SUPPORTING INFORMATION

Synthetic Aminopyrrolic Receptors Have Apoptosis Inducing Activity

Seong-Hyun Park,[1] Yoon Pyo Choi,[1] Jinhong Park,[2] Andrew Share,[3] Oscar Francesconi,[4] Cristina Nativi,[4] Wan Namkung,[2] Jonathan L. Sessler,[3] Stefano Roelens,[5] and Injae Shin[1,*]

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Material and Methods

Measurement of intracellular Cl^- concentrations. FRT cells expressing the mutant YFP were washed three times with PBS buffer and 100 μL HEPES-buffered solutions (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM $CaCl_2$, 10 mM D-glucose, pH 7.4) were added to the cells. Cells were incubated with each compound for 2 h at 37 ^oC. The YFP fluorescence was measured using an Infinite® 200 PRO multimode microplate reader (TECAN, Austria) (λ_{ex} = 480 nm, λ_{em}= 530 nm).

Measurement of intracellular Na^+ concentrations. FRT cells were incubated in culture media containing 10 μM SBFI-AM and 0.04% Pluronic F-127 for 1.5 h at 37 ^oC. After washing with PBS to remove the remaining SBFI-AM, 100 μL HEPES-buffered solutions were added to the cells. Cells were incubated with each compound for the indicated times at 37 ^oC. The SBFI-AM fluorescence was measured using an Infinite® 200 PRO multimode microplate reader (λ_{ex} = 340 nm, λ_{em}= 500 nm).

Measurement of intracellular K^+ concentrations. Calu-3 cells were incubated in culture media containing 10 μM PBFI-AM and 0.04% Pluronic F-127 for 1.5 h at 37 ^oC. After washing with PBS to remove the remaining PBFI-AM, 100 μL HEPES-buffered solutions were added to the cells. Cells were incubated with 10 μM of each compound for 2 h at 37 ^oC. The PBFI fluorescence was measured using an Infinite® 200 PRO multimode microplate reader (λ_{ex} = 344 nm, λ_{em} = 500 nm).

Measurement of intracellular Ca^{2+} concentrations. FRT cells were loaded with Fluo-4 NW according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). After washing with PBS to remove the remaining Fluo-4, 200 μL HEPES-buffered solutions were added to each well. Cells were incubated with 10 μM of each compound for 2 h at 37 ^oC. Fluo-4 fluorescence was measured using an Infinite® 200 PRO multimode microplate reader (λ_{ex} = 485 nm, λ_{em} = 538 nm).

Effect of methyl α-mannopyranoside on receptor-induced cell death. HeLa and PLC/PRF/5 cells, pretreated with methyl α-mannopyranoside (0–7.5 mM) for 1 h, were incubated with **6** and **7** at various concentrations (0–1 μM) for 18 h. MTT assays were performed according to standard procedures. The absorbance at 570 nm was measured using an Infinite® 200 PRO multimode microplate reader.

cDNA microarray analysis. HeLa cells were treated with 5 μM of **6** for 6 h. The total RNA of the treated cells was isolated using RNeasy Mini Kit columns (Qiagen). RNA (300 ng) from each sample was converted to double-strand cDNA. The cDNA was hybridized to the GeneChip® Human Gene 2.0 ST arrays for 17 h. After hybridization, the chips were stained and washed in a Genechip Fluidics Station 450(Affymetrix) and scanned using a Genechip Array scanner 3000

7G (Affymetrix). The expression intensity data were extracted from the scanned images using Affymetrix Command Console software version 1.1 and stored as CEL files. The intensity values of CEL files were normalized to remove bias between the arrays by means of the Robust Multi-array Average (RMA) algorithm implemented in the Affymetrix Expression Console software (version 1.3.1.). The normalized data were imported into the Statistical Programming Environment R (version 3.0.2) for further analysis using the tools available from the Bioconductor Project (http://www.bioconductor.org) To determine whether genes were differentially expressed between control groups and treatment groups, genes showing over 2-fold difference between the average signal values of the two groups were selected. The web-based tool DAVID (the Database for Annotation, Visualization, and Integrated Discovery) was used to perform the biological interpretation of the differentially expressed genes.

Table S1. Binding affinities (BC_{50}, mM) of **1-7** for octyl mannopyranosides in CD_3CN.[a]

Receptor	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Oct α-Man	n.d.	22.0	12.0	8.20	n.d.	0.13	0.44
Oct β-Man	n.d.	20.6	5.90	8.00	n.d.	0.87	0.57

BC_{50} values = intrinsic median binding concentrations

n.d. = non-detectable

[a]Data from previous studies (see ref. 17).

Table S2. Expression analysis of apoptosis-related genes seen after treatment of HeLa cells with **6** for 6 h.

