Supporting Information

Application of HIV-1 viral protein R-derived-peptides as new E3 ligase-binding components of BRD4 degraders

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I. General methods for synthesis and characterization of compounds

I-I. General methods for synthesis

All reactions utilizing air- or moisture-sensitive reagents were performed in dried glassware under an atmosphere of nitrogen or argon (Ar), using commercially supplied solvents and reagents purchased from Sigma-Aldrich, Tokyo Chemical Industry Co., Ltd. (TCI), FUJIFILM Wako Pure Chemical Corporation, KANTO CHEMICAL CO.,INC., NACALAI TESQUE, INC., WATANABE CHEMICAL INDUSTRIES, LTD., KOKUSAN CHEMICAL Co.,Ltd., BLDpharm, Ambeed, Combi-Blocks, and Matrix Scientific without further purification unless otherwise noted. Preparative RP-HPLC was performed using a Cosmosil 5C_{18}-ARII column (20 × 250 mm, Nacalai Tesque, Inc., Japan) on a JASCO PU-2086 plus, PU-2087 plus, and PU-4086-Binary (JASCO Corporation, Ltd., Japan) in a linear gradient of MeCN containing 0.1% TFA (Solvent B) in H_2O containing 0.1% (v/v) TFA (Solvent A) at a flow rate of 10 cm^3 min^{-1}, and eluting products were detected by UV at 220 nm using JASCO UV-2075 plus and UV-4075 (JASCO Corporation, Ltd., Japan).

I-II. Characterization methods

Low-resolution mass spectra were recorded on a Bruker Daltonics micrOTOF focus in the positive and negative detection mode. For analytical HPLC, a Cosmosil 5C_{18}-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc.) was employed with a linear gradient of MeCN containing 0.1% (v/v) trifluoroacetic acid (TFA) (Solvent B) in H_2O containing 0.1% (v/v) TFA (Solvent A) at a flow rate of 1.0 cm^3 min^{-1} on a PU-2089 plus (JASCO Corporation, Ltd.), and eluting products were detected by UV at 220 nm using JASCO UV-2075 plus.

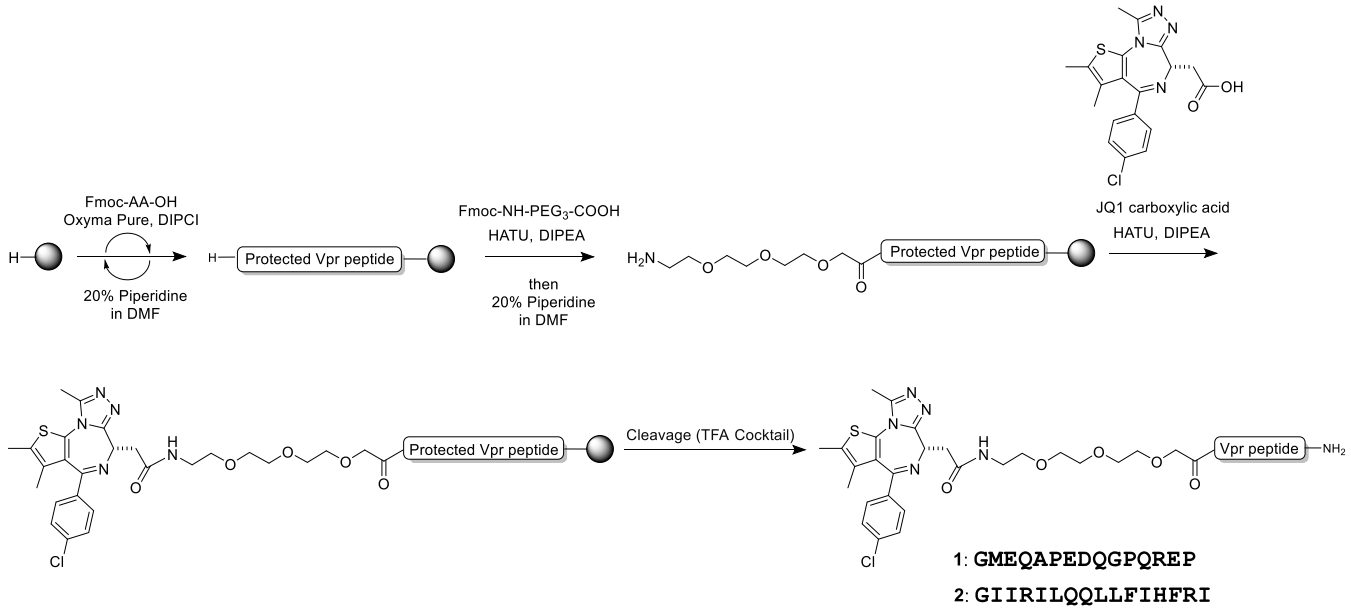
I-III. General Fmoc-based solid phase peptide synthesis

Peptides were synthesized using NovaSyn® TGR resin (0.23 or 0.25 mmol/g). 9-Fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase peptide syntheses (SPPS) were manually and automatically performed (PurePrepChorus, Gyros Protein Technologies, AZ, USA). The following side chain protected amino acids were used: *tert*-butoxycarbonyl (Boc) and 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) for Lys, (2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl (Pbf) for Arg, O^{t}Bu for Asp and Glu, trityl (Trt) for Asn, Cys, Gln, and His, ^{t}Bu for Ser, Thr, and Tyr.

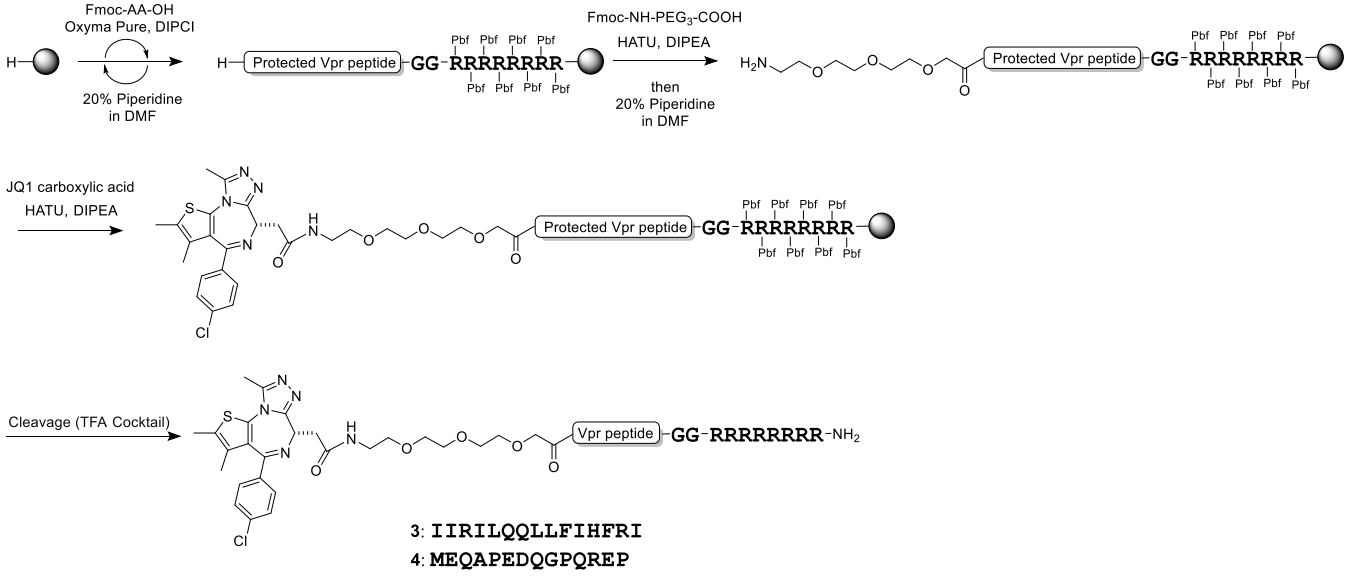
In the automated peptide synthesis procedure (0.1 mmol scale) using NovaSyn® TGR resin, each cycle of SPPS involves (i) 2 min shaking for Fmoc removal (20% piperidine/DMF (6 mL) twice at 50 ºC) and (ii) 5 min shaking for coupling (Fmoc-AA-OH (5 equiv.), Oxyma Pure (5.5 equiv.), and *N*,*N*'-diisopropylcarbodiimide (DIPCI, 5 equiv.) in DMF (6 mL) at 90 ºC). The elongated peptide resins were treated with Ac_2O (20 equiv.), pyridine (20 equiv) in DMF for 45 min for acetylation if necessary. Deprotection of the ivDde group on the Lys ε-amine group was performed by treatment with 2% (v/v) hydrazine monohydrate in NMP (two times for 3 h to overnight each). The resin was coupled with Fmoc-NH-PEG3-COOH (1.0 equiv. based on resin loading) using 1-

((dimethylamino)(dimethyliminio)methyl)-1*H*-[1,2,3]triazolo[4,5-*b*]pyridine 3-oxide hexafluorophosphate (HATU, 0.95 equiv. relative to the amino acid) and *N*,*N*-diisopropylethylamine (DIPEA, 2.0 equiv. relative to the amino acid) at room temperature for 2 h with shaking, or Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-miniPEG-OH, 3 equiv. based on resin loading) using HATU (2.85 equiv. relative to the amino acid) and DIPEA (6.0 equiv. relative to the amino acid) at room temperature for 1.5 h with shaking, and JQ-1 carboxylic acid (1.0 equiv. based on resin loading) using HATU (0.95 equiv. relative to the amino acid) and DIPEA (2.0 equiv. relative to the amino acid) at room temperature for 2 h with shaking. After construction of protected peptides on the resins, the resins were extensively washed with DMF, dichloromethane (DCM), and Et_2O and then dried *in vacuo*. The protected peptide was cleaved from the resin with the deprotection of all the protecting groups on their side chain functional groups by treatment with a mixture of trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/H_2O = 95:2.5:2.5 (v/v), TFA/TIPS/H_2O/ethanedithiol (EDT) = 95/2.5/2.5/7.5 (v/v), or TFA/anisole/H_2O = 95:2.5:2.5 (v/v) at room temperature for 2 h. The resin was then filtered off and washed with TFA (3 times). The volatile was removed by nitrogen gas flow, and the residue was precipitated by the addition of cold Et_2O. After centrifugation, the supernatant was removed, and the precipitate was washed with cold Et_2O (3 times). The crude peptide was purified by preparative RP-HPLC. The purified peptide was identified by ESI-TOF MS and analytical RP-HPLC, and was lyophilized to obtain as white powder.

