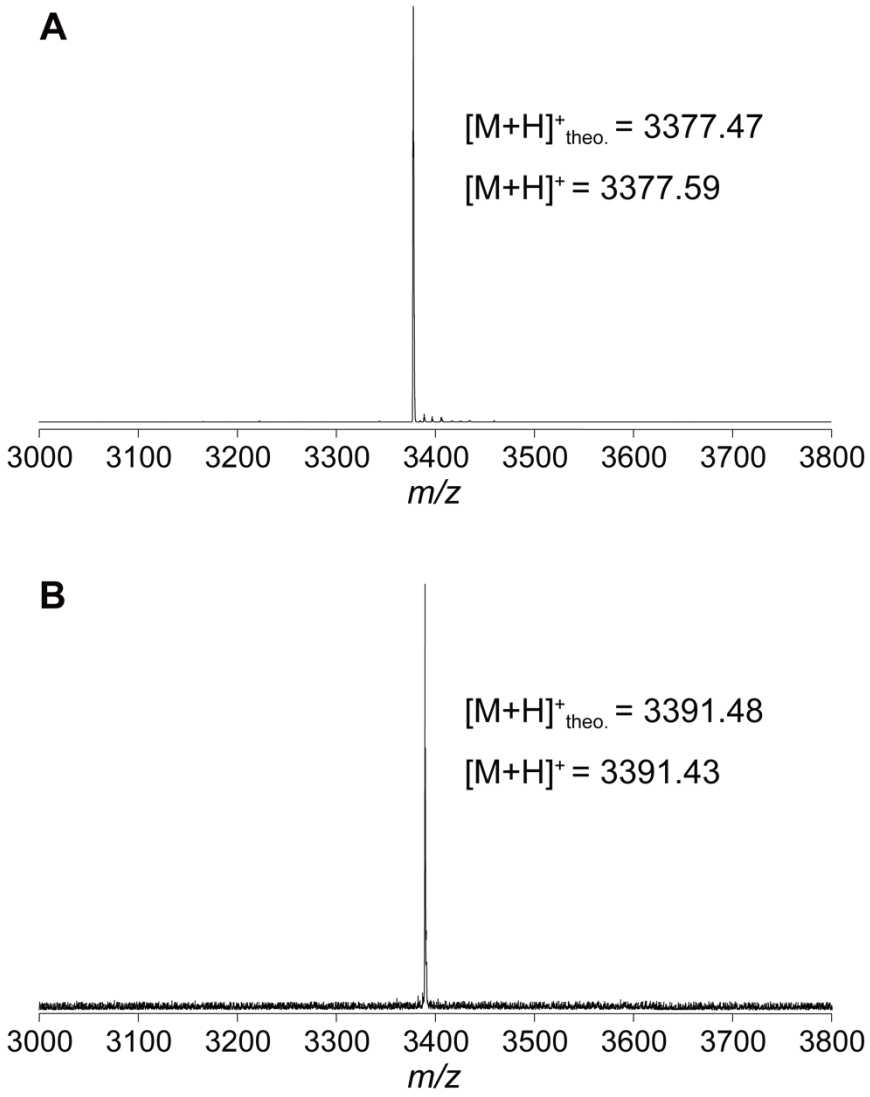

Figure S2. MALDI-TOF MS spectra of (A) βh-RGD and (B) βh-RGE peptides. Matrix: DHBA.