No.	Regulation	Gene symbol	Gene name	GeneBank no.	Log_2
1	Apoptosis	TNF	tumor necrosis factor	NM_000594	1.832853
2	Apoptosis	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	NM_001270508	2.698783
3	Apoptosis	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	NM_003842	1.364135
4	Apoptosis	TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	NM_003811	2.026444
5	Apoptosis	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	NM_005118	1.713136
6	Apoptosis	IGFBP3	insulin-like growth factor binding protein 3	NM_001013398	2.176074
7	Apoptosis	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_001220778	1.760625
8	Apoptosis	GADD45B	"growth arrest and DNA-damage-inducible, beta "	NM_015675	1.08855
9	Apoptosis	PPP1R15A	protein phosphatase 1, regulatory subunit 15A	NM_014330	2.683734
10	Apoptosis	DDIT3	DNA-damage-inducible transcript 3	NM_001195056	3.742109
11	Apoptosis	JUN	jun proto-oncogene	ENST00000371222	2.621887
12	Apoptosis	DEDD2	death effector domain containing 2	NM_133328	1.053597
13	Apoptosis	FOS	FBJ murine osteosarcoma viral oncogene homolog	NM_005252	1.365331
14	Anti-apoptosis	CAV1	caveolin 1, caveolae protein, 22kDa	NM_001172895	-1.106092
15	Anti-apoptosis	DOCK2	dedicator of cytokinesis 2	NM_004946	-1.196864
16	Anti-apoptosis	DLX1	distal-less homeobox 1	ENST00000361725	-1.161235
17	Anti-apoptosis	LGR4	leucine-rich repeat containing G protein-coupled receptor 4	NM_018490	-1.152357

Log_2; $log_2(T/C)$ where C is the gene expression level in an untreated sample, T is the gene expression level in a treated sample.

Genes 1-13 shown in Table S2 are positive regulators of apoptosis, and genes 14-17 are negative regulators of apoptosis. After treatment with **6**, positive regulators of apoptosis are upregulated, while negative regulators of apoptosis are down-regulated. As noted in the text, this is taken as an indication that **6** induces apoptosis.