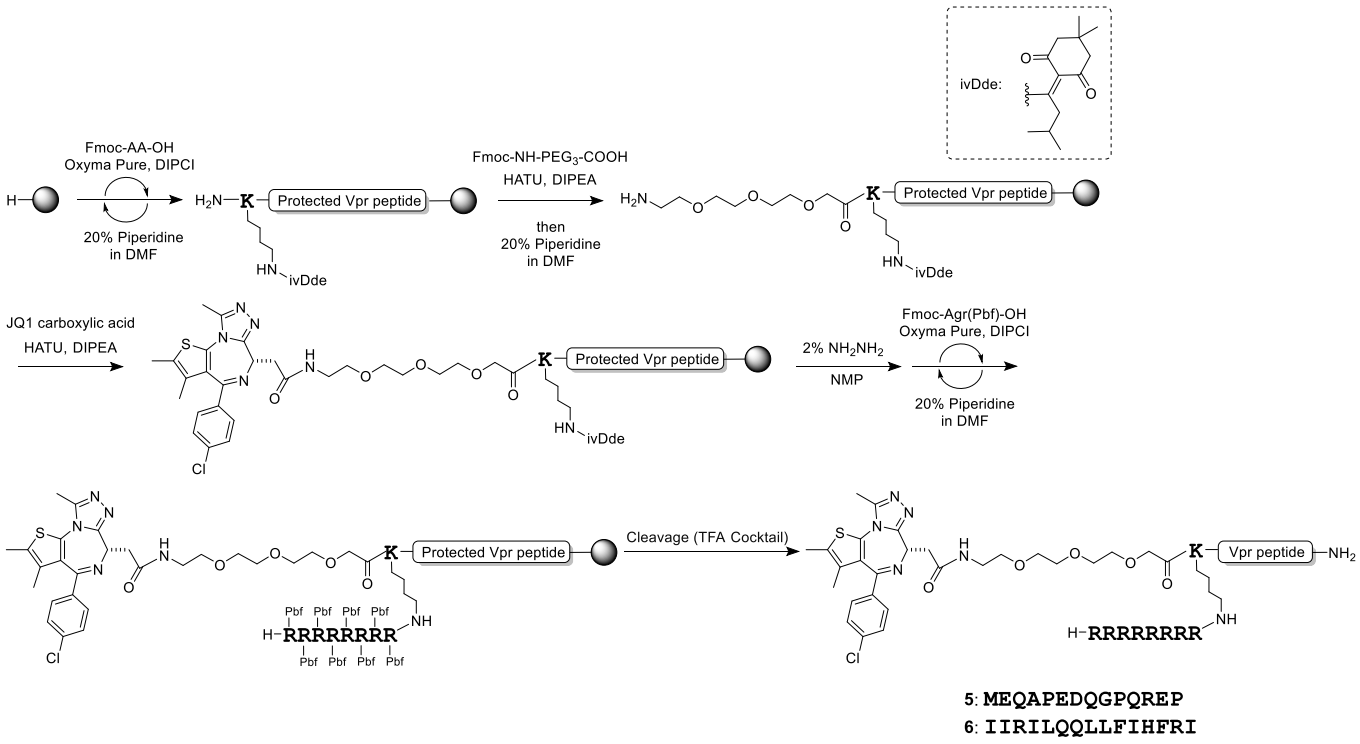
I-IV. Synthesis of BRD4-PROTACs 1–15



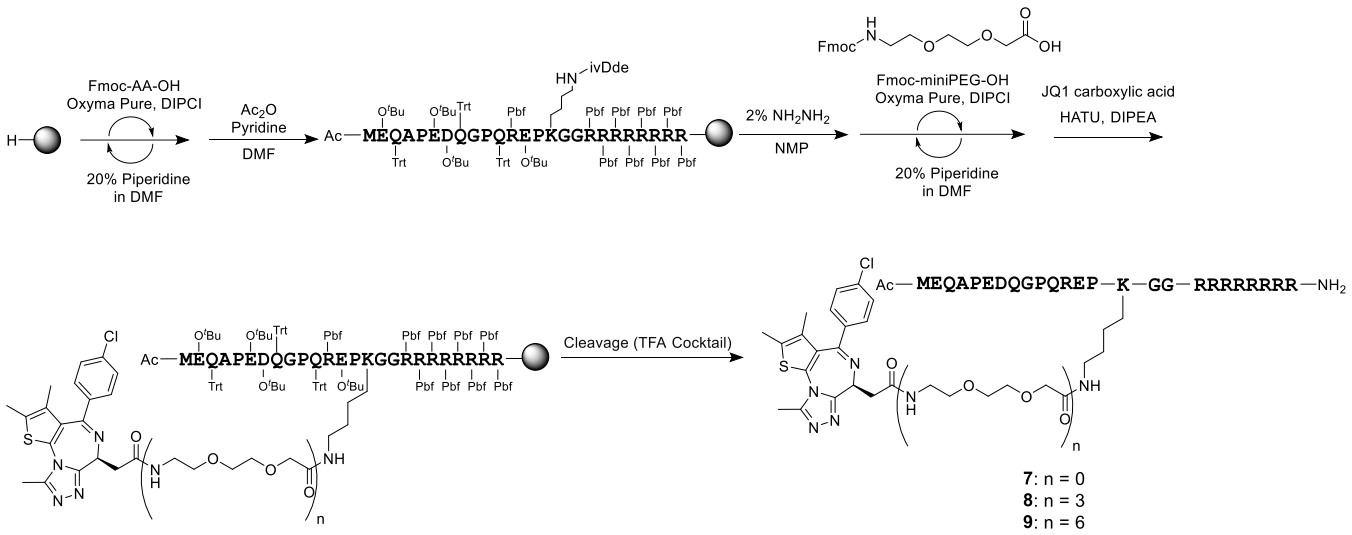
Scheme S1. Synthesis of BRD4-PROTACs **1** and **2** possessing Vpr (1-14) and Vpr (60-74), respectively, as the E3 ligase ligands.



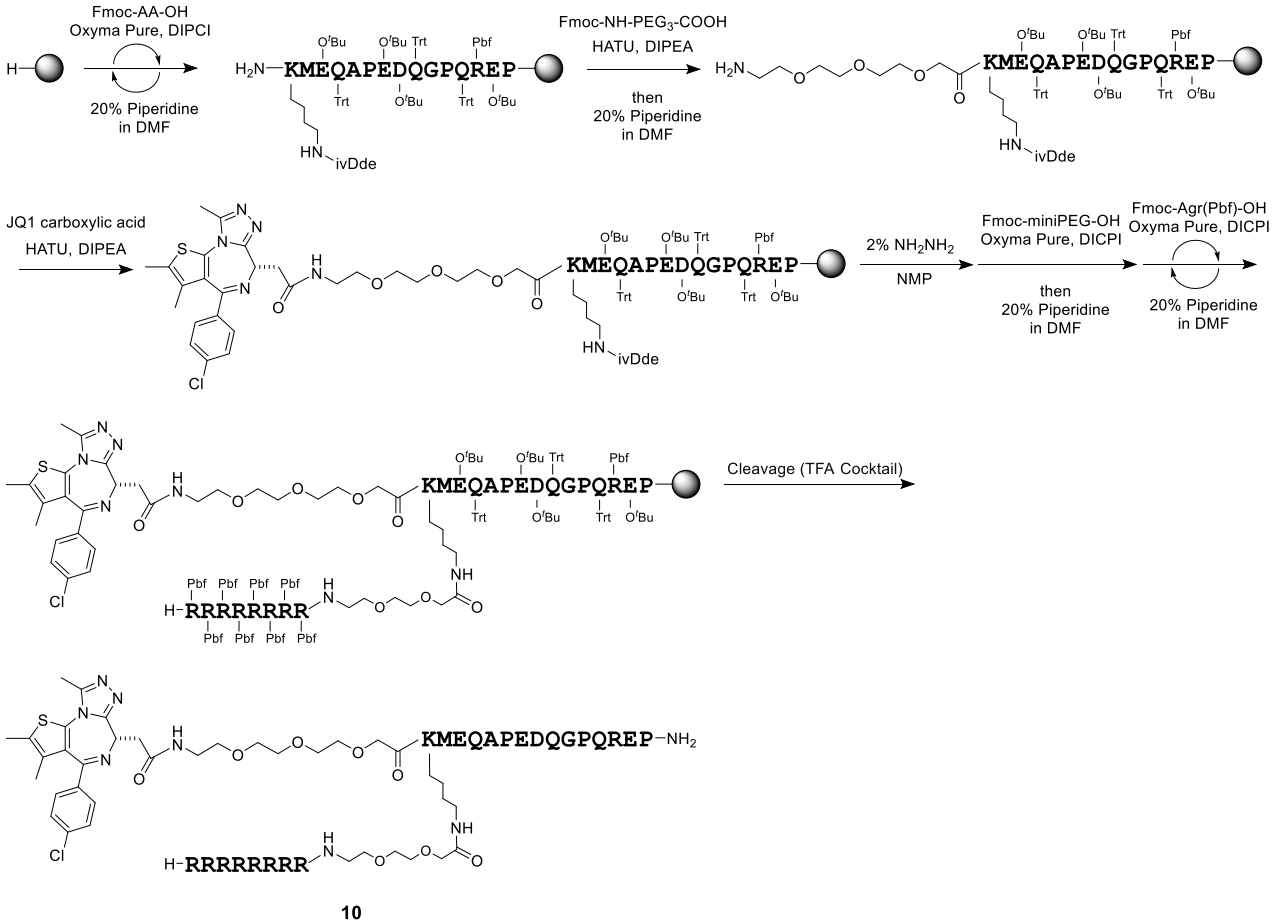
Scheme S2. Synthesis of **3** and **4** possessing Vpr (60-74) and Vpr (1-14), respectively, with octa-arginine at their C-termini.



