Supplementary information for

Facile transformation of low-potency small molecules into photochemical protein knock-out reagents

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Supplementary Methods

General remarks

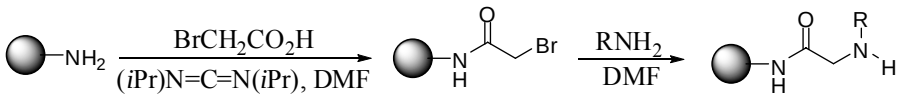
All chemicals and solvents, unless stated, were purchased from Aldrich Chemical Co., and were used without further purification. Preparative HPLC was performed on a Waters Breeze HPLC system with a Vydac C18 preparative column (Flow rate = 10 mL / min.). Mass spectra were obtained with a Voyager-DE^{TM} PRO (Applied Biosystems) for MALDI-TOF with α-cyano-4-hydroxycinnamic acid matrix.

Cell culture

PAE/KDR cells (Sibtech, Inc.) and HeLa cells (ATCC, CCL-2) were maintained in DMEM (Invitrogen) supplemented with 110 mg / L sodium pyruvate, 2mM L-glutamine and 10% (v/v) fetal calf serum at 37˚C in a 5% CO_2 environment. H441 cells were maintained in RPMI-1640 (Invitrogen) supplemented as above. For irradiation experiments, phenol red-free DMEM or RPMI-1640 was used.

Peptoid synthesis

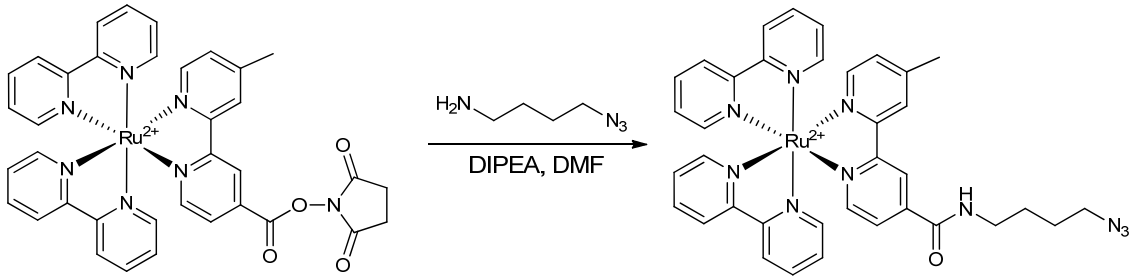
All peptoids were synthesized by on Rink Amide AM resin (Nova Biochem) using the sub-monomer approach (outline shown below) by a microwave-assisted protocol [(1)].



Peptoid synthesis outline: Coupling of bromoacetic acid followed by alkylation with amine completes addition of one residue in the sequence.

Preparation of Ruthenium-peptoids

Compound 7: To a stirred solution of Bis-(2,2′-bipyridin)-4′-methyl-4-carboxy-bipyridin-ruthenium-N-succinimidylester-bis-(hexafluorophosphate) (5.0 mg, 0.005 mmol) was added 4-azidobutylamine (0.025 mmol) [(2)] and DIPEA (0.025 mmol). After 3 hr at room temperature, the reaction mixture was diluted with dH_2O (0.1 % TFA, 3 mL) and directly purified by preparative HPLC and fractions containing product were lyophilized to afford compound **7** (0.004 mmol) as a red solid. MALDI / TOF: $[M]^+$ calculated 724.2, observed 723.4