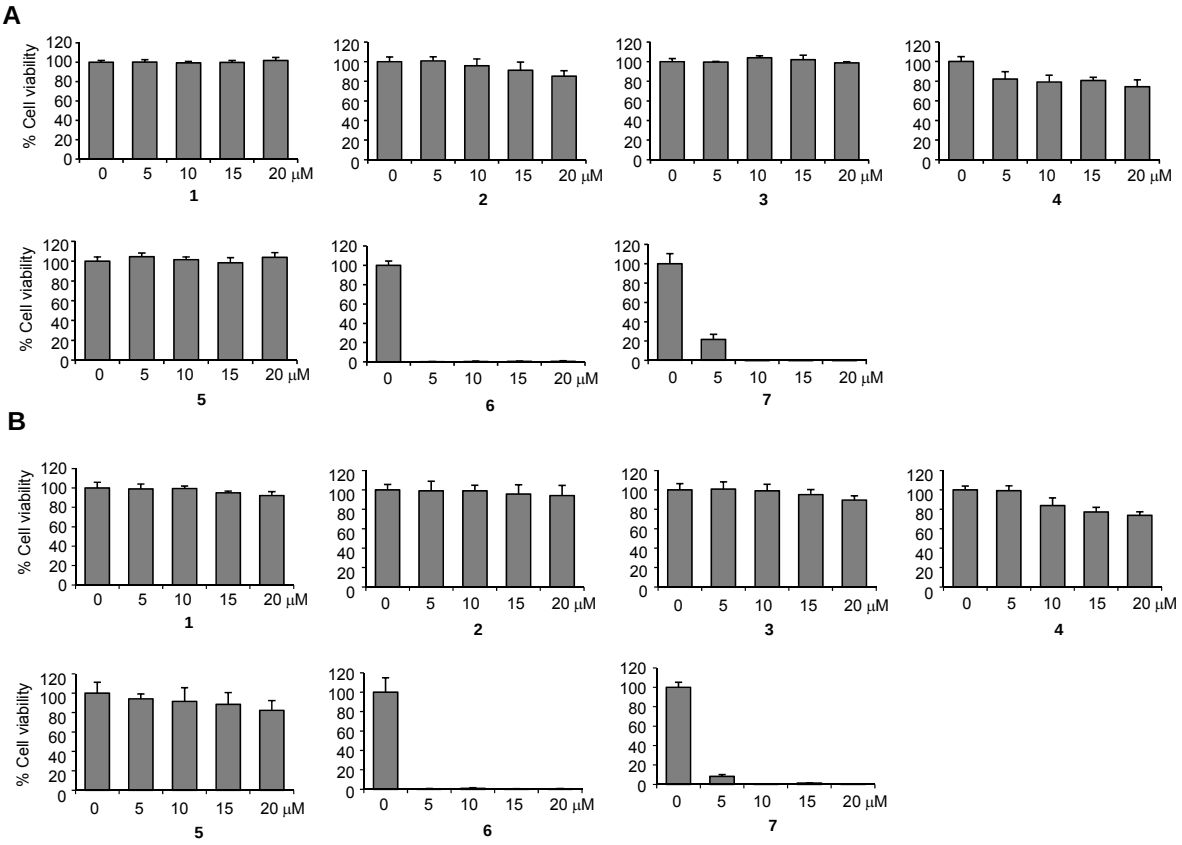

Figure S1. Cytotoxicity of compounds. (A) HeLa and (B) PLC/PRF/5 cells were treated with 0–20 μM of each of the indicated compounds for 18 h. Cell death was then measured by using an MTT assay (mean ± s.d., n = 3).

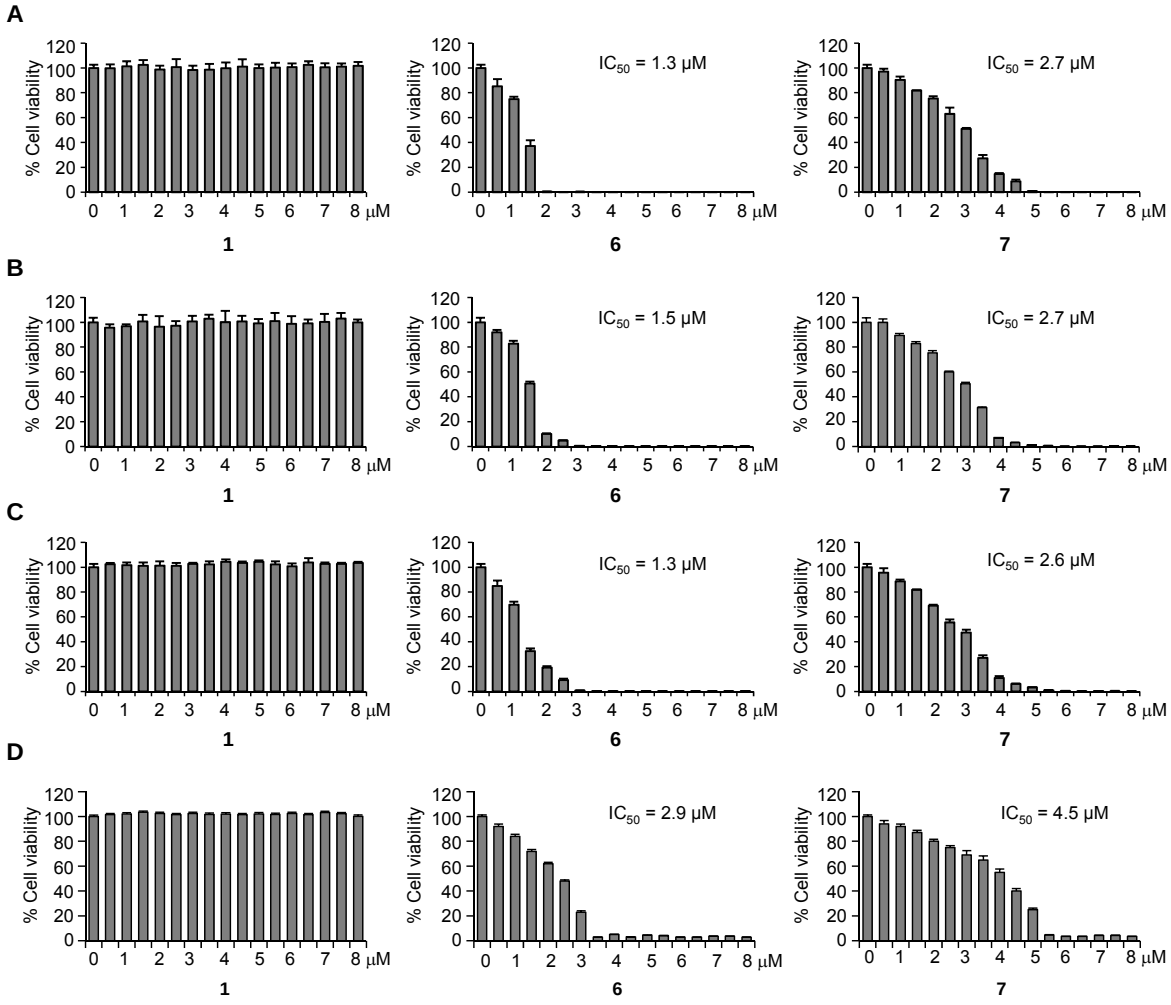

Figure S2. Cytotoxicity of compounds. (A) HeLa, (B) PLC/PRF/5, (C) A549 and (D) KG-1 cells were treated with various concentrations of each of the indicated compounds for 18 h. Cell death was then measured by using an MTT assay (mean ± s.d., n = 3).

