Supporting Information

Bcl-X_L-binding helical peptides possessing D-Ala residues at their C-termini with the advantage of long-lasting intracellular stabilities

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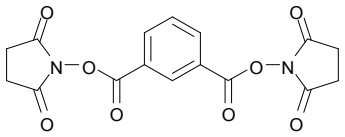
Contents:

Experimental procedures

General procedures and materials: MALDI TOF MS spectra were recorded with 2,5-dihydroxy benzoic acid and α-cyano-4-hydroxycinnamic acid as a matrix by use of a Brüker Autoflex. CD measurements were performed on a J-720WI spectropolarimeter.

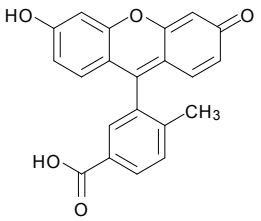
Solid Phase Peptides Synthesis (SPPS): All peptides were synthesized by using standard Fmoc chemistry. Peptides were constructed on an Fmoc-NH-SAL Peg-resin (capacity 0.22 mmol/g WATANABE CHEMICAL IND., Ltd.). The dry resin was swelled with DMF before use. The Fmoc group was removed by treatment with 30% piperidine in DMF (2 × 2 min). Amino acids were coupled for 60 min using 4 equiv of N-[1-(cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino(morphorino)]uronium hexafluorophosphate (COMU) as the activating agent, 4 equiv of Fmoc-protected amino acid, and 8 equiv of diisopropyl ethylamine (DIPEA) in 1-methyl-2-pyrrolidinone (NMP). The N-terminal amino groups of the peptides were acetylated with Ac_2O and *N*-methylmorpholine in DMF for 15 min at room temperature. Peptide cleavage and deprotection of amino acids side chains were performed simultaneously by treating with the TFA/triisopropylsilane/ethanedithiol/H_2O (94:2.5:2.5:1) over 90 min at room temperature. When cross-linking, labeling, and biotinylation were required, these reactions were carried out on the synthetic resin, prior to the peptide cleavage and the side-chain deprotection.

Cross-linking reaction on synthetic resins: After the peptide elongation and N-terminal acetylation, the protecting groups (Dde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl groups) in the Orn residues were selectively removed with hydrazine monohydrate (3% v/v) in DMF for 1 h at room temperature, and this treatment was repeated twice. The deprotected peptide on the synthetic resin was cross-linked with an isophthalic crosslinking agent (5 equiv, see below) over 8 h at room temperature. The isophthalic crosslinking agent was prepared according to the method previously reported.[1]



isophthalic crosslinking agent

Labeling of peptides with TG: After peptide elongation, TokyoGreen (TG) were introduced to the N-terminal amino groups of the peptides instead of conventional Ac capping in SPPS. Resins were incubated in DMF including a TG derivative (5 equiv, see below), PyBOP (10 equiv), HOBt (10 equiv), and *N*-methylmorpholine (10%, v/v) for 8 h at room temperature. The TG derivative was prepared according to the published procedure. [3]



TokyoGreen (TG)

Biotinylation on synthetic resins: Before N-terminal biotinylation, Fmoc-8-amino-3,6-dioxaoctanoic acid was introduced to the N-terminal amino groups of the peptides by the same procedure as SPPS. Resins were incubated in DMF including D-Biotin (10 equiv), PyBOP (10 equiv), HOBt (10equiv), and *N*-methylmorpholine (10%, v/v) for 1 h at room temperature.

Peptide purification: Peptides were purified by reversed-phase HPLC (column; COSMOSIL 5C_{18}-AR-II nakalai tesque, 10 × 150 mm) and were eluted with 0.1 % TFA buffer and CH_3CN, including 0.1% TFA, linear gradients at a flow rate of 2.0 mL/min; 20-70% (0-50 min).