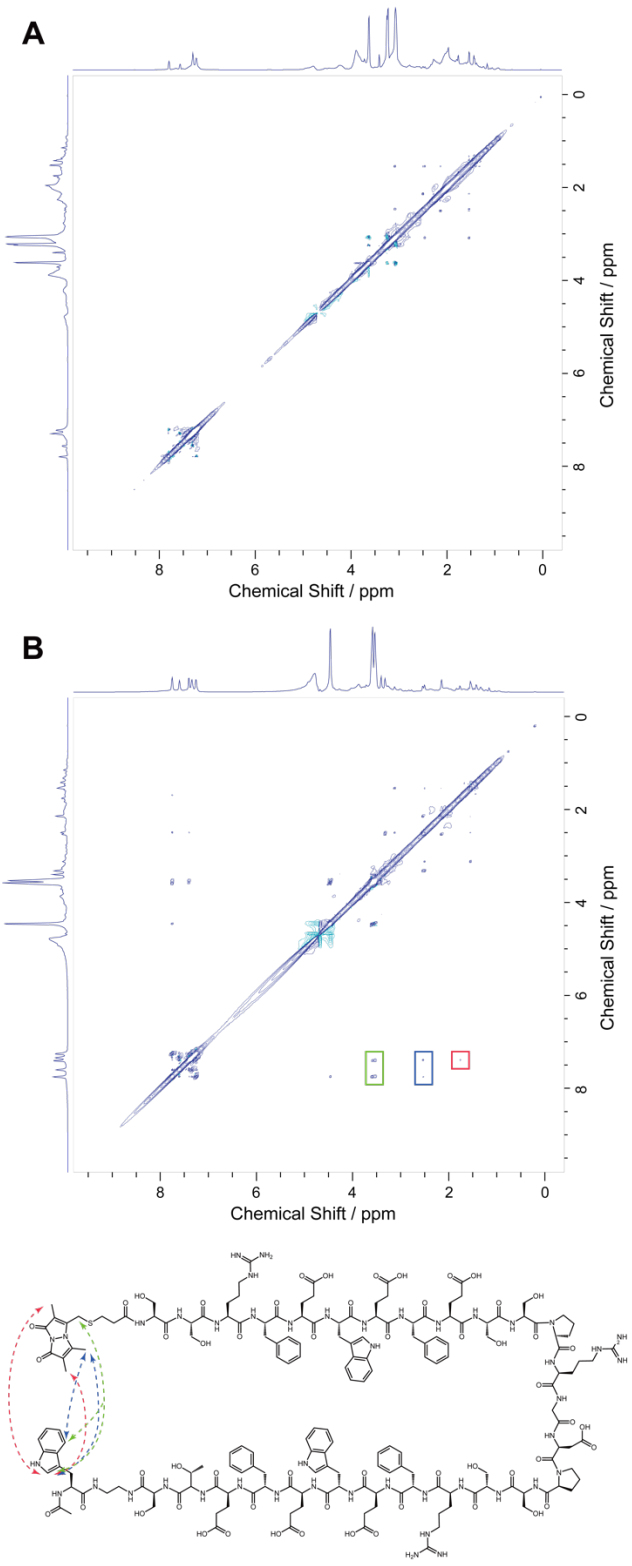

Figure S3. NOESY spectra of Bimane/Trp-βh-RGD peptide in D_2O at 37°C. (A) Natural condition. (B) Weak acidic condition.

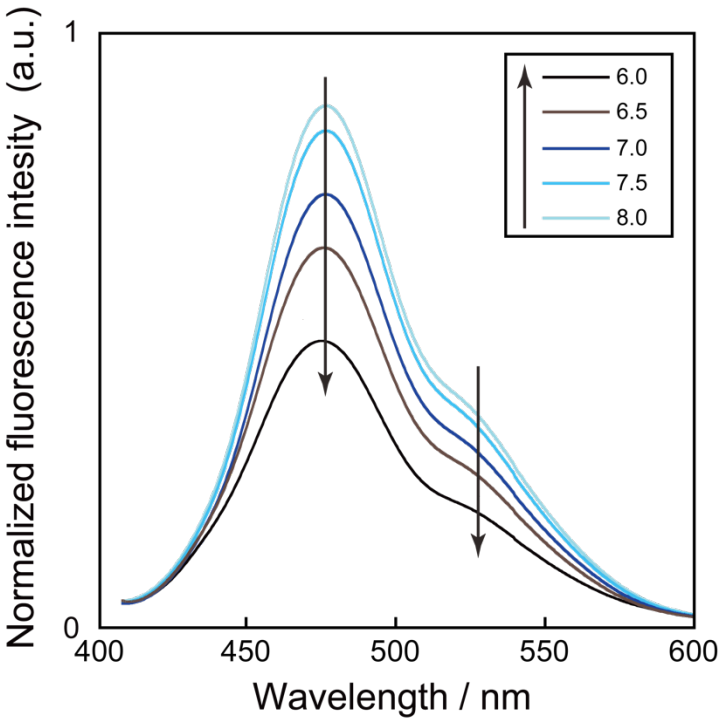

Figure S4. Fluorescence spectral changes of Bimane/Trp-βh-RGD aqueous solution depending on pHs at 37°C. [peptide] = 40 μM.