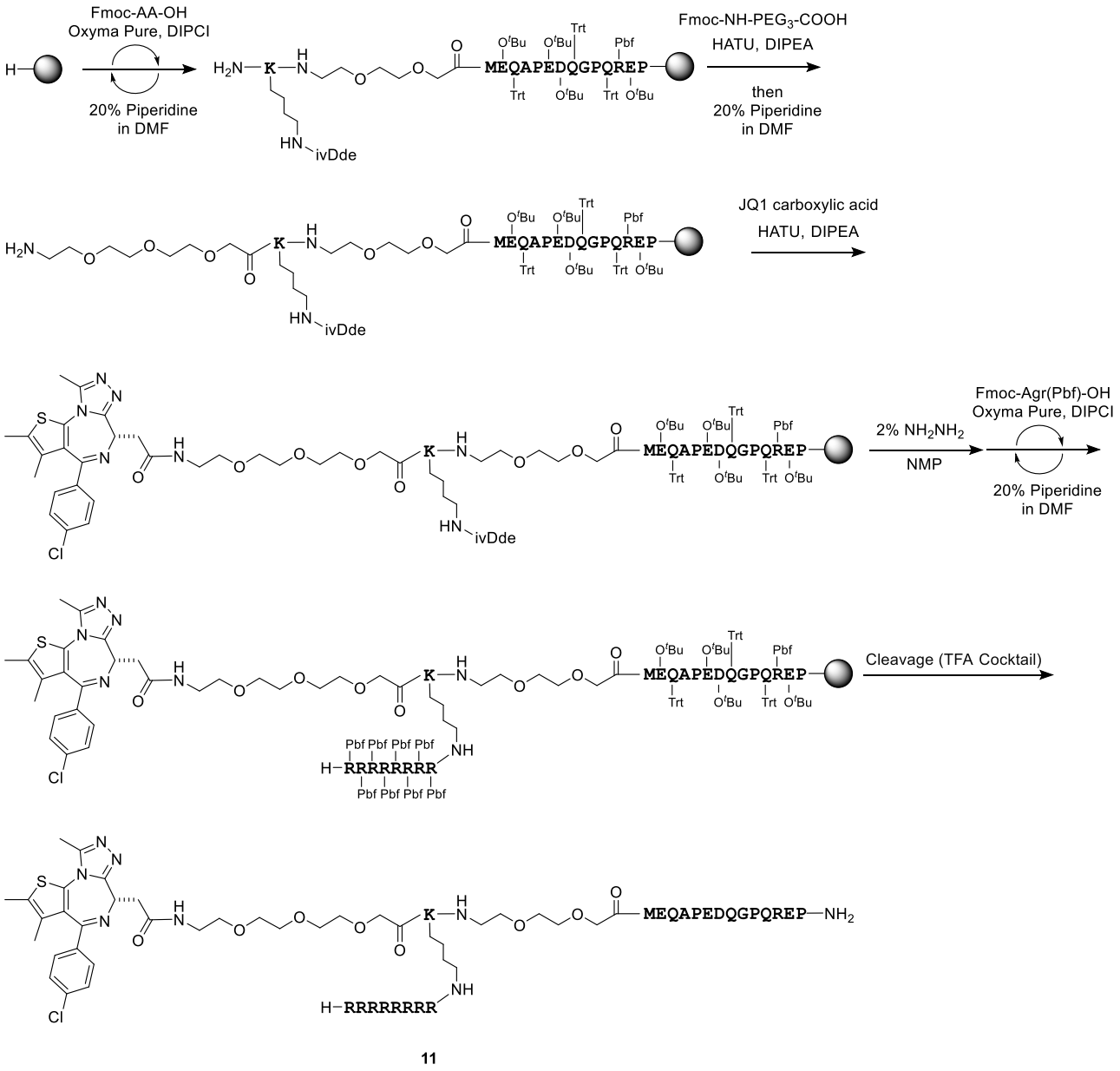
Scheme S3. Synthesis of **5** and **6** possessing Vpr (1-14) and Vpr (60-74), respectively, with octa-arginine at their N-termini.



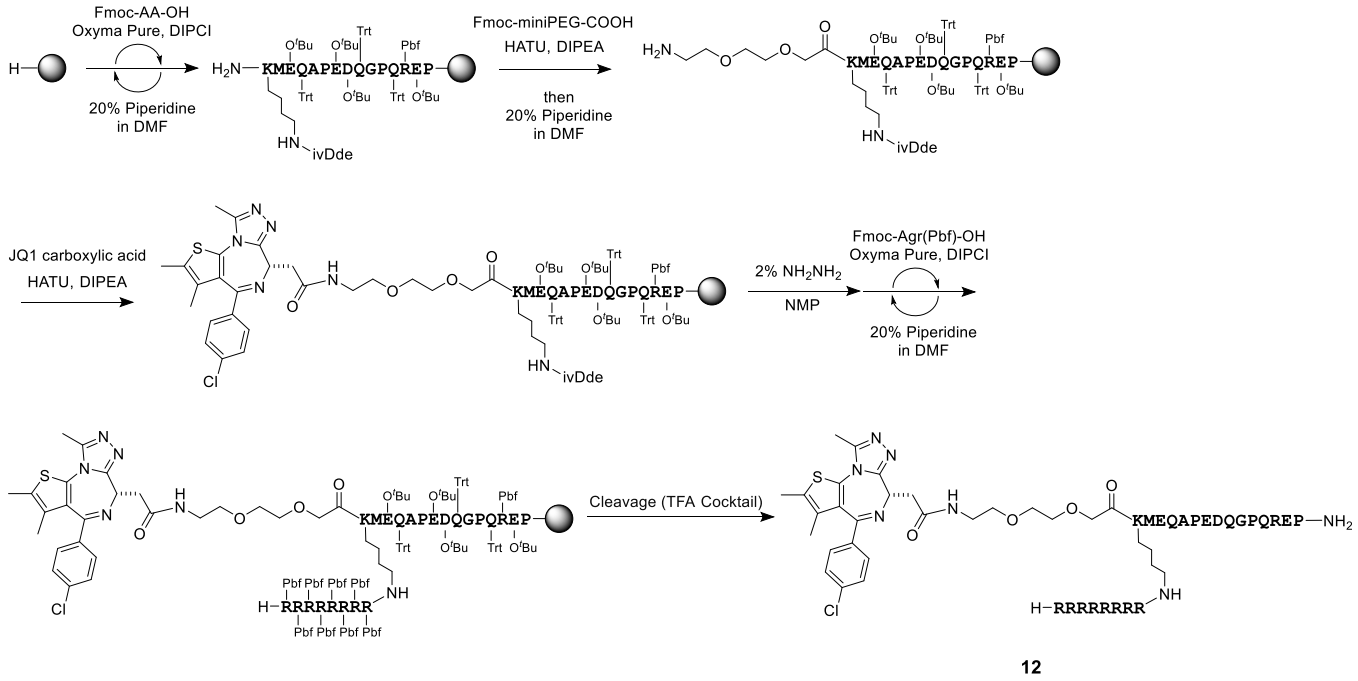
Scheme S4. Synthesis of **7** (n = 0), **8** (n = 3), and **9** (n = 6) possessing Vpr (1-14) peptide conjugated with JQ1 at their C-termini.



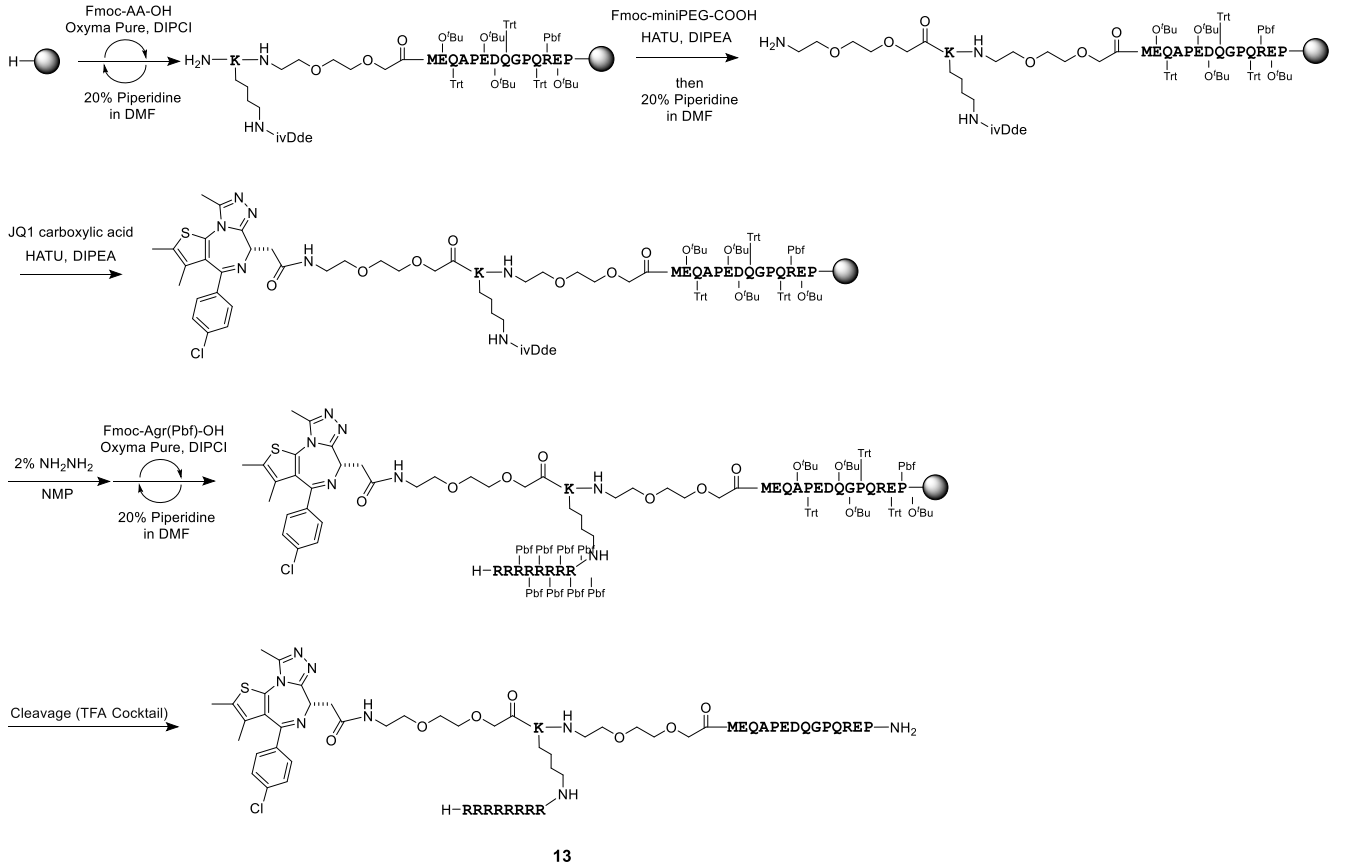
Scheme S5. Synthesis of **10** possessing a miniPEG unit between Lys ε-amino group and octa-arginine.