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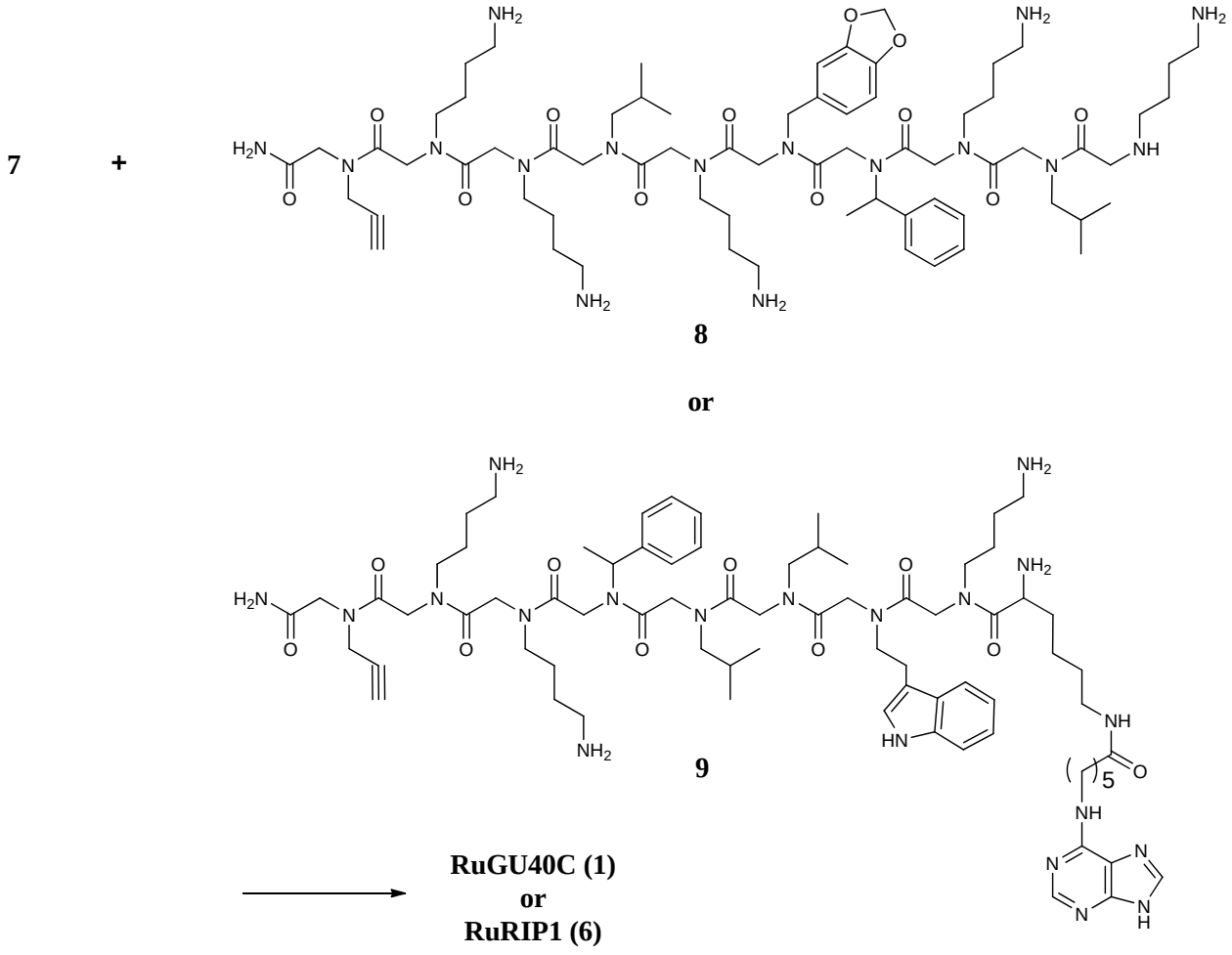
Compound 8. MALDI/TOF: $[M+H]^+$ calculated 1331.8, observed 1332.1

Compound 9. MALDI/TOF: $[M+Na]^+$ calculated 1465.9, observed 1466.7

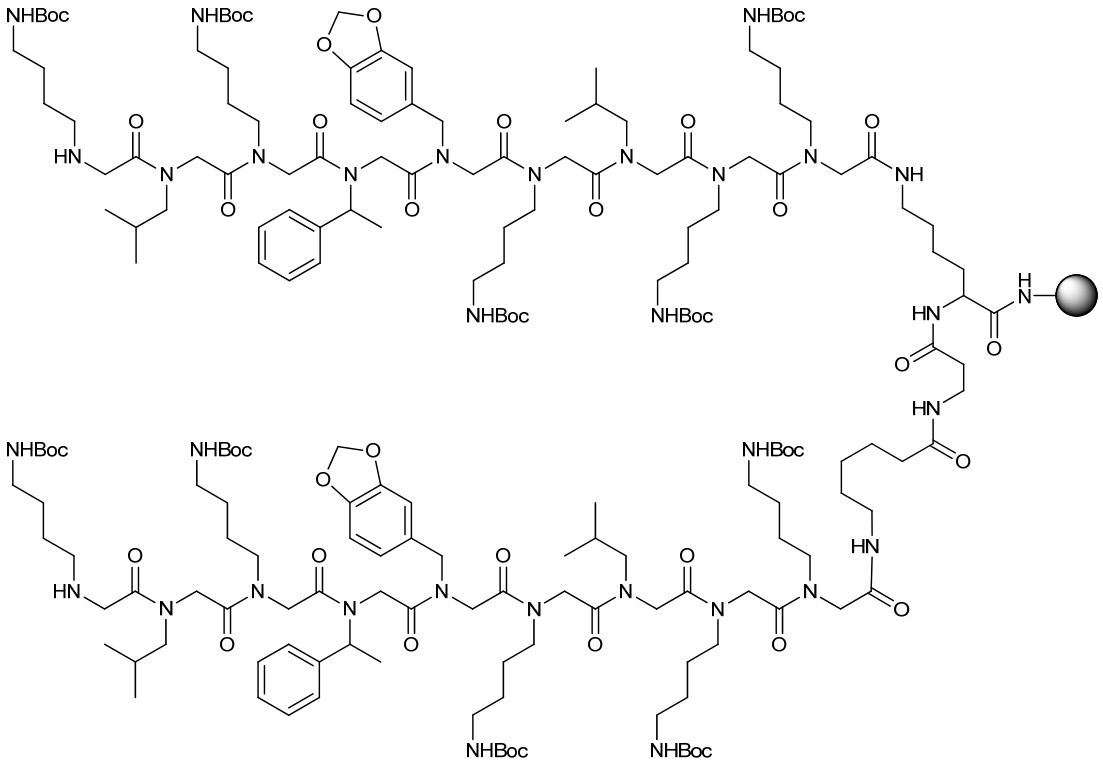
RuGU40C (1) To a stirred solution of compound **7** (0.004 mmol) in DMSO (150 μL) was added compound **8** (0.004 mmol) and Tetrakis(acetonitrile)copper hexafluorophophate (0.0004 mmole, 10 mole %). The reaction mixture was microwaved (100 % power) for 45 sec, diluted with dH_2O (0.1 % TFA, 3 mL), and directly purified by preparative HPLC to give 0.0022 mmol of RuGU40C. MALDI / TOF: $[M]^+$ calculated 2055.0, observed 2054.0 With same method, Scr-RuGU40C (**3**) was prepared. MALDI / TOF: $[M]^+$ calculated 1971.3, observed 1971.9