MS data for the peptides:

D0: *m/z*: calcd for $[M+H]^+$, $C_{141}H_{218}N_{41}O_{41}S$: 3173.60; found 3173.84
D1: *m/z*: calcd for $[M+H]^+$, $C_{144}H_{223}N_{42}O_{42}S$: 3244.63; found 3244.81
D2: *m/z*: calcd for $[M+H]^+$, $C_{147}H_{228}N_{43}O_{43}S$: 3315.67; found 3316.02
D3: *m/z*: calcd for $[M+H]^+$, $C_{150}H_{233}N_{44}O_{44}S$: 3386.71; found 3386.96
D4: *m/z*: calcd for $[M+H]^+$, $C_{153}H_{238}N_{45}O_{45}S$: 3457.74; found 3457.87
D5: *m/z*: calcd for $[M+H]^+$, $C_{156}H_{243}N_{46}O_{46}S$: 3528.78; found 3528.85
L3: *m/z*: calcd for $[M+H]^+$, $C_{150}H_{233}N_{44}O_{44}S$: 3386.71; found 3387.08
TG-D0: *m/z*: calcd for $[M+H]^+$, $C_{160}H_{228}N_{41}O_{44}S$: 3459.66; found 3460.08
TG-D1: *m/z*: calcd for $[M+H]^+$, $C_{163}H_{233}N_{42}O_{45}S$: 3530.70; found 3530.87
TG-D2: *m/z*: calcd for $[M+H]^+$, $C_{166}H_{238}N_{43}O_{46}S$: 3601.73; found 3601.91
TG-D3: *m/z*: calcd for $[M+H]^+$, $C_{169}H_{243}N_{44}O_{47}S$: 3672.77; found 3672.81

TG-D4: *m/z*: calcd for $[M+H]^+$, $C_{172}H_{248}N_{45}O_{48}S$: 3743.81; found 3744.43
TG-D5: *m/z*: calcd for $[M+H]^+$, $C_{175}H_{253}N_{46}O_{49}S$: 3814.84; found 3815.17
TG-L3: *m/z*: calcd for $[M+H]^+$, $C_{169}H_{243}N_{44}O_{47}S$: 3672.77; found 3672.32
Biotin-D0: *m/z*: calcd for $[M+H]^+$, $C_{155}H_{241}N_{44}O_{45}S_2$: 3502.74; found 3503.18

CD measurement for the peptides in buffer solution (Fig. S1, Table S1): CD measurements were performed with a quartz cell having 1.0 mm path length. The peptides were dissolved in a 100 mM phosphate buffer (pH 6.5), and their spectra were obtained at 25 °C.

$$\text{Helical content (\%)} = -([\theta]_{222}+2340)\times 100 / 30300$$

Binding assay based on Surface plasmon resonance (SPR) (Table S1): The Biacore system (GE Healthcare, Chicago, United States) was utilized for exploring the bindings of peptides to Bcl-X_L according to the published procedure.[4] Streptavidin was immobilized onto a sensor chip CM5 that possesses dextran-carboxylic acids on its surface using running buffer (pH 7.4) consisting of 10 mM HEPES and 150 mM NaCl at 25 °C. After the activation of the carboxy groups on the sensor chip with 200 mM *N*-[3-(dimethylamino)propyl]-*N*'-ethylcarbodiimide hydrochloride and 50 mM *N*-hydroxy succinimide for 7 min, streptavidin dissolved in a 10 mM acetate buffer (pH 5.0) was injected into the flow cell. Next, **biotin-D0** dissolved in a 10 mM HEPES buffer (pH7.4) including 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20 was injected into the flow cell. Bcl-X_L proteins (final concentration 5 nM) were incubated with tested peptides (final concentration from 0 to 100 nM) for at least 2 h prior to injection onto a **biotin-D0-**immobilized sensor chip CM5. Response units were measured at 200 seconds in the association phase and the specific binding response to the surface was quantified by subtracting the signal obtained on the control channel from the signal obtained on the **D0** immobilizing channel. IC_{50} values were calculated by simultaneous non-linear regression using BIA evaluation software (GE Healthcare, Chicago, United States).