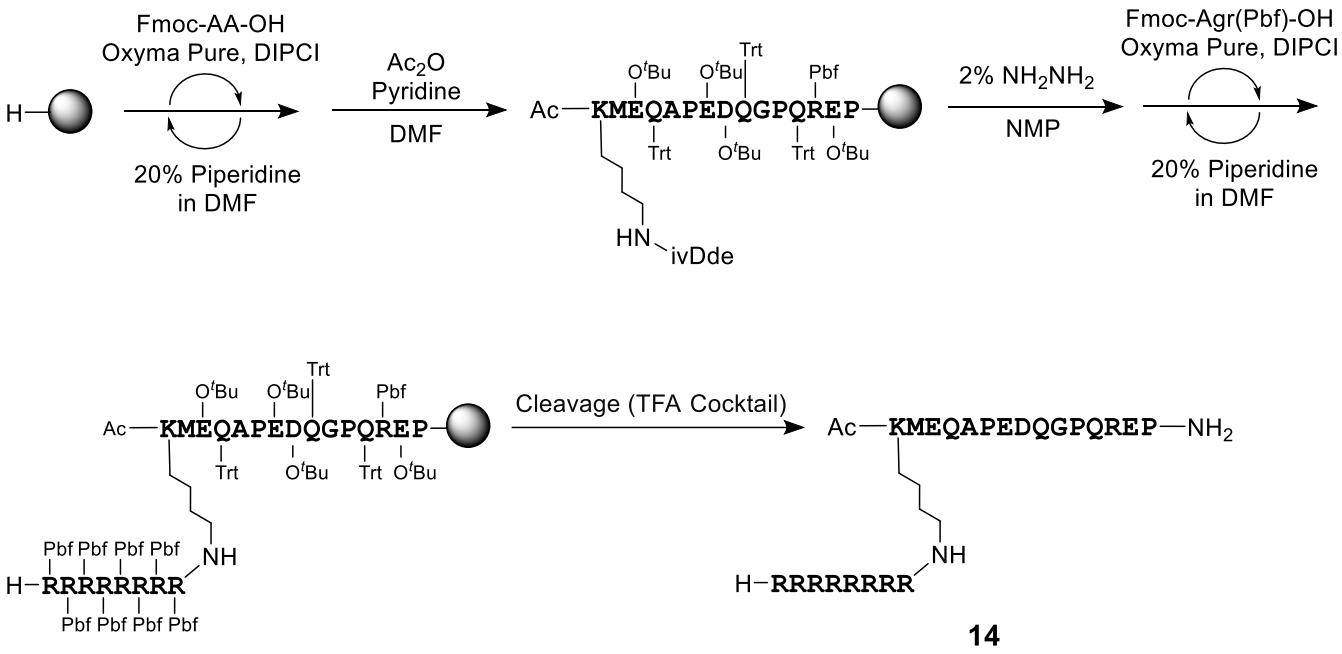
Scheme S6. Synthesis of **11** possessing a miniPEG unit between Lys and Vpr (1-14).



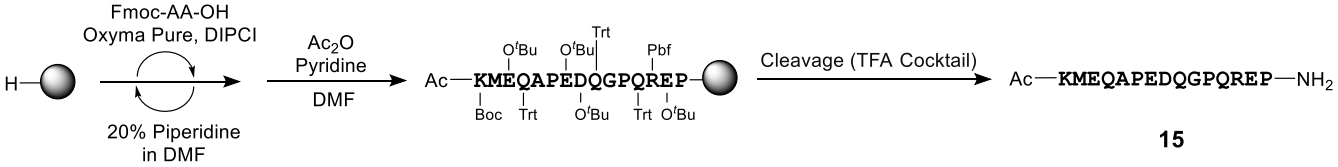
Scheme S7. Synthesis of **12** possessing a miniPEG unit between JQ1 and Lys.



Scheme S8. Synthesis of **13** possessing two miniPEG unit between JQ1 and Lys, and Lys and Vpr (1-14), respectively.



Scheme S9. Synthesis of **14** possessing octa-arginine on Lys ε-amino group.



Scheme S10. Synthesis of Ac-Lys-Vpr (1-14) **15**.

1 to **15** were synthesized following the method mentioned in section **I-III** (Schemes S1–S10), and characterization data of the synthesized peptides were summarized in Table S1. The results of peptides' purity check by analytical RP-HPLC were shown in section **I-V**.

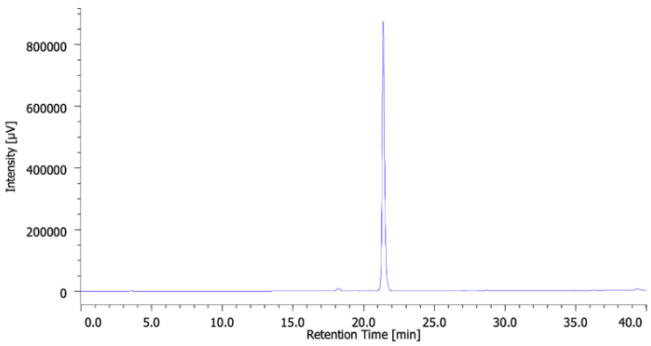
Table S1. Characterization data of synthesized peptides **1**–**15**.

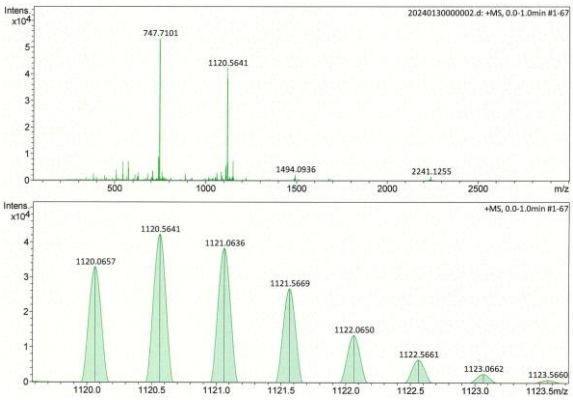
Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield[d] (%)
	[c] t_R (min)	Gradient	Gradient	Calcd.	Found	
1	21.8	15% - 55% / 40 min	25% - 45% / 40 min	1120.0 $[M + 4H]^{4+}$	1120.0	28.7
2	17.0	35% - 75% / 40 min	45% - 65% / 40 min	850.8 $[M + 3H]^{3+}$	850.8	8.2
3	22.1	25% - 65% / 40 min	35% - 55% / 30 min	964.8 $[M + 4H]^{4+}$	964.9	13.3
4	24.5	15% - 55% / 40 min	25% - 45% / 40 min	886.9 $[M + 4H]^{4+}$	887.0	14.2
5	21.9	10% - 50% / 40 min	20% - 40% / 40 min	890.5 $[M + 4H]^{4+}$	890.5	18.4
6	19.0	25% - 65% / 40 min	35% - 55% / 40 min	968.3 $[M + 4H]^{4+}$	968.4	3.3
7	20.2	10% - 50% / 40 min	20% - 40% / 40 min	882.2 $[M + 4H]^{4+}$	882.2	14.3
8	22.0	10% - 50% / 40 min	20% - 40% / 40 min	991.0 $[M + 4H]^{4+}$	991.0	9.4,
9	22.6	10% - 50% / 40 min	20% - 40% / 40 min	1099.8 $[M + 4H]^{4+}$	1099.8	7.9
10	22.4	10% - 50% / 40 min	20% - 40% / 40 min	926.7 $[M + 4H]^{4+}$	926.7	7.2
11	20.9	10% - 50% / 40 min	20% - 40% / 40 min	926.7 $[M + 4H]^{4+}$	926.7	14.7
12	21.8	10% - 50% / 40 min	20% - 40% / 40 min	879.4 $[M + 4H]^{4+}$	879.4	18.5
13	20.6	10% - 50% / 40 min	20% - 40% / 40 min	915.7 $[M + 4H]^{4+}$	915.7	16.4
14	19.4	1% - 41% / 40 min	5% - 25% / 40 min	758.2 $[M + 4H]^{4+}$	758.2	59.8
15	18.0	1% - 41% / 40 min	5% - 25% / 40 min	890.9 $[M + 2H]^{2+}$	890.9	67.4

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution.
[a]Cosmosil C_{18}-AR-II analytical column was employed with a linear gradient of solvent B in solvent A.
[b]Cosmosil C_{18}-AR-II preparative column was employed with a linear gradient of solvent B in solvent A.
[c]Retention time. [d]from NovaSyn®TGR resin.