RuRIP1 (6) To a stirred solution of compound **7** (0.004 mmol) in MeOH (150 μL) was added compound **9**, CuI (0.012 mmol), and DIPEA (0.02 mmol). The reaction mixture was stirred overnight at room temperature and diluted with dH_2O (0.1 % TFA, 3 mL), and directly purified by preparative HPLC to give 0.0013 mmol of RuRIP1. MALDI / TOF: $[M]^+$ calculated 2167.1, observed 2167.1



RuGU40C4 (5) To a resin containing GU40C4 (0.0025 mmol) in DMF was added N-Fmoc-6-aminohexanoic acid (0.0075 mmol), HOBt (0.0075 mmol), HBTU (0.0075 mmol), and DIPEA (0.0075

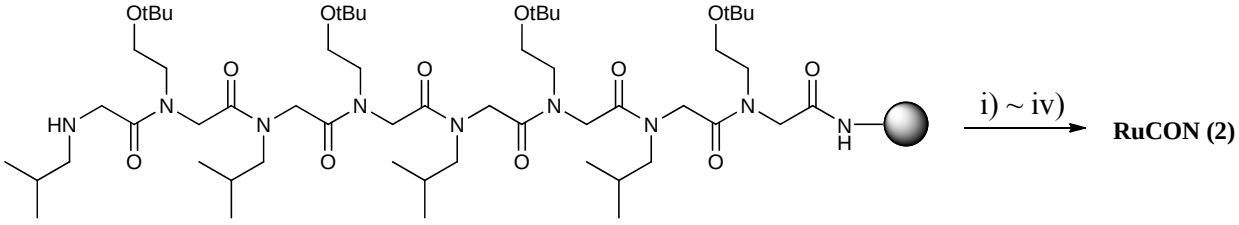
mmol). The suspension was shaken overnight at room temperature. The resin was washed with DMF (2 mL × 8) and treated with 20% piperidine in DMF for 1hr at room temperature. After washing with DMF (2 mL × 8), the resin was treated with Bis-(2,2′-bipyridin)-4′-methyl-4-carboxy-bipyridin-ruthenium-N-succinimidylester-bis-(hexafluorophosphate) (5.0 mg, 0.005 mmol) and DIPEA in DMF. After 2 hr, the resin was washed with DMF (2 mL × 8) and CH_2Cl_2 (2 mL × 8). The conjugate was cleaved from the resin by cleavage cocktail (1 mL, 97.5% TFA, 2.5% water) at room temperature for 2hr. TFA was removed and the crude produce was purified by preparative HPLC. MALDI / TOF: $[M]^{4+}$ calculated 1053.5, observed 1054.5



i) ~iv) → **RuGU40C4 (5)**

i) N-Fmoc-6-aminohexanoic acid, HOBT, HBTU, DIPEA, DMF ii) 20% piperidine in DMF
iii) $Ru(bpy)_2$(mcbpy-OSu), DIPEA, DMF iv) TFA, H_2O