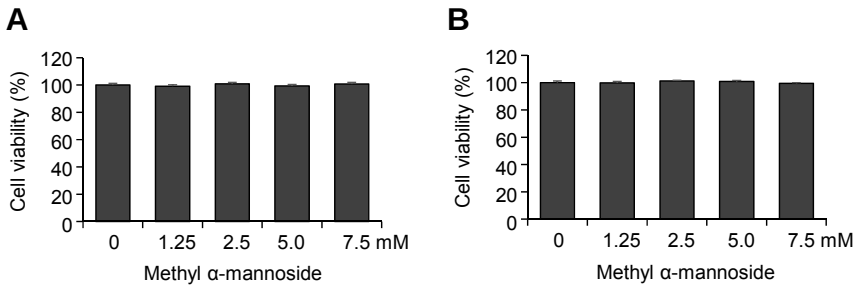

Figure S3. Cytotoxicity of methyl α-mannopyranoside. (A) HeLa and (B) PLC/PRF/5 cells were treated with various concentrations of methyl α-mannopyranoside for 18 h. Cell death was then measured by using an MTT assay (mean ± s.d., n = 3).

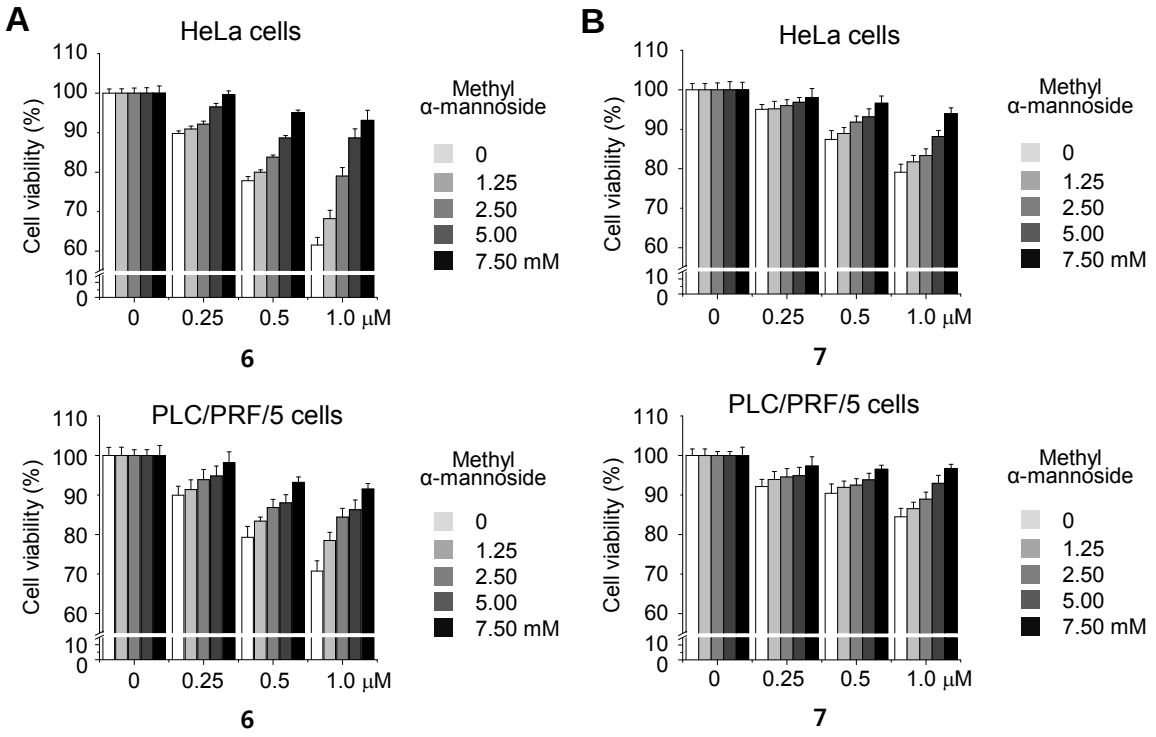

Figure S4. Effect of methyl α-mannopyranoside on cell death induced by compounds. HeLa and PLC/PRF/5 cells, pre-incubated with a competitor methyl α-mannopyranoside (0-7.5 mM) for 1 h, were treated with (A) **6** and (B) **7** for 18 h. Cell death was then measured by using an MTT assay (mean ± s.d., n = 3).