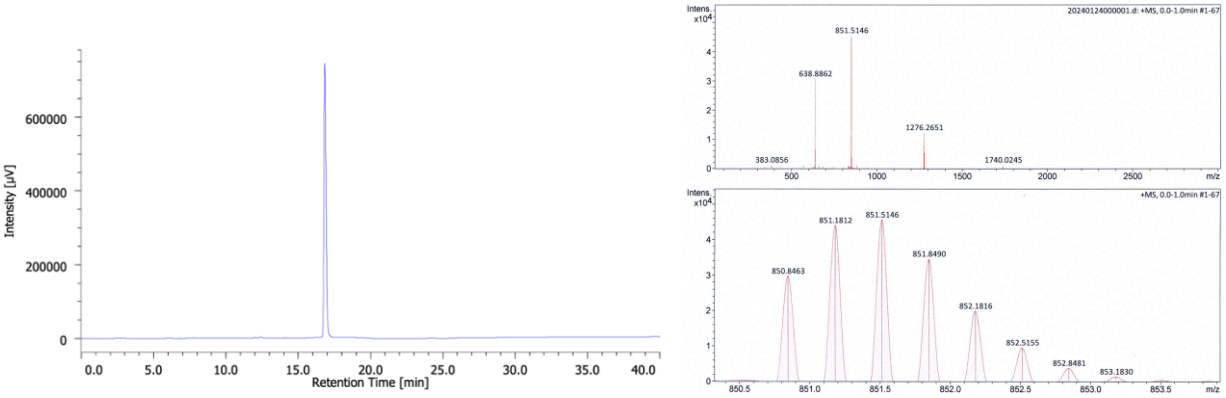
I-V. Analytical HPLC and LRMS data for the synthesized peptides