Cleavage of peptides by carboxypeptidaseY (Fig. 2, Table S1): Carbxypeptidase Y was purchased from Oriental Yeast Co., Ltd. A peptide (**D0, D1, D2, D3, D4, D5, L3**; 30 μM), peptidase (1 μM), and tryptophan (30 μM; internal control) were incubated in phosphate buffer (100 μL; pH 6.5) at 37 °C. Before HPLC analysis, the mixture was treated with aqueous TFA (2%, 100 μL).

Microscope analysis (Fig. 3-4, S2-S4): HCT-116 cells were provided from RIKEN Cell Bank. The cells (1.5×10^6 cells) were aliquoted in six-well plates and treated with **TG-D0**, **TG-D1**, **TG-D2**, **TG-D3, TG-D4, TG-D5, TG-L3** (1 μM) in DMEM containing FBS (10%). Samples were washed with the peptide-free media after incubation. Nuclear DNA was stained with a Hoechst 33342 (Invitrogen/Life Technologies), as described previously.[5] Mitochondria were stained with Mito-Tracker RED CMXRos (Invitrogen/Life Technologies), according to the manufacture's protocol. Cells were analyzed after 5 h or 48 h with an IX71 inverted microscope equipped with a DP30BW CCD camera (Olympus). Images were obtained through a 60×/1.42 NA Plan Apo oil immersion objective lens and analyzed with MetaMorph software (version 7; Molecular Devices). Dots from the whole cell and from mitochondria were quantified in eleven individual confocal layers per cell.

Annexin V binding assay (Fig. 5): HCT-116 cells (1.5×10^6 cells) were aliquoted in 6-well plates and treated with **D0, D1, D2, D3, D4** and **D5** (10 μM) in DMEM containing FBS (10%). Samples were washed with PBS after 48 h and then fluorescein—annexin V and propidium iodide (Annexin-V-FLUOS Staining Kit; Roche) were added. The mixtures were incubated for 15 min at room temperature in the dark, according to the manufacturer's protocol. The cells were analyzed by using an IX71 inverted microscope (Olympus) equipped with a DP30BW CCD camera (Olympus). We analyzed four hundred cells in one experiment and performed independent three experiments to obtain the data in Fig. 5.

Expression and purification of His-tag fusion proteins: The recombinant protein Bcl-X_L for *in vitro* experiments was fused to His6-tag (MAHHHHHHM) at its *N*-terminus. The DNA encoding the Bcl-X_L was inserted into an *Escherichia coli* expression plasmid according to the published procedure. [6] The protein was expressed in C43(DE3)RIPL cells. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.3mM. After 5h cultivation, the cells were centrifuged at 4500 rpm for 10 min at 4 °C. The cells were resuspended in a Tris-HCl buffer (20mM Tris-HCl, 100mM NaCl and 20mM Imidazole at pH 8.0), and then sonicated on ice. The cell lysate was centrifuged at 35000 rpm for 20 min at 4 °C. The supernatant of the cell lysate containing the protein was collected and purified with a nickel-charged resin (Qiagen, Hilden, Germany). Further purification of the protein was performed by gel filtration on a superdex 75 16/60 columm (GE healthcare

Bio-sciences, Pittsburgh, PA, USA) equilibrated with Tris-HCl buffer (20mM Tris-HCl, 100mM NaCl, and 1mM dithiothreitol at pH 8.5). The purity of the protein was checked by SDS-PAGE. The molecular weight of the protein was confirmed by MALDI-TOF MS. Protein concentration was determined with a Nano Drop (Thermo Scientific).