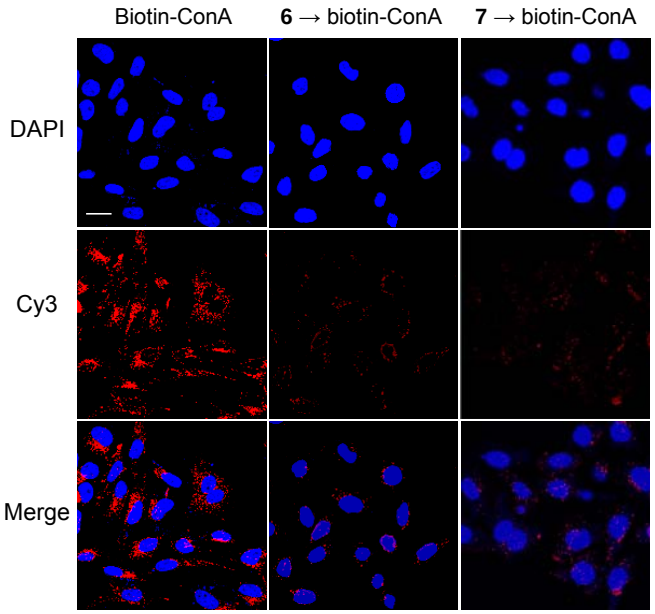

Figure S5. HeLa cells pre-incubated with 10 μM of each compound for 2 h were treated with 5 μM biotin-ConA for 1 h. The treated cells were then stained with Cy3-streptavidin. DAPI was used to stain the nucleus (scale bar = 20 μm).

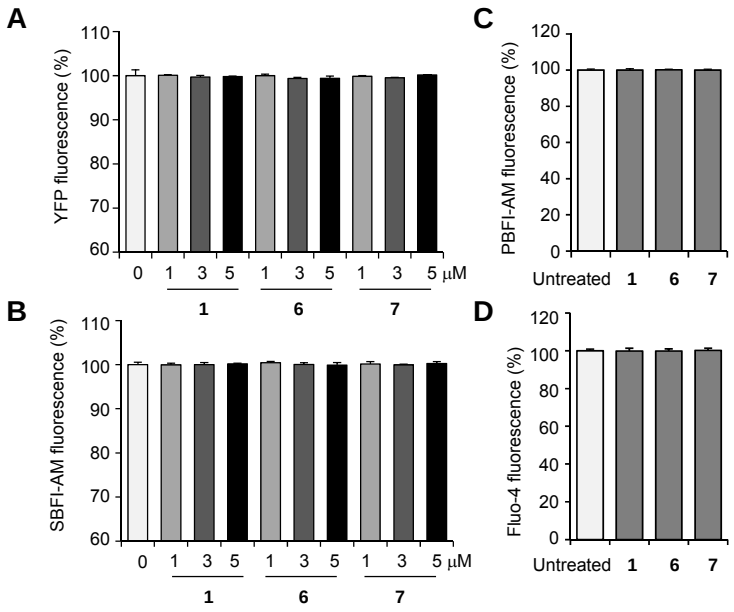

Figure S6. (A) FRT cells were incubated with 10 μM of each of the indicated compounds for 2 h and the YFP fluorescence was measured to monitor changes in the intracellular chloride ion concentration (mean ± s.d., n = 3). (B) FRT cells pretreated with 10 μM SBFI-AM for 1.5 h were incubated with 10 μM of each of the indicated compounds for 2 h. The SBFI-AM fluorescence was then measured to probe changes in the intracellular sodium ion concentration (mean ± s.d., n = 3). (C) Calu-3 cells pretreated with 10 μM PBFI-AM for 1.5 h were incubated with 10 μM of each of the indicated compounds for 2 h. The PBFI fluorescence was then measured to probe changes in the intracellular potassium ion concentration (mean ± s.d., n = 3). (D) FRT cells pretreated with Fluo-4 NW for 1 h were incubated with 10 μM of each of the indicated compounds for 2 h. The Fluo-4 fluorescence intensity was then measured to monitor changes in the intracellular calcium ion concentration (mean ± s.d., n = 3).