Peptide **1**

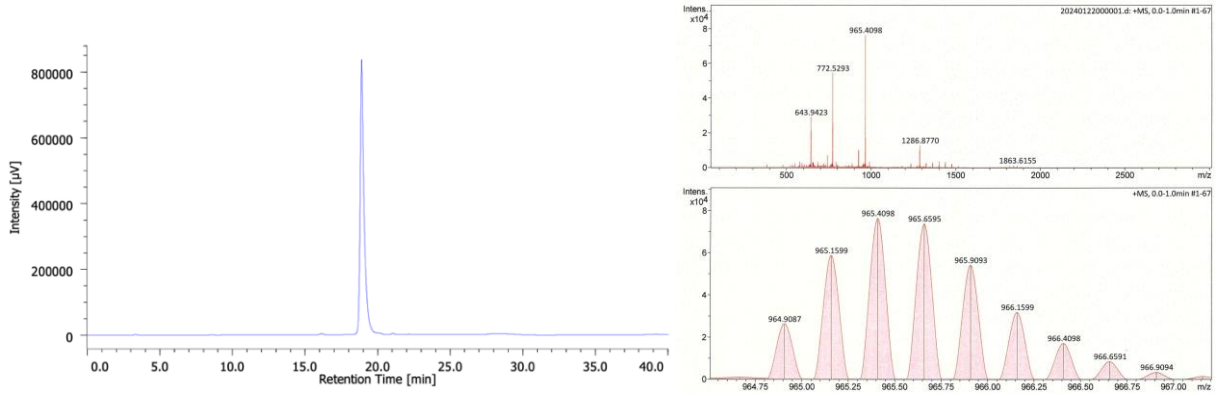




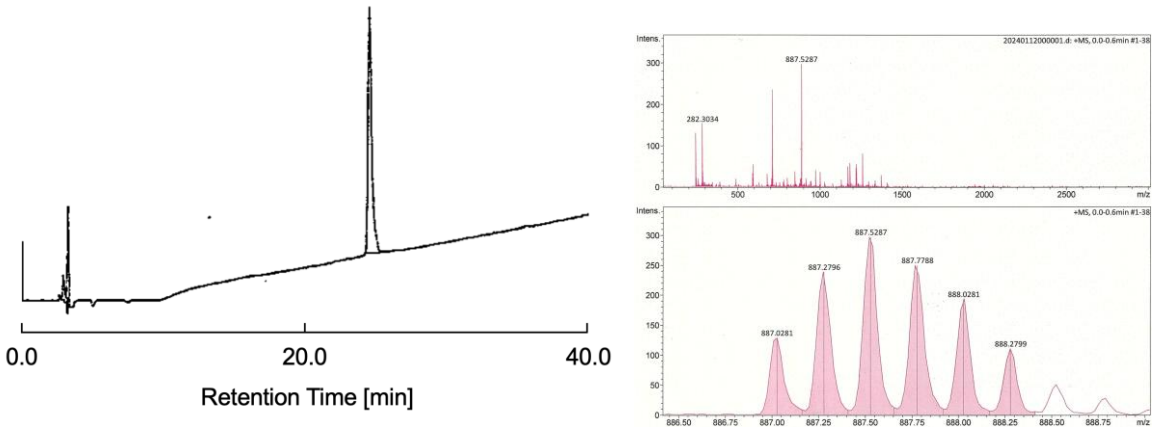
Peptide **2**



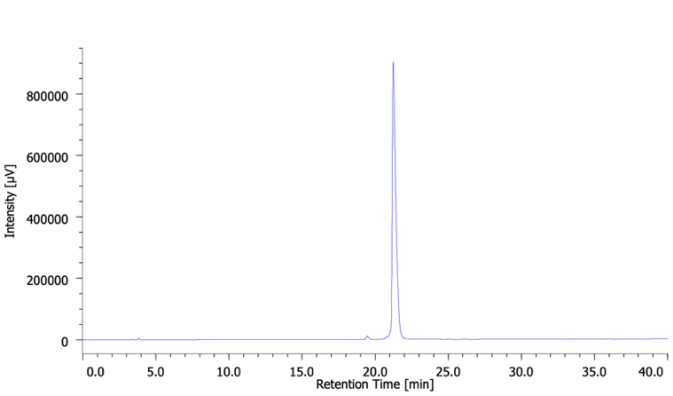
Peptide **3**

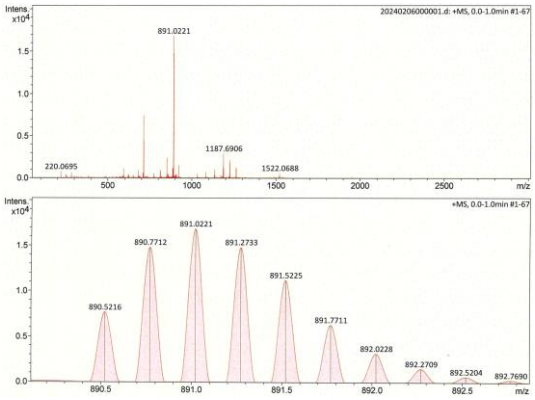


Peptide **4**

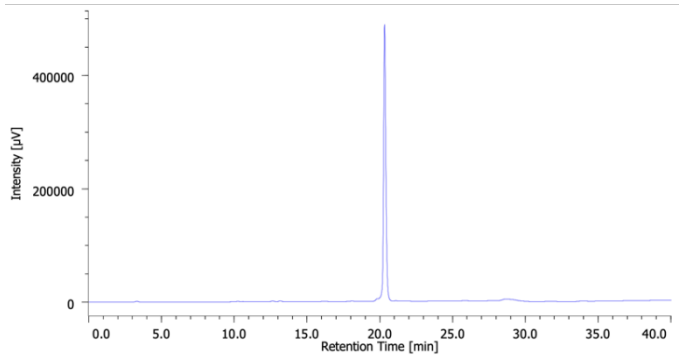


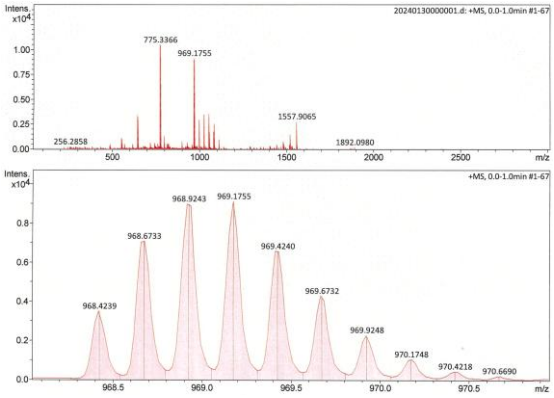
Peptide **5**



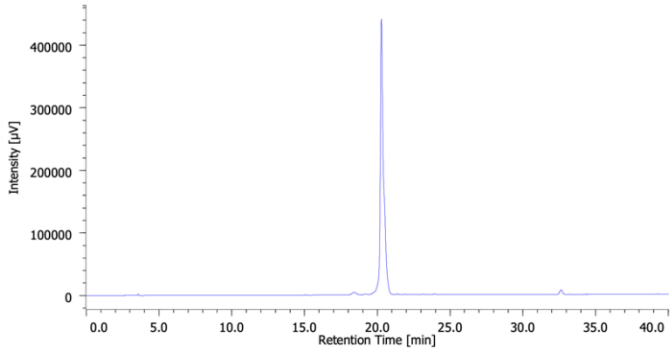


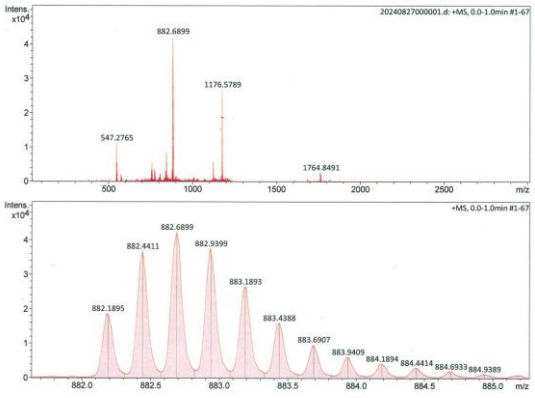
Peptide **6**



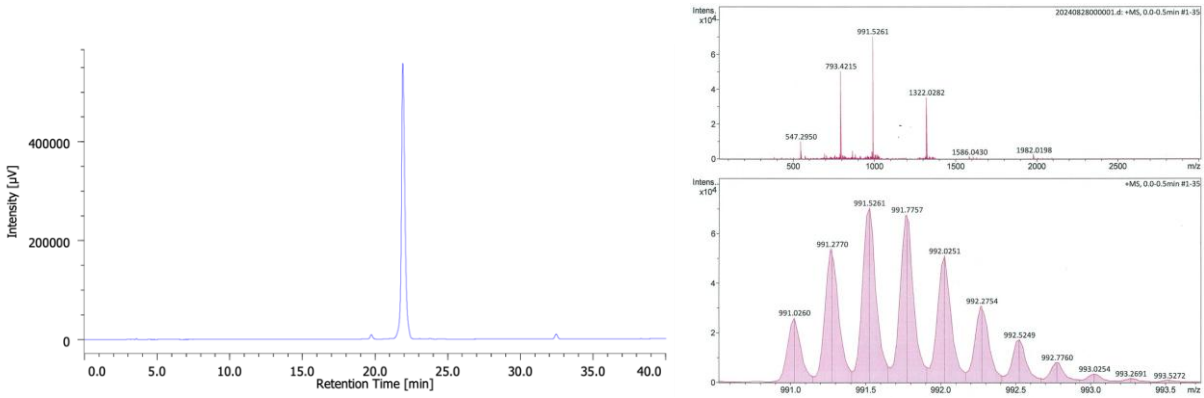


Peptide **7**

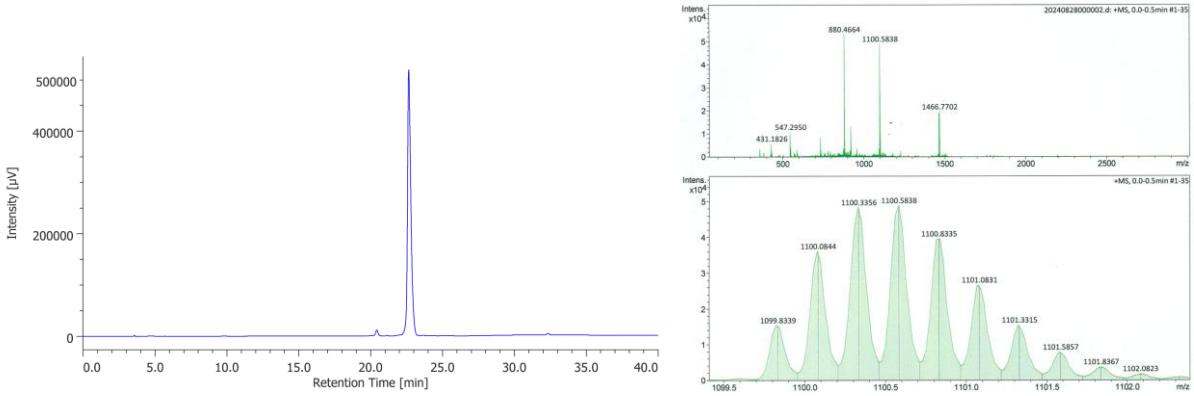




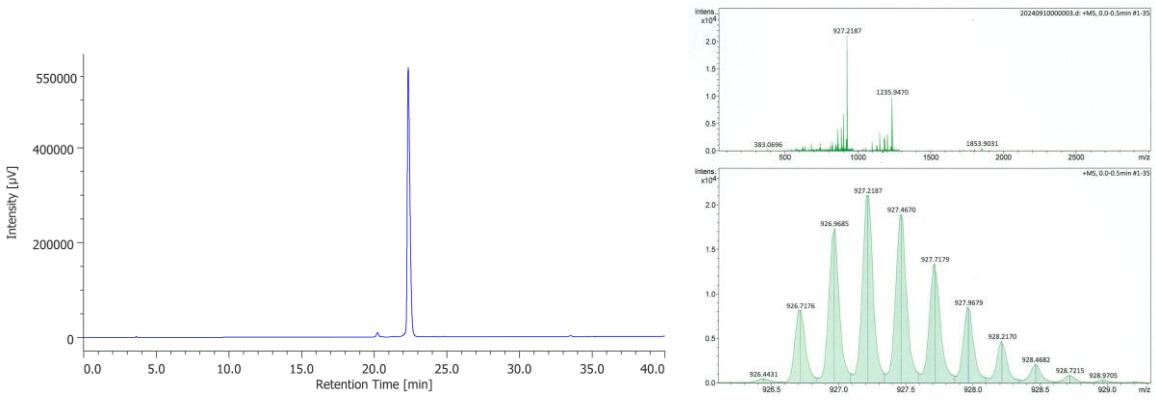
Peptide **8**



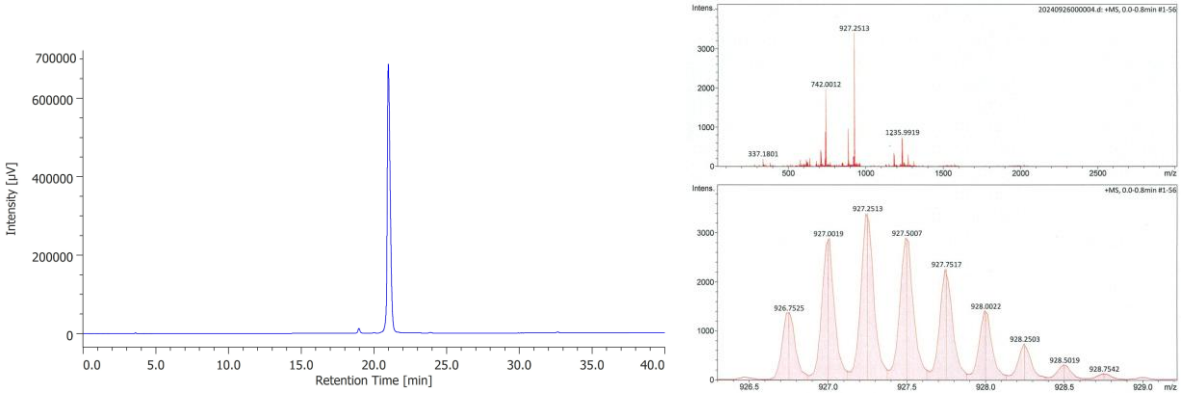
Peptide **9**



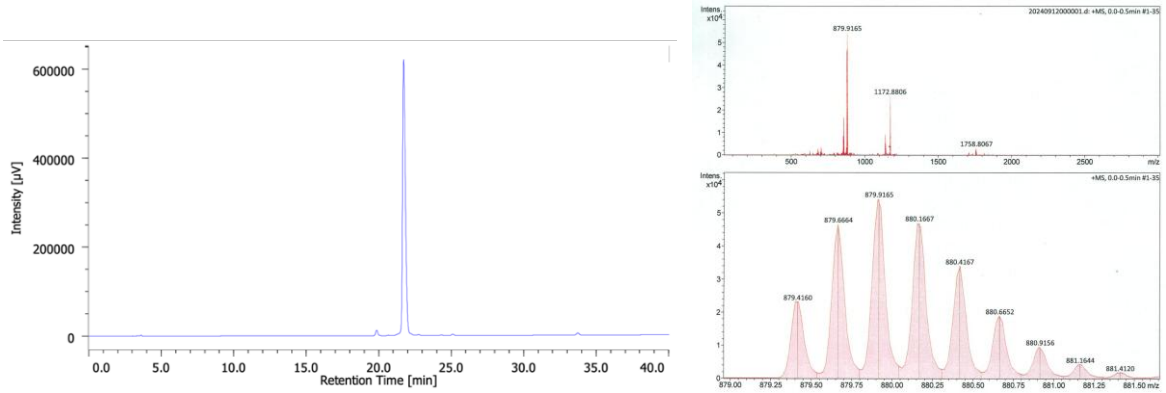
Peptide **10**



Peptide **11**



Peptide **12**



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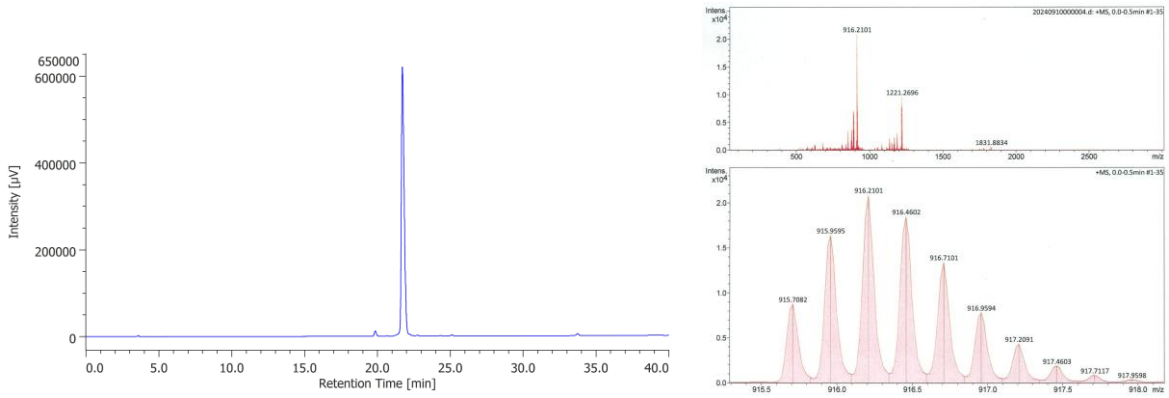
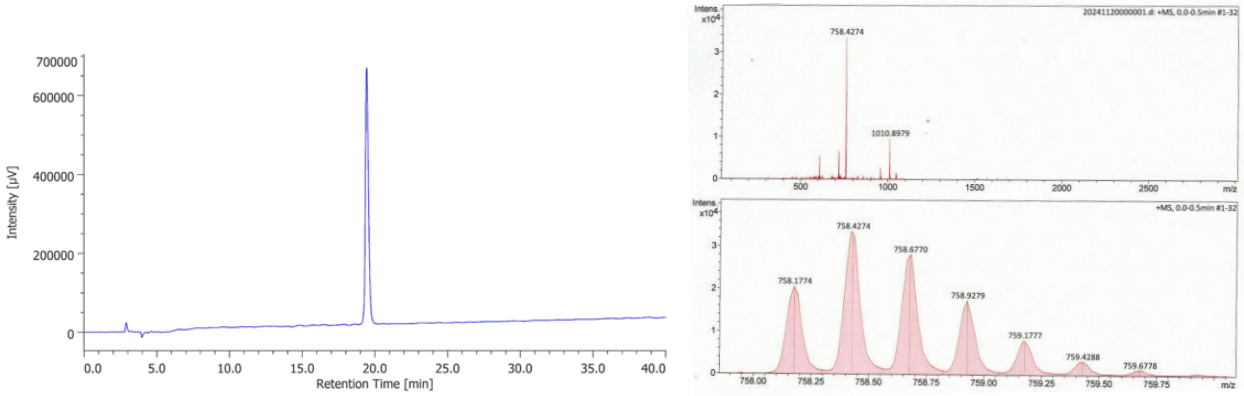
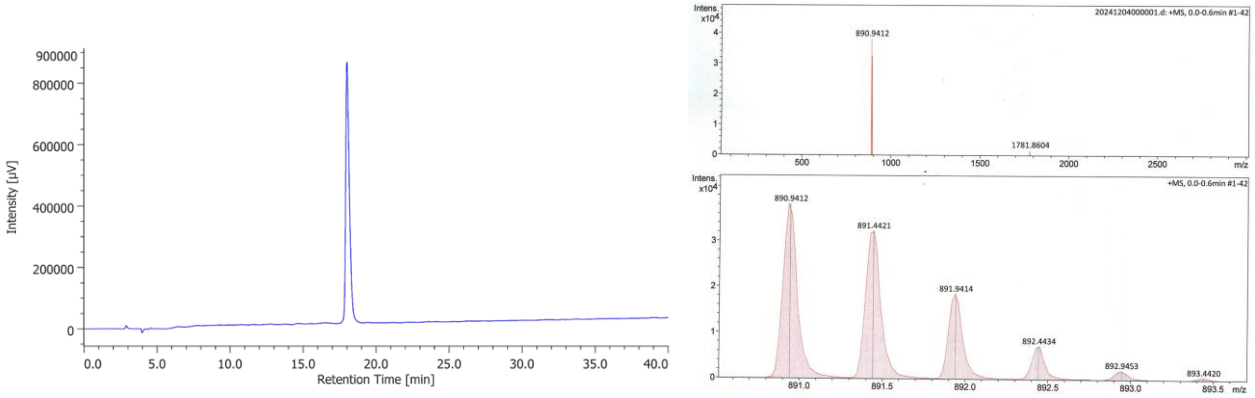