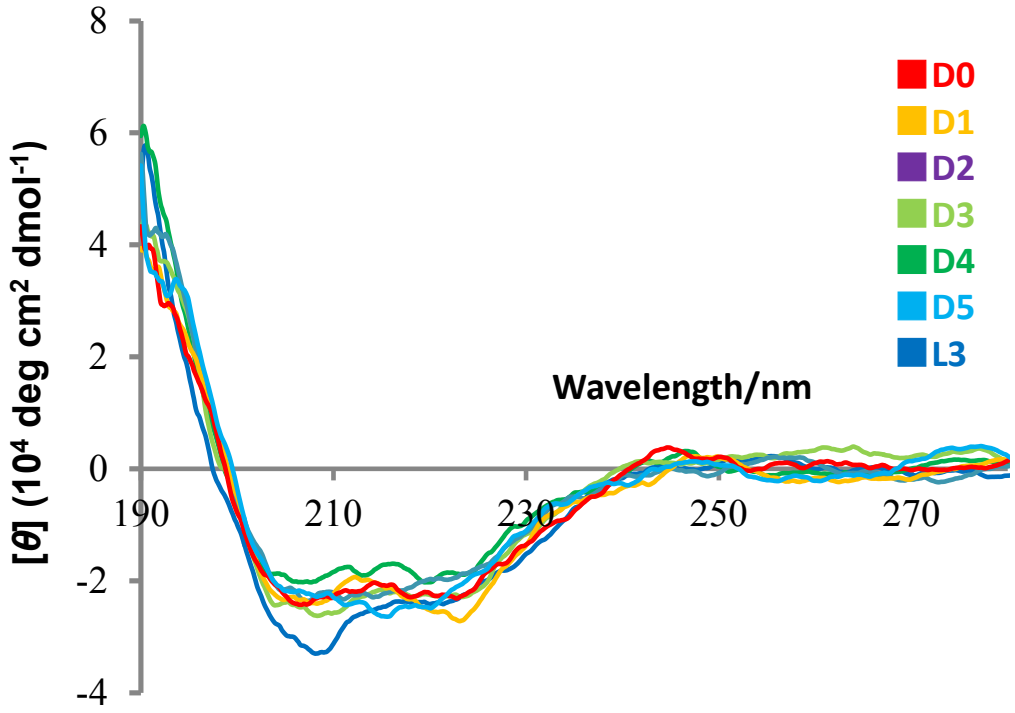


Fig. S1. CD spectra of peptides

	Helical content (%)	IC_{50}（nM）	Half-life（h）
D0	**57±7.3**	**1.86±0.14**	**1.3**
D1	**64±10**	**2.09±0.49**	**10**
D2	**53±1.7**	**2.14±0.06**	**10**
D3	**59±6.0**	**1.94±0.03**	**12**
D4	**50±2.1**	**2.09±0.39**	**12**
D5	**56±6.6**	**2.23±0.11**	**12**
L3	**70±1.2**	**2.14±0.13**	**0.18**

Table S1. Helical content (25°C), IC_{50} and proteolysis data for peptides.