RuCON (2) To a resin containing control peptoid (0.0025 mmol) in DMF was added N-Fmoc-γ-aminobutyric acid (0.0075 mmol), HOBt (0.0075 mmol), HBTU (0.0075 mmol), and DIPEA (0.0075 mmol). The suspension was shaken at room temperature for 2 hr. The resin was washed with DMF (2 mL × 8) and was treated with 20% piperidine in DMF for 2hr at room temperature. After washing with DMF (2 mL × 8), the resin was treated with Bis-(2,2′-bipyridin)-4′-methyl-4-carboxy-bipyridin-ruthenium-N-succinimidylester-bis-(hexafluorophosphate) (5.0 mg, 0.005 mmol) and DIPEA in DMF. After 2 hr, the resin was washed with DMF (2 mL × 8) and CH_2Cl_2 (2 mL × 8). The conjugate was cleaved from the resin by cleavage cocktail (1 mL, 97.5% TFA, 2.5% water) at room temperature for 2hr. TFA was removed and the crude produce was purified by preparative HPLC to give 0.0019 mmol of RuCON. MALDI / TOF: $[M]^+$ calculated 1568.7, observed 1568.7



i) N-Fmoc-aminobutyricacid, HOBT, HBTU, DIPEA, DMF ii) 20% piperidine in DMF
iii) $Ru(bpy)_2$(mcbpy-OSu), DIPEA, DMF iv) TFA, H_2O

Visible light irradiation

Irradiation was done using 150-W xenon arc lamp (Oriel). Light was filtered first through distilled water (10 cm) and then through a 380- to 2,500-nm cut-on filter (Oriel). Samples were positioned at 25 cm distance from the light source and light intensity at the distance was 50 mW / cm^2.

Autophosphorylation assay

Cells were grown (~75% confluency) in 6-well plates, serum-starved overnight (0.1% FBS) and treated with compounds for 15 min. After irradiation or dark incubation for 10 min, cells were incubated for 37˚C for 10 min before addition of VEGF (37 ng / mL) or EGF (20 ng / mL). After 8 min, cells were lysed with nuclear lysis buffer (125 μL) for 10 min at 4˚C. The collected lysate was mixed with 2 × SDS sample buffer and was heated for 5 min at 95˚C. The samples were separated by SDS-PAGE and transferred to PDVF membranes (Immobilon, Millipore). The membranes were probed with anti-phospho VEGFR2, anti-phospho-EGFR, anti-VEGFR2, or anti-EGFR primary antibodies (Cell signaling) and subsequently developed with appropriate HRP-conjugated secondary antibody (BioRad) followed by chemiluminescent detection using SuperSignal® West dura substrate (PIERCE). Quantifications of blot bands were performed using "Image J" software.

Tube-formation assay

HUVECs (2.5×10^4 cells/well) in phenol red-free Endothelial Cell Medium (ECM) basal media (ScienCell Research Lab.) containing 0.2% FBS were mixed with DMSO, RuCON, or RuGU40C and then dispensed in growth-factor reduced Matrigel (BD biosciences)-coated 96-well plates and were incubated at 37˚C for 15 min. After irradiation or dark incubation for 10 min, cells were treated with VEGF (1.3 nM) and incubated for 16 hr. Images were taken under the light microscope and analyzed for quantitation of tube formation using software "AngioQuant".

26S proteasomal peptidase assay

The proteasomal peptidase activity was measured using fluorogenic substrates Suc-LLVY-AMC (Bachem), Cbz-ARR-AMC (Calbiochem), and Cbz-LLE-AMC (Calbiochem) to analyze the chymotrypsin-like, trypsin-

like, and caspase-like activities of the 26S proteasome, respectively. 26S proteasome (2 nM) was incubated at 25^{o}C for 10 min with DMSO, MG132, RuCON, or RuRIP1 in the reaction buffer containing 50 mM Tris (pH 8.0) and 20 μM β-mercaptoethanol (100 μL of final reaction volume). Samples were irradiated or incubated in the dark for indicated time. 10 min after additional dark incubation, fluorogenic substrate (50 μM) was added and the peptidase activity was monitored after 30 min by measuring the fluorescence of the released 7-amido-4-methylcoumarin (Ex: 365 nm, Em: 460 nm).

Cell-based proteasomal peptidase assay

The Effect of RIP1 and RuRIP1 on the proteasomal chymotrypsin-like peptidase activity was determined using a chemiluminescent assay (Proteasome Glo Cell-based Assay, Promega). HeLa cells were dispensed into white, clear-bottom 96-well plate at a density of 1×10^4 cells in 100 μL phenol red-free DMEM (10% FBS). After 6hr incubation, media was replaced with 100 μL serum-deficient, phenol red-free DMEM (0.1% FBS) and incubated for 4 hr. Cells were treated with compounds and incubated for 2 hr and then were irradiated or incubated in the dark for 30 min. After incubation at 37 ^{o}C for 2hr, cells were equilibrated to room temperature for 30 min and then assay buffer (100 μL) containing luminogenic substrate Suc-LLVT-aminoluciferin and a recombinant Luciferase were added. After 10 min incubation at room temperature, chemiluminescence was measured using plate reader (Molecular device) and expressed as relative light units (RLU) after subtraction of background luminescence (100 μL media + 100 μL assay buffer).