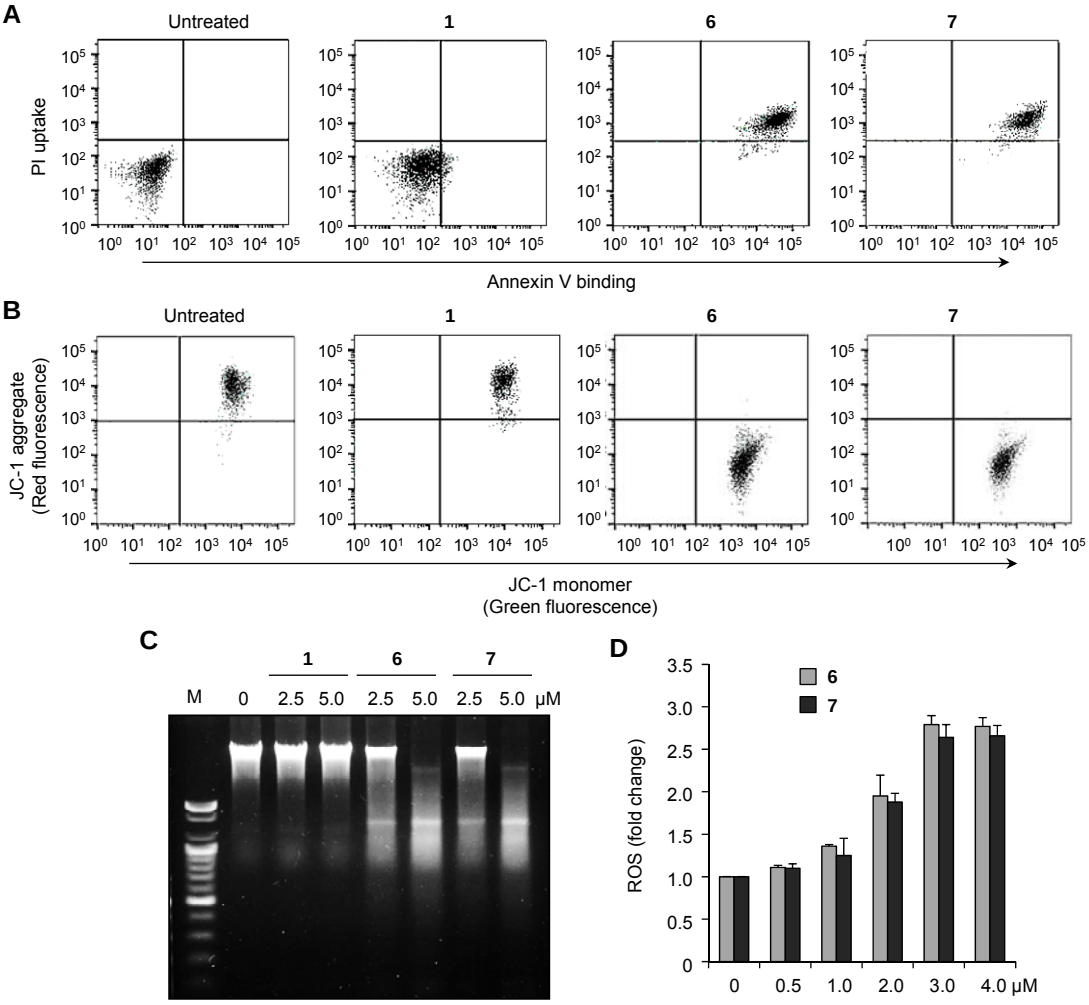

Figure S7. Synthetic receptors induce apoptosis. (A) Flow cytometry of PLC/PRF/5 cells treated with 5 μM of each compound for 18 h and then stained with a mixture of fluorescein-annexin V and PI (annexin V binding versus PI uptake). (B) Flow cytometry of PLC/PRF/5 cells treated with 5 μM of each compound for 18 h and stained with JC-1. Shown is a dot plot of red fluorescence (FL2, JC-1 aggregate) versus green fluorescence (FL1, JC-1 monomer). (C) PLC/PRF/5 cells were treated with each compound for 18 h. The DNA fragments were then visualized by staining with RedSafe™ Nucleic Acid Staining Solution. (D) PLC/PRF/5 cells were treated with various concentrations of **6** or **7** for 8 h and then stained with 10 μM PF1 for 1 h to monitor the ROS production (mean ± s.d., n = 3).