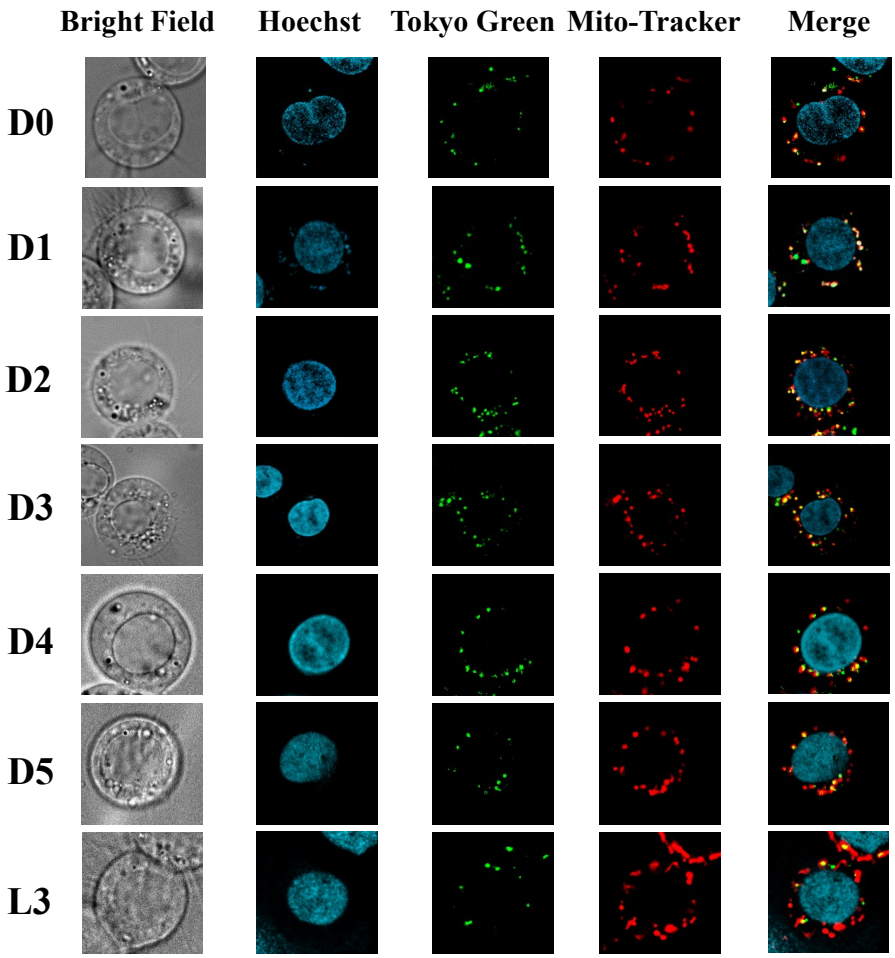


Fig. S2. Cellular uptake and localization of peptides. HCT-116 cells were exposed to each TG-labeled peptide (1 μM) for 5 h. Mitochondria were stained with Mito-Tracker, and nuclei were stained with Hoechst 33342. All images were obtained for an optical section by deconvolution microscopy.