Peptide **14**



Peptide **15**



II. Results from biological experiments

II-I. General methods for western blot assays using MCF-7 cells

Western blot assays using MCF-7 cells to evaluate compounds' BRD4 degradation ability were performed following previously reported method.[S1] Briefly, human breast carcinoma MCF-7 cells (purchased from ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan). The cells were seeded in a 6 well plate (2.0 x 10^5 cells/well) and harvested at 37 °C for 1 day in 5% CO_2 incubator. Then cells were added test compounds and incubated for 1 day. When MG132 (10 μM), MLN7243 (10 μM), or JQ1 (100 μM) was co-treated with the test compounds, the cells were incubated for 8 hours. In the case of MLN4924, MLN4924 (2 μM) was pre-incubated for 1 hour, and then test compounds were added and incubated for 8 hours. The cells were lysed with SDS lysis buffer (0.1 M Tris·HCl at pH 8.0 containing 10% glycerol and 1% SDS) and immediately boiled for 5 min to obtain clear lysates. Protein concentrations of the lysates were determined by the BCA method (Pierce, Washington, USA), and the lysates containing appropriate amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes (Merck) for western blotting. The

transferred membranes were cut for BRD4 and β-actin according to their molecular weight based on the marker bands. Immunoreactive proteins were visualized using the Clarity Western ECL substrate (Bio-Rad, California, USA); light emission intensity was quantified using a ChemiDoc MP Imaging System equipped with Image Lab™ Software (Bio-Rad). The antibodies used in this study were: anti-BRD4 rabbit monoclonal antibody (1:2000, Cell Signaling Technology, Danvers, MA, USA, 13440), and anti-β-actin mouse monoclonal antibody (1:2000, Sigma-Aldrich, 2228).

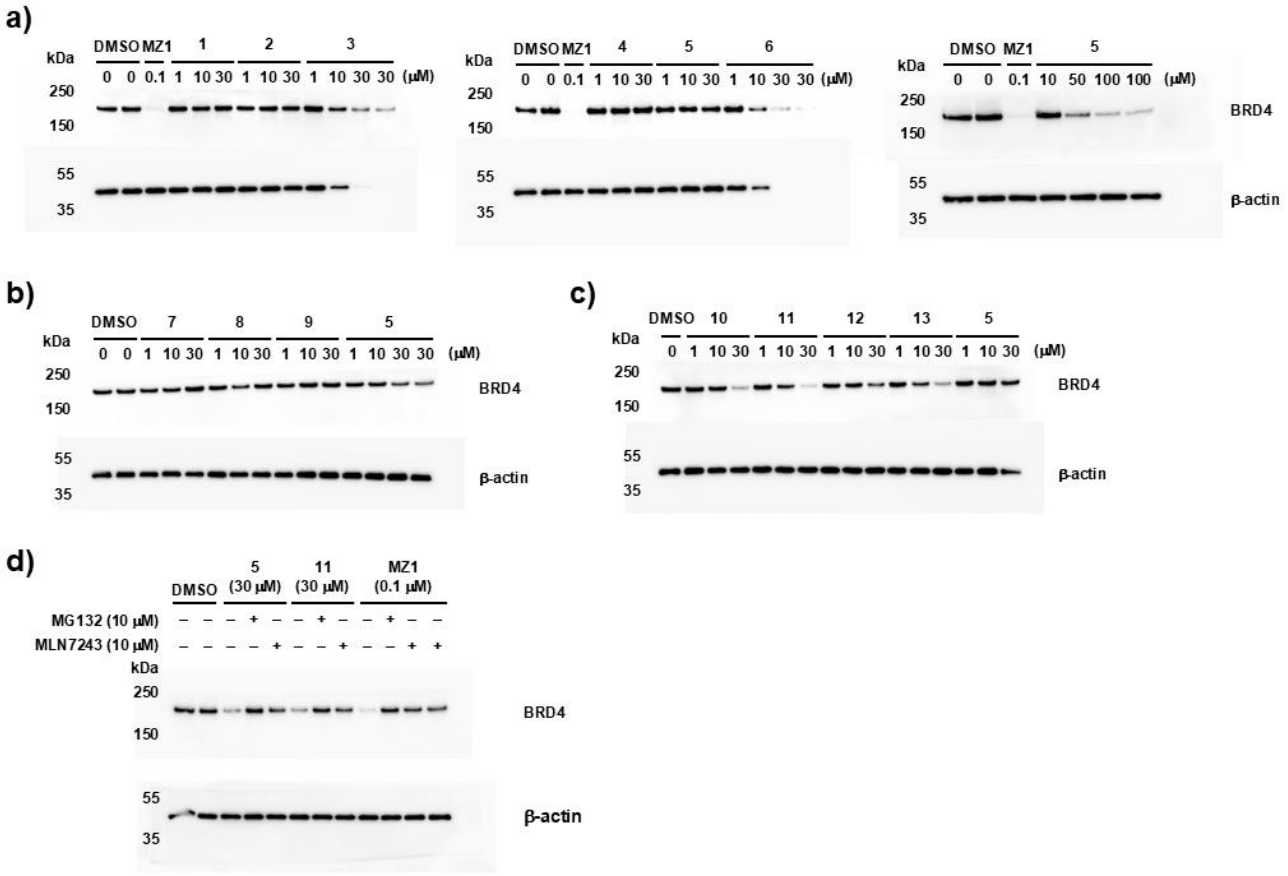


Fig. S1. Raw data of western blot assays to evaluate BRD4 degradation ability of the synthesized compounds shown in Figs. 4, 6, 8, and 9. (a) Data for Fig. 4; (b) data for Fig. 6; (c) data for Fig. 8; (d) data for Fig. 9.

II-II. Results from western blot assays in the presence of MLN4924, JQ1, or Vpr peptides

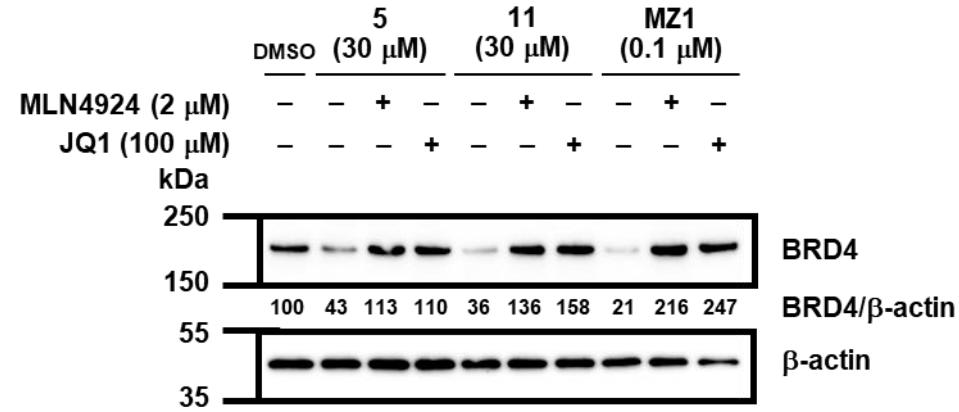


Fig. S2. Results from western blot assays to evaluate BRD4 degradation ability of BRD4-PROTACs **5** and **11** in the presence of MLN4924 (2 μM)) or JQ1 (100 μM).

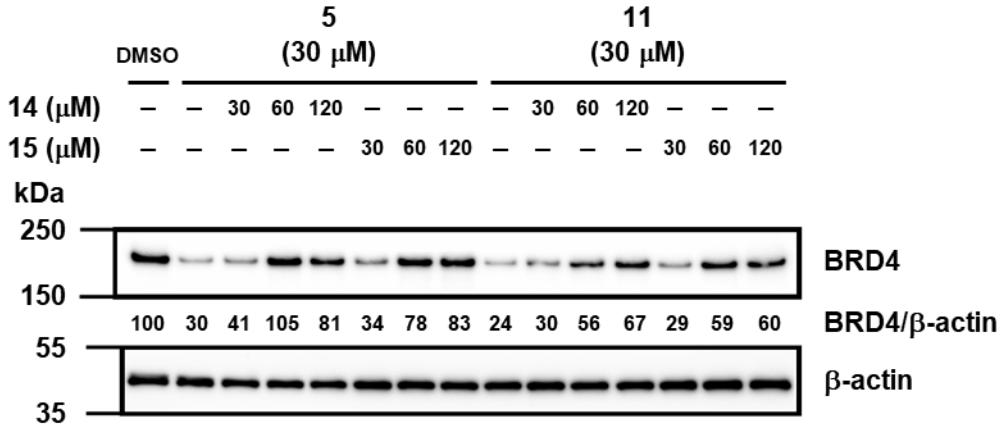


Fig. S3. Results from western blot assays to evaluate BRD4 degradation ability of BRD4-PROTACs **5** and **11** in the presence of Vpr (1-14)-derived peptides **14** or **15** at 30, 60, or 120 μM.