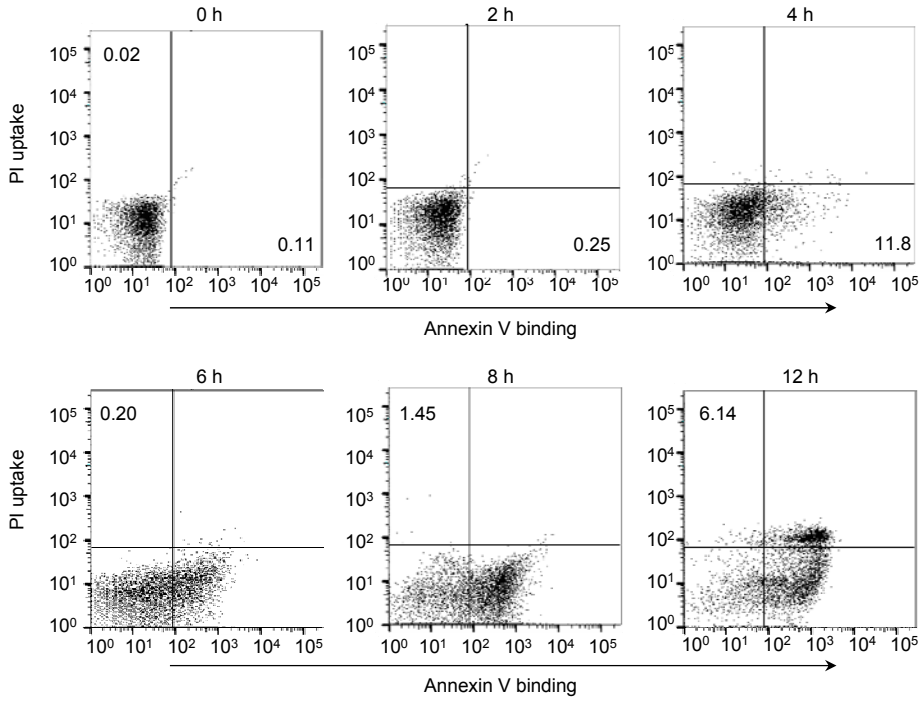

Figure S8. Flow cytometry of HeLa cells treated with 5 μM **6** for indicated times and then stained with fluorescein-annexin V and PI (annexin V binding versus PI uptake). Annexin V staining precedes PI staining, indicating that cells treated with a compound undergo apoptosis.

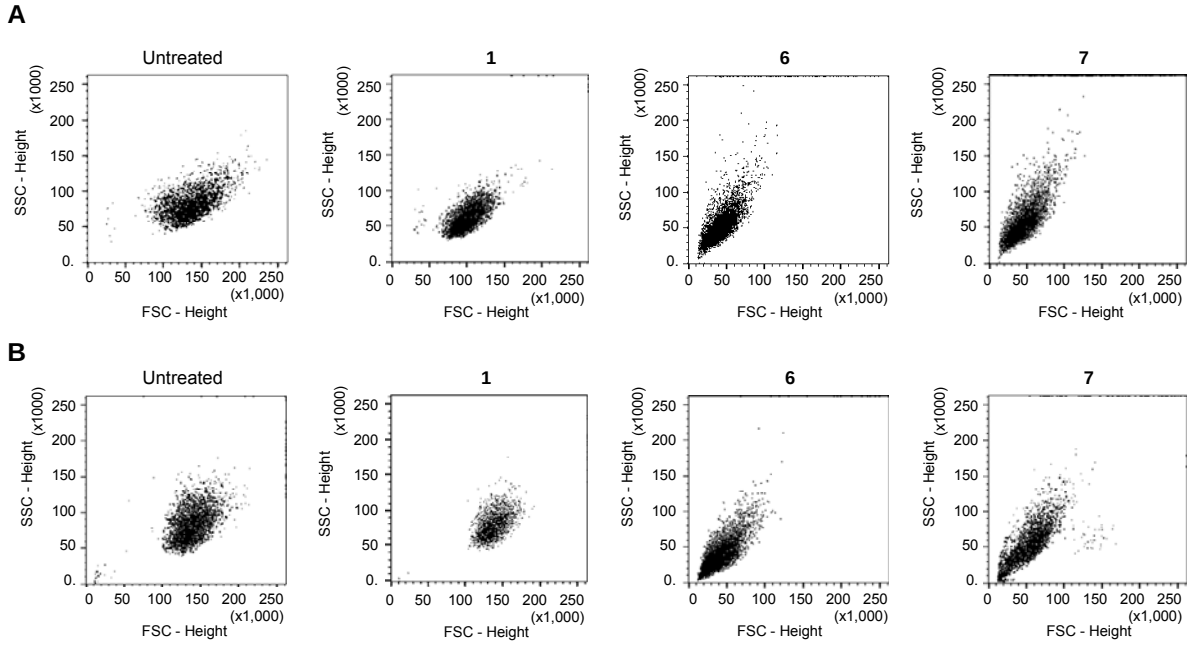

Figure S9. Synthetic receptors lead to cell shrinkage. (A) HeLa and (B) PLC/PRF/5 cells were treated with 5 μM of each of the indicated compounds for 18 h and shrunk cells were determined by using flow cytometry (FSC; forward scatter, SSC; side scatter).