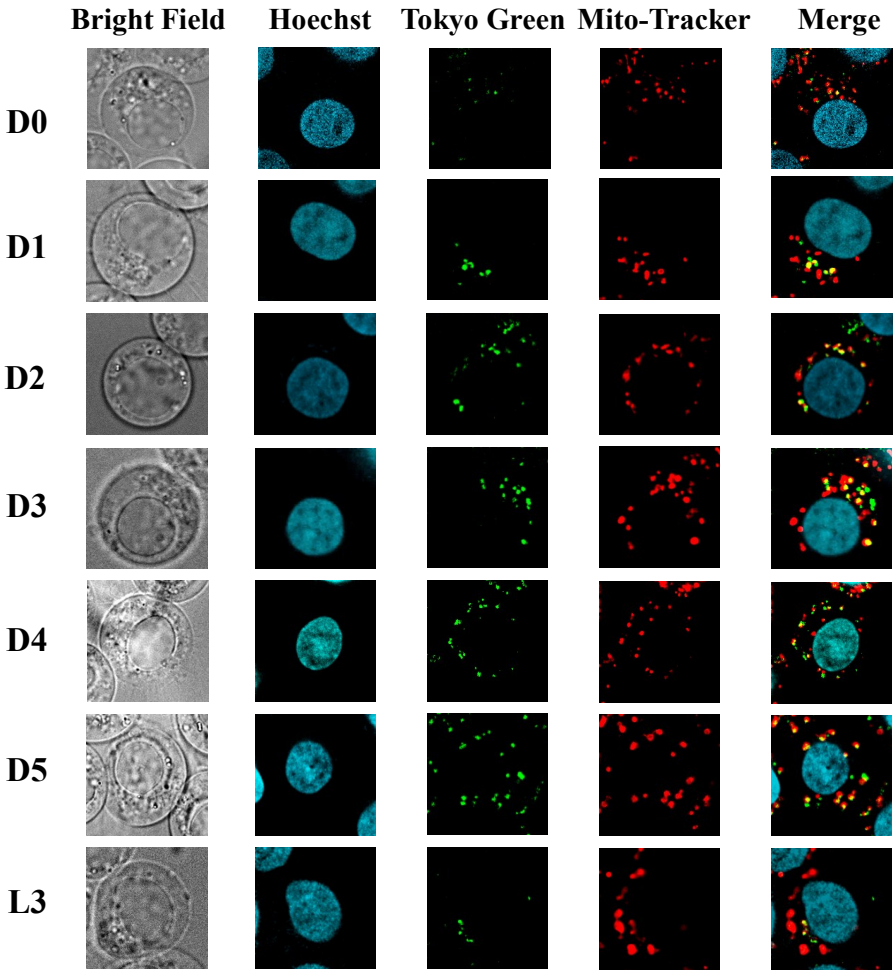


Fig. S3. Cellular uptake and localization of peptides. HCT-116 cells were exposed to each TG-labeled peptide (1 μM) for 48 h. Mitochondria were stained with Mito-Tracker, and nuclei were stained with Hoechst 33342. All images were obtained for an optical section by deconvolution microscopy.

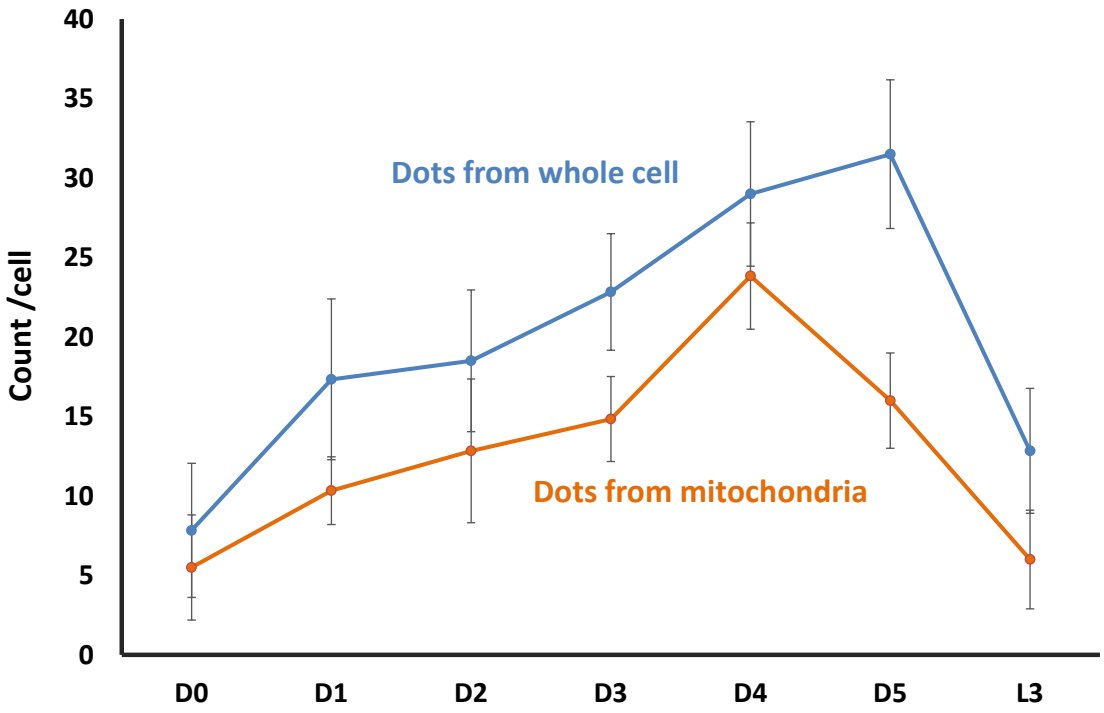


Fig. S4. The number of cellular dots and mitochondrial dots of peptides. HCT-116 cells were exposed to each TG-labeled peptide (1 μM) for 48 h. Mitochondria were stained with Mito-Tracker. All images were obtained for an optical section by deconvolution microscopy and fluorescent dots were counted.

References

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