Cell viability assay

Cell viability was determined by the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). PAE/KDR cells (2×10^4 cells / well) in phenol red-free DMEM (10% FBS) in 96-well plate (clear bottom)

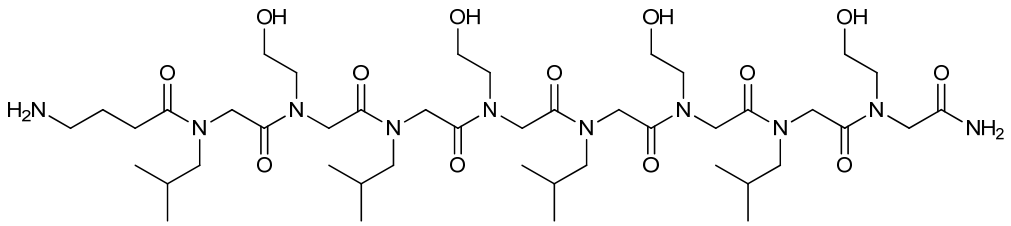
were incubated with DMSO, RuCON, or RuGU40 for 15 min and irradiated or incubated in the dark for 10 min. After 2 days incubation at 37˚C, CellTiter96® AQ_{ueous} One Solution Reagent (20 μL) was added to culture wells and incubated for 3 hr and then absorbance at 490nm was measured.

ROS detection assay

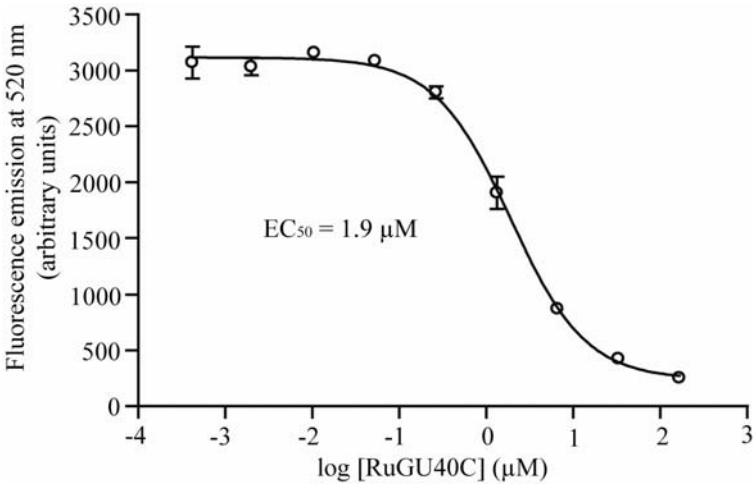
HeLa cells (1×10^4 cells) in 96-well plate (black wall, clear bottom) in phenol red-free DMEM (10% FBS) was incubated with DMSO or RuRIP1 and ROS probe (Carboxy-H_2DCFDA, Invitrogen) at 37˚C for 20 min. Cells were irradiated for 30 min. To examine the effect of exogenous H_2O_2, 1 mM H_2O_2 was added to cells at this time. Cells were incubated for additional 20 min. After washing with PBS three times, fluorescence emission intensity at 529 nm (excitation: 494 nm) was measured with plate reader (Molecular device) and subtracted by the fluorescence intensity of medium only.