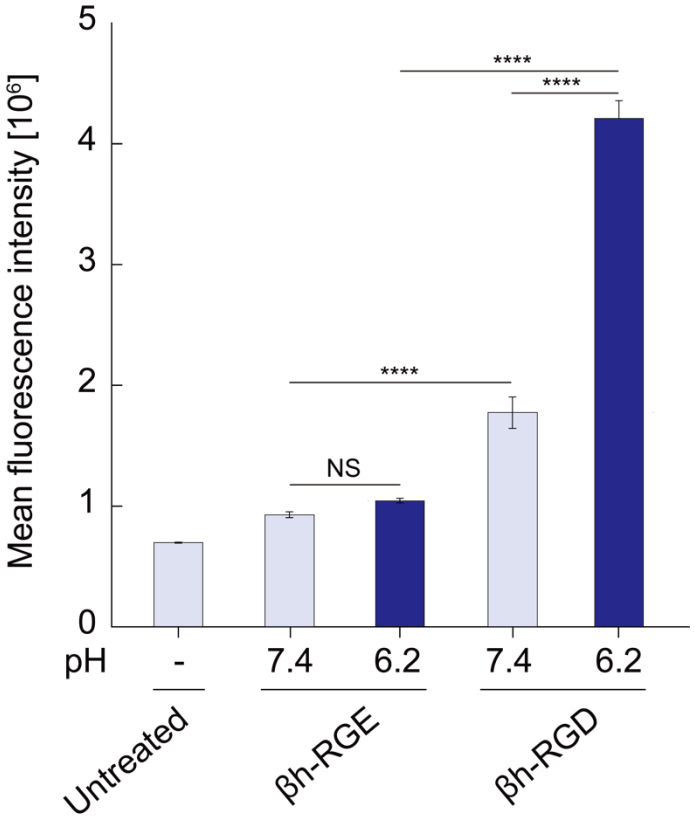

Figure S5. Mean fluorescence intensity of MDA-MB-231 cells with the FITC-βh-peptides at pH 7.4 and 6.2 for 6 h (n = 3). **** $p < 0.001$; NS, not significant.

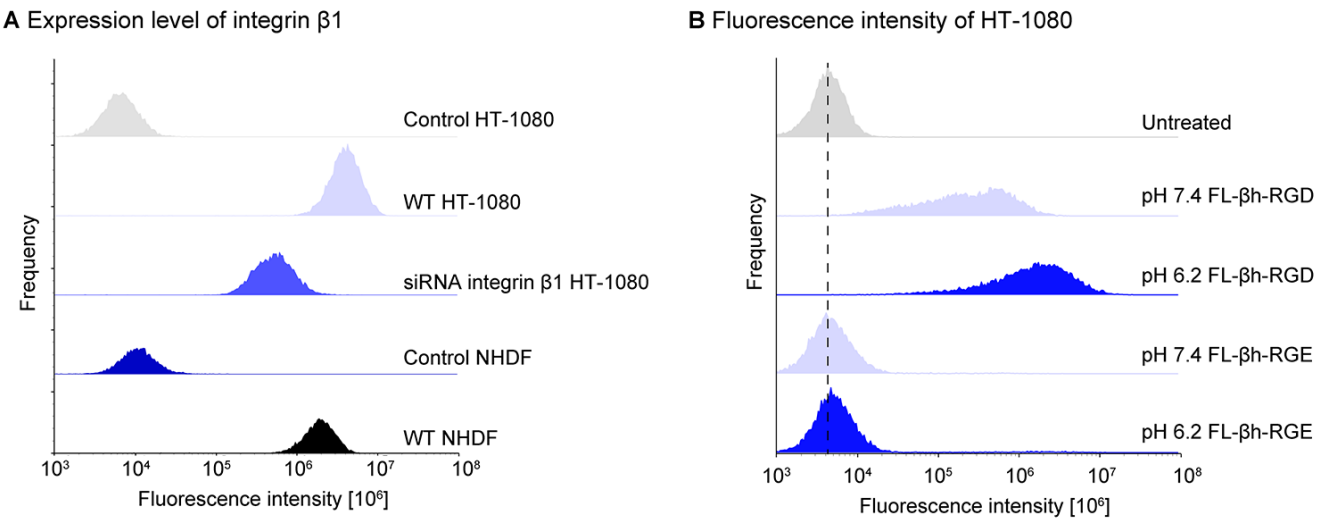

Figure S6. A) Histogram for fluorescence intensity of HT-1080 and NHDF cells treated with APC-anti-human integrin β1. Control and wild type (WT) were ascribed to siRNA-nontreated cells with and without the APC-anti-human integrin β1, respectively. Integrin β1 expressed in HT-1080 was down-regulated with siRNA treatment (siRNA integrin β1 HT-1080) B) Fluorescence intensity histogram of HT-1080 treated with FITC-βh-RGD and FITC-βh-RGE at pH 7.4 and 6.2.