II-III. General methods for MTT assays

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays using MCF-7 cells to evaluate cell viability were performed following previously reported procedure.[S2] MCF-7 cells (kindly provided from Dr. Shinya Fujii, Institute of Science Toyko) were seeded in a 96-well plate (8 x 10^3 cells/well) with 100 μL of Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% FBS, 2 mM L-Gln, 100 μg/mL of penicillin, and 100 μg/mL of streptomycin for each well. After 1 day incubation at 37 °C, the media were replaced with the serially diluted compounds containing media, and cells were incubated at 37 °C for additional 2 days. The cells were washed with PBS (200 μL x1) and the generated formazan was dissolved in 200 μL of 4 M HCl aq./2-

propanol (0.1:10). The absorbance of each well at 565 nm were read using iMark Microplate Reader (BIO-RAD). The absorbance values were obtained in triplicate and normalized to 100% (no inhibitor) and 0% viability (no cell) controls. Normalized values were plotted versus concentration and analyzed using non-linear regression in GraphPad Prism 10 [log(inhibitor) vs response – variable slope (four parameter) model]. IC_{50} values represent average ± standard error of the mean (SEM).

MTT assays using J-Lat 10.6 cells and THP-1 NLuc#225 cells were performed as following previously reported fashion.[S3] J-Lat 10.6 cells and THP-1 NLuc#225 cells were maintained in RPMI-1640 (Nacalai Tesque) supplemented with 10% FBS and penicillin/streptomycin. The cells were seeded at 2×10^4 cells/well in 96-well culture plates and incubated 24 h at 37 °C. Cell viability was assessed in the presence of two- or three-fold serially diluted test compounds. After 24 h incubation from the addition of test compounds, MTT was added to each well and incubated for 5 h. Cell lysis buffer (40 mM HCl, 5% Triton X-100 containing 2-propanol) was added to each well and absorbance at 560 nm was measured using GloMax multidetection system (Promega Corp.).

II-IV. Results from MTT assays

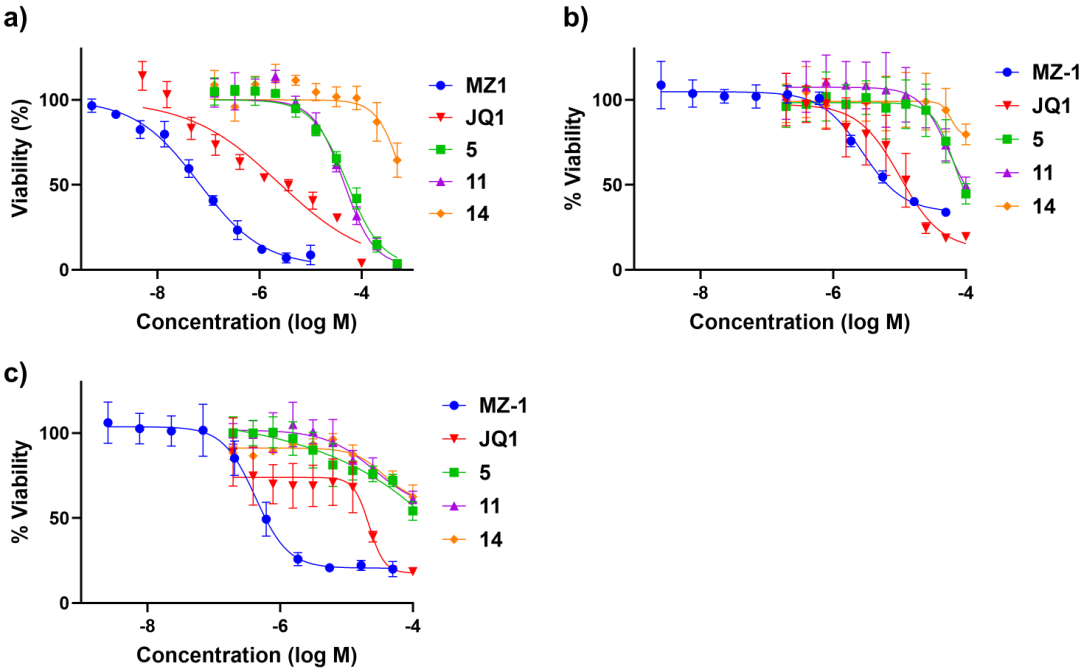


Fig. S4. Results from MTT assays in Table 1 to evaluate cytotoxicity of JQ1, BRD4-PROTACs MZ1, **5**, and **11**, and Vpr (1-14)-derived peptide **14** using (a) MCF-7 cells, (b) J-Lat 10.6 cells, and (c) THP-1 NLuc#225 cells. The X axis represents compound concentration (log M), and the Y axis represents relative cell viability based on DMSO control. Data points represent average ± standard error of the mean (SEM) from at least three independent experiments and fit using non-linear regression in GraphPad Prism 10.

II-V. General methods for evaluation of LRA activity and western blot assays using J-Lat 10.6 cells and THP-1 NLuc#225 cells

LRA activity of the compounds were evaluated using previously reported procedures.[S3] J-Lat 10.6 cells and THP-1 NLuc#225 cells were maintained in RPMI-1640 (Nacalai Tesque) supplemented with 10% FBS and penicillin/streptomycin. J-Lat 10.6 cells and THP-1 NLuc#225 cells (5 x 10^5 cells) were aliquoted and seeded in 48 well plate (0.5 mL/well) and treated with test compounds. After 1 day incubation, cells from the 2 wells treated with the same compound and concentration were combined and then divided for RNA extraction and western blot assays.

To evaluate HIV-1 Tat mRNA expression, quantitative RT-PCR was performed with a THUNDERBIRD Probe One-step qRT-PCR Kit (Toyobo, Osaka, Japan). Primers 5'-CAGGAAGAAGCGGAGACAG-3' and 5'-ATTGGGAGGTGGGTTGC-3' and probe 5'-FAM-ACGAAGAGCTCATCAGAACAGTCAGACT-TAMRA-3' were used to amplify the HIV-1 doubly spliced form of HIV-1 NL4-3 associated RNA (tat). For standardization, *18S rRNA*-specific primers 5'-GTAACCCGTTGAACCCCATT-3' and 5'-CCATCCAATCGGTAGTAGCGG-3' and probe 5'-FAM-TGCGTTGATTAAGTCCCTGCCCTTTGTA-TAMRA-3' were used to quantify *18S rRNA* transcripts. Real-time RT-qPCR was carried out using a StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Relative mRNA levels were determined using the ΔΔCt quantification method.[S4] Results are presented as the average of at least three independent experiments with error bars indicating standard error of the mean (SEM). Statistical significance between means was determined using unpaired two-tailed Student's t-tests. All statistical analyses were performed using Prism 9 software (GraphPad Software, Inc). *P*-values below 0.05 were considered significant.

For western blot assays, 1 x 10^6 cells were lysed in 100 μL of 1 x SDS sample Buffer containing 2-mercaptoethanol. Proteins were solubilized by heating for 10 min at 95 °C. The sample solutions (15 μL) were applied to Mini-Protean TGX 4-15% Gel (BIO-RAD) and run electrophoresis. The separated proteins were transferred to PVDF membranes for western blotting. Immunoreactive proteins were visualized using Western Lighting Ultra or Chemi-Lumi One, Chemiluminescent Substrate (Nacalai Tesque, INC. Kyoto, Japan) using Odyssey imaging system (SCRUM Inc., Tokyo, Japan). The antibodies used in this study were: anti-BRD4 antibody (1:1000, a-BRD4 (E1Y1P), Cat#83375S, Cell Signaling technology, Danvers, MA), HRP-conjugated anti-Rabbit IgG antibody (1:5000, Cat#7074, Cell Signaling technology, Danvers, MA), anti-α-tubulin antibody (1:10000, Clone#DM1A, Sigma-Aldrich, Cat#T9026, St. Louis, MO), and HRP-conjugated anti-Mouse IgG antibody (1:10000, cat#NA931, Amersham Biosciences, Piscataway, NJ).

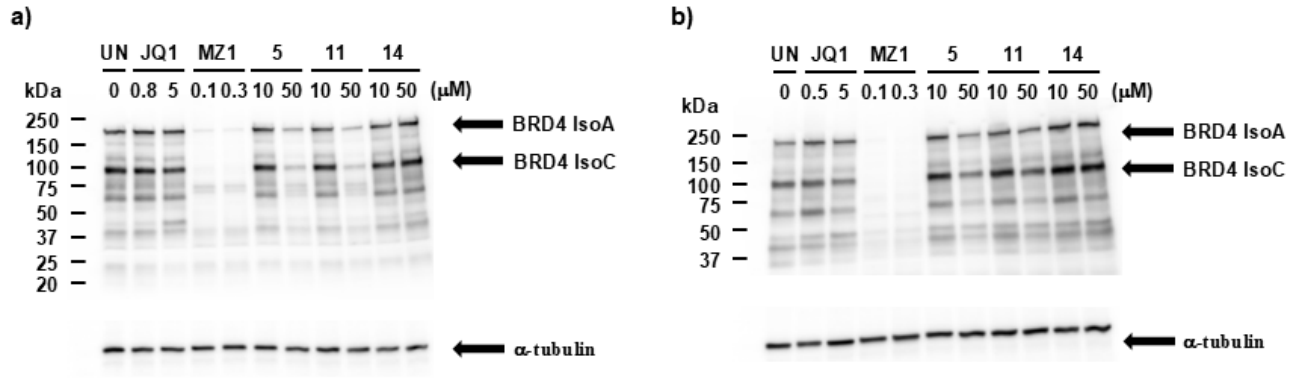


Fig. S5. Raw data of western blot assays to evaluate BRD4 degradation ability of the synthesized compounds shown in Figs. 10c (a, J-Lat 10.6 cells) and 10d (b, THP-1 NLuc#225 cells).

III. References

S1. H. Xu, T. Kurohara, N. Ohoka, G. Tsuji, T. Inoue, M. Naito and Y. Demizu, *Bioorg. Med. Chem.*, 2023, **86**, 117293.

S2. K. Tsuji, D. Hymel, B. Ma, H. Tamamura, R. Nussinov and T. R. Burke, *RSC Chem. Biol.*, 2022, **3**, 1111–1120.

S3. H. Kitamura, S. Sukegawa, K. Matsuda, K. Tanimoto, T. Kobayakawa, K. Takahashi, H. Tamamura, K. Tsuchiya, H. Gatanaga, K. Maeda and H. Takeuchi, *Biochem. Biophys. Res. Commun.*, 2023, **641**, 139–147.

S4. K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402–408.