MS spectrometry for photostability measurement of Ruthenium-peptoids

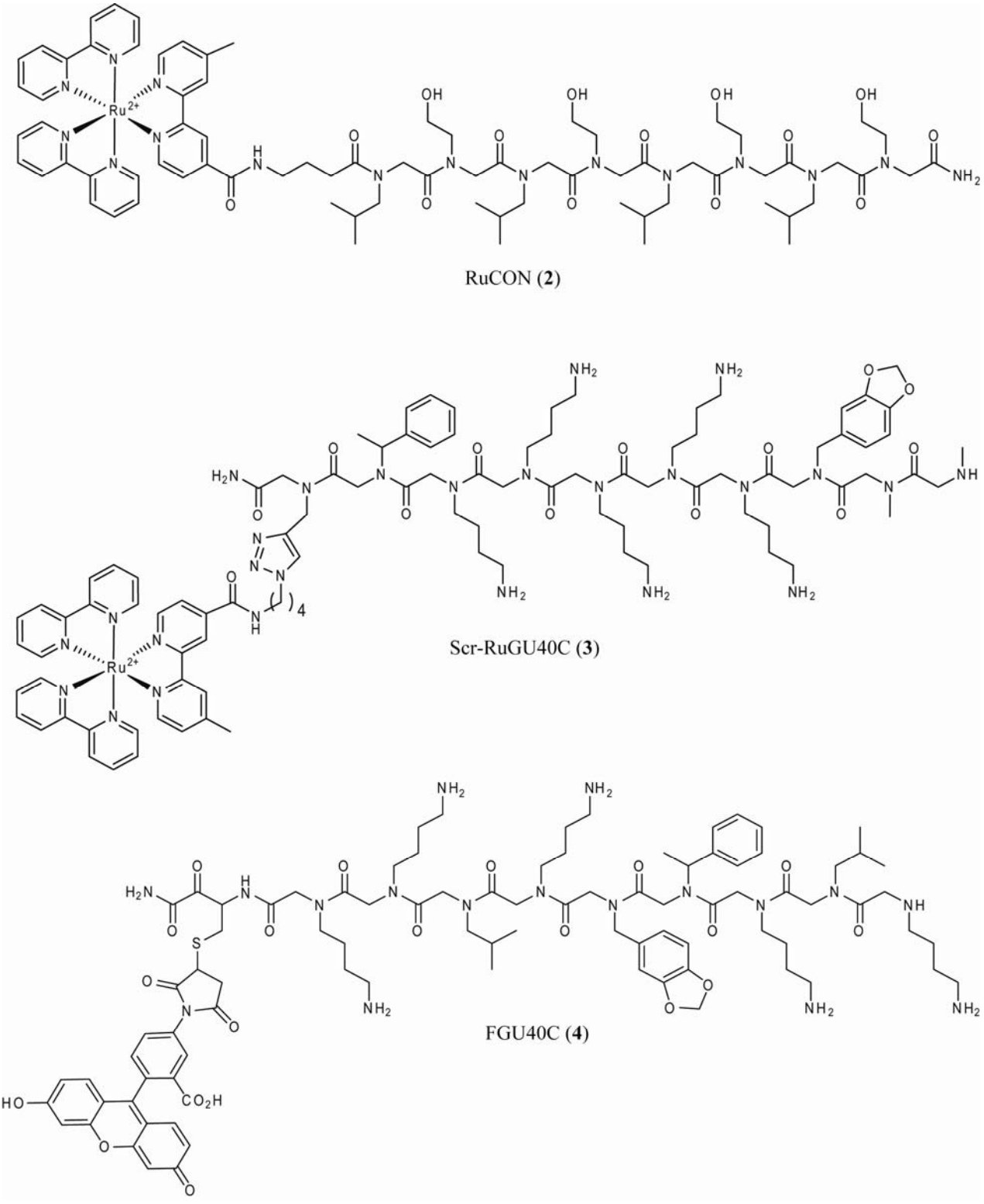
RuGU40C (2.5 μM) or RuRIP1 (2.5 μM) in 100 μL phenol red-free DMEM (0.1% FBS) containing 2.5 μM internal standard peptoid (structure shown below) were irradiated. At different time points, sample (3.5 μL) was taken and mixed with MALDI/TOF matrix solution (7 μL). 1 μL of the mixed solution was subjected to MALDI / TOF analysis and intensity of molecular ion peak of RuGU40C or RuRIP1 was normalized with that of internal standard peptoid and expressed as percentage of initial value at 0 min irradiation.