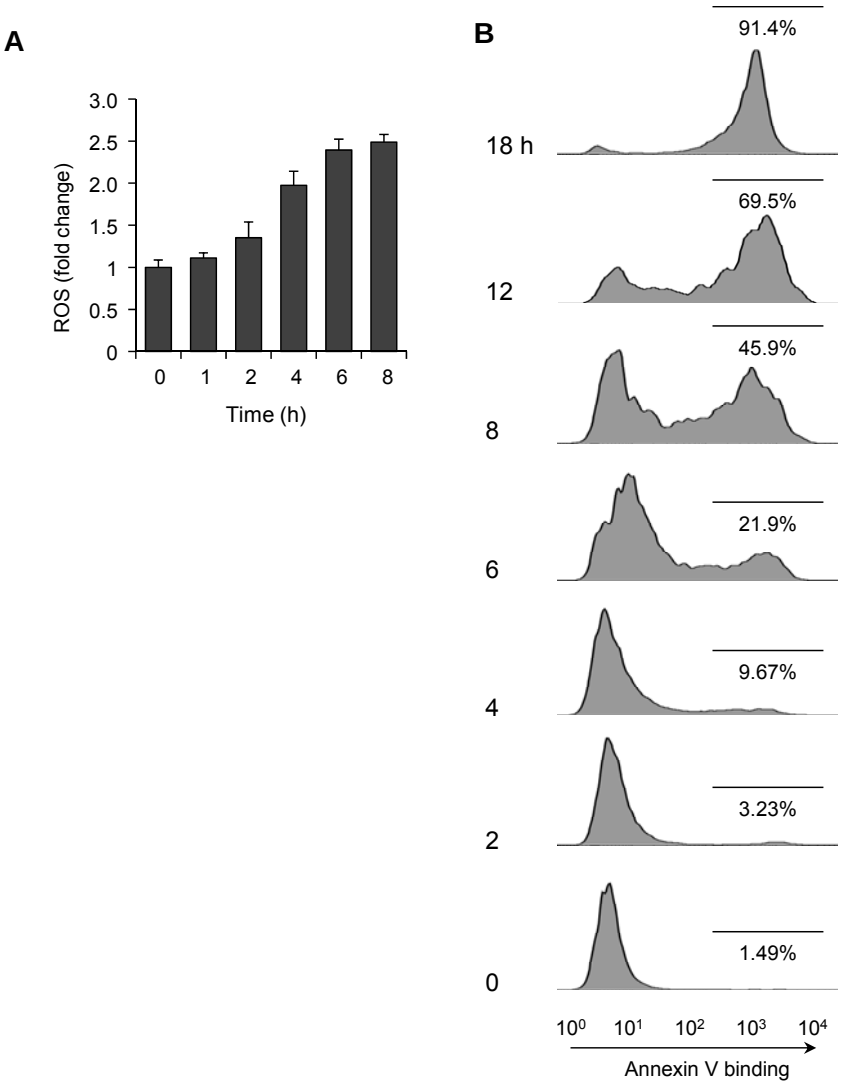

Figure S10. Compounds enhance ROS production and thus induce apoptosis. (A) HeLa cells were treated with 5 μM **6** for the indicated times. The cells were then treated with 10 μM PF1 to monitor ROS production (mean ± s.d., n = 3). (B) HeLa cells were incubated with 5 μM **6** for the indicated times and then treated with fluorescein-annexin V.

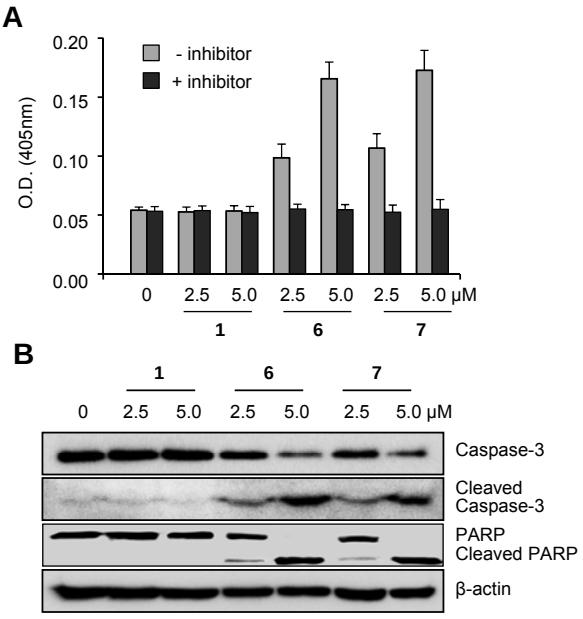

Figure S11. Synthetic receptors induce caspase activation. (A) Caspase activities of lysates of PLC/PRF/5 cells treated with each of the indicated compounds for 18 h were measured using acetyl-DEVD-pNA in the absence (grey) or presence (black) of 200 μM Ac-DEVD-CHO (mean ± s.d., n = 3). (B) PLC/PRF/5 cells were treated with each of the indicated compounds for 18 h and the indicated proteins were immunoblotted using appropriate corresponding antibodies.