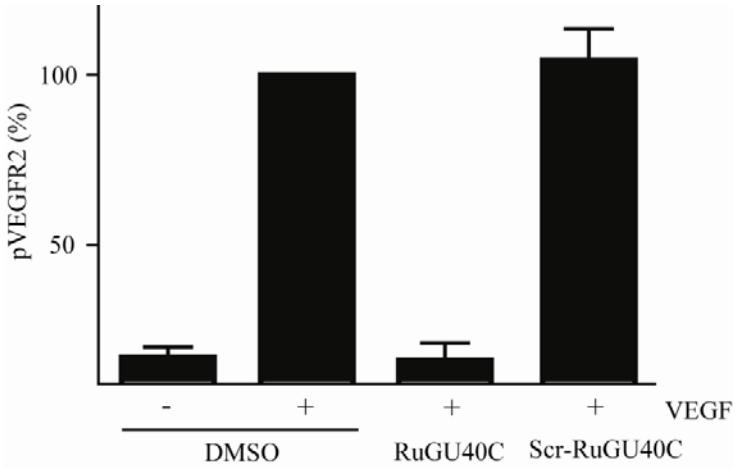
Supplementary Results



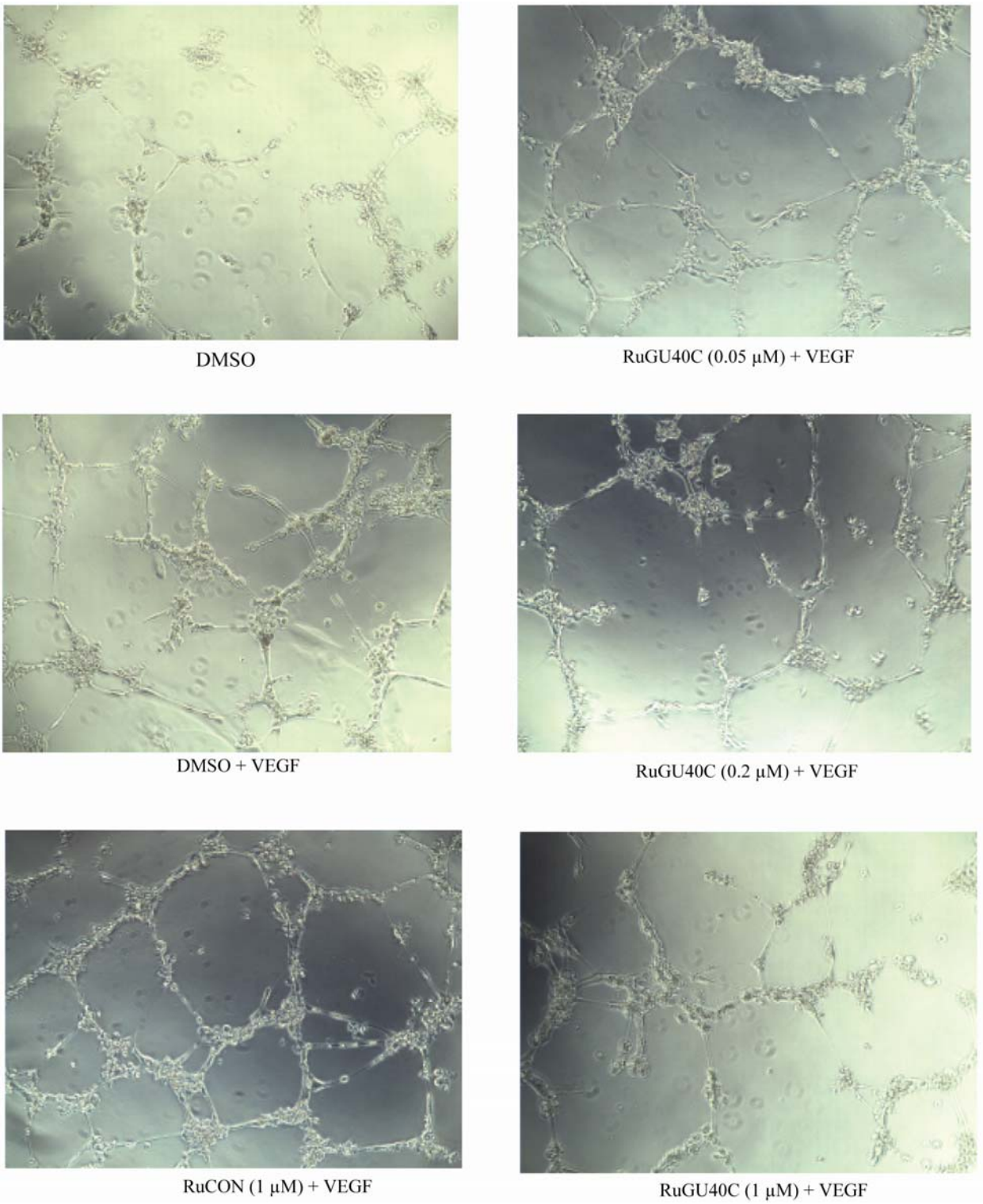
Supplementary Figure 1. Competitive binding assay of RuGU40C. Displacement of FGU40C (see Supplementary Figure 2) on immobilized VEGFR2 extracellular domain (ECD) by increased concentration of RuGU40C was assessed by measuring fluorescence emission at 520 nm.



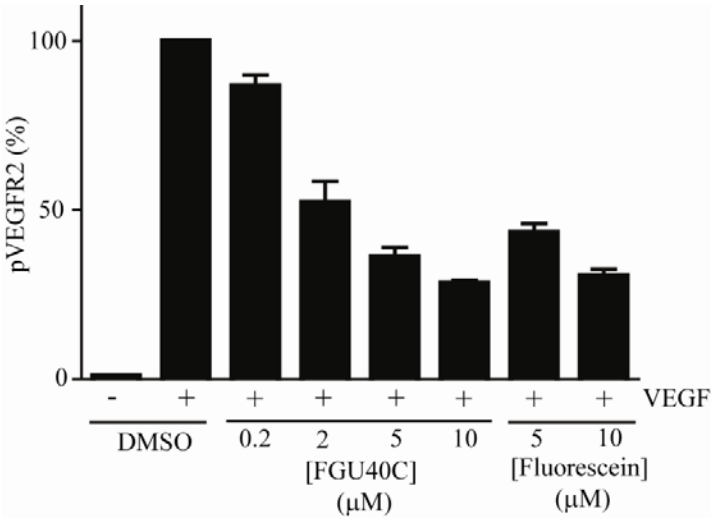
Supplementary Figure 2. Chemical structure of RuCON, Scr-RuGU40C, and FGU40C



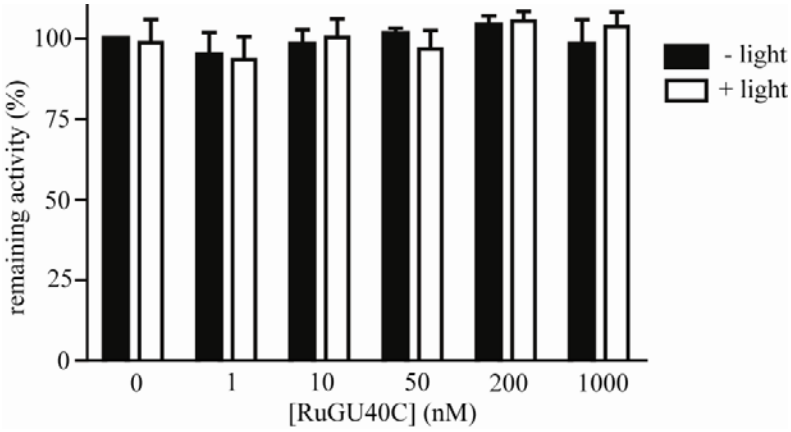
Supplementary Figure 3. Effect of RuGU40C and Scr-RuGU40C on the VEGF-induced autophosphorylation of VEGFR2 (PAE/KDR cell) was examined by western blot and evaluated by quantitative analysis (Image J).



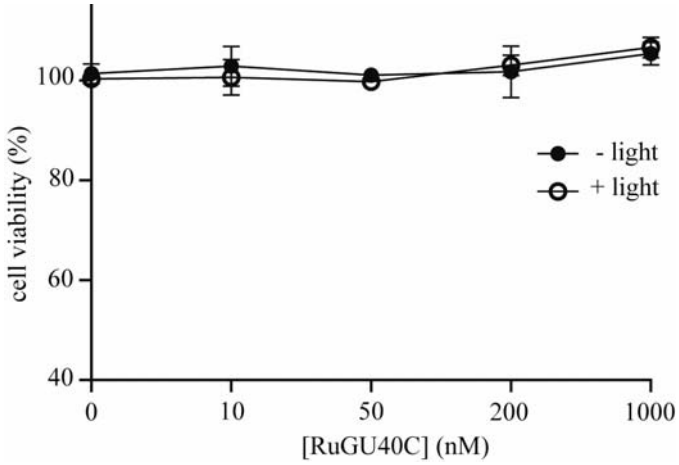
Supplementary Figure 4. Effect of RuGU40C on VEGF-induced tube formation by HUVECs. HUVECs on Matrigel-coated 96-well plate were incubated under the conditions indicated and irradiated (10 min). 16 hr after VEGF addition, light microscopic images were taken.



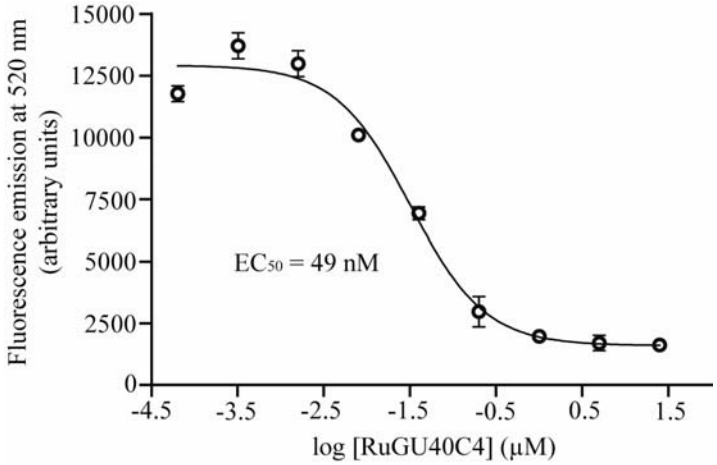
Supplementary Figure 5. Effect of FGU40C and Fluorescein (Fluorescein-6-carboxylicacid) on the VEGF (37 ng / mL)-induced autophosphorylation of VEGFR2 (PAE/KDR cell) was examined by western blot and evaluated by quantitative analysis (Image J).



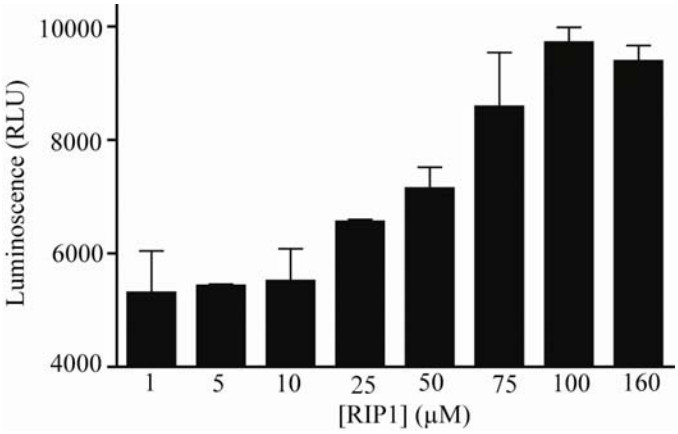
Supplementary Figure 6. Effect of RuGU40C on recombinant Luciferase activity. Activity of recombinant Luciferase with indicated concentration of RuGU40C was measured after irradiation or dark incubation (10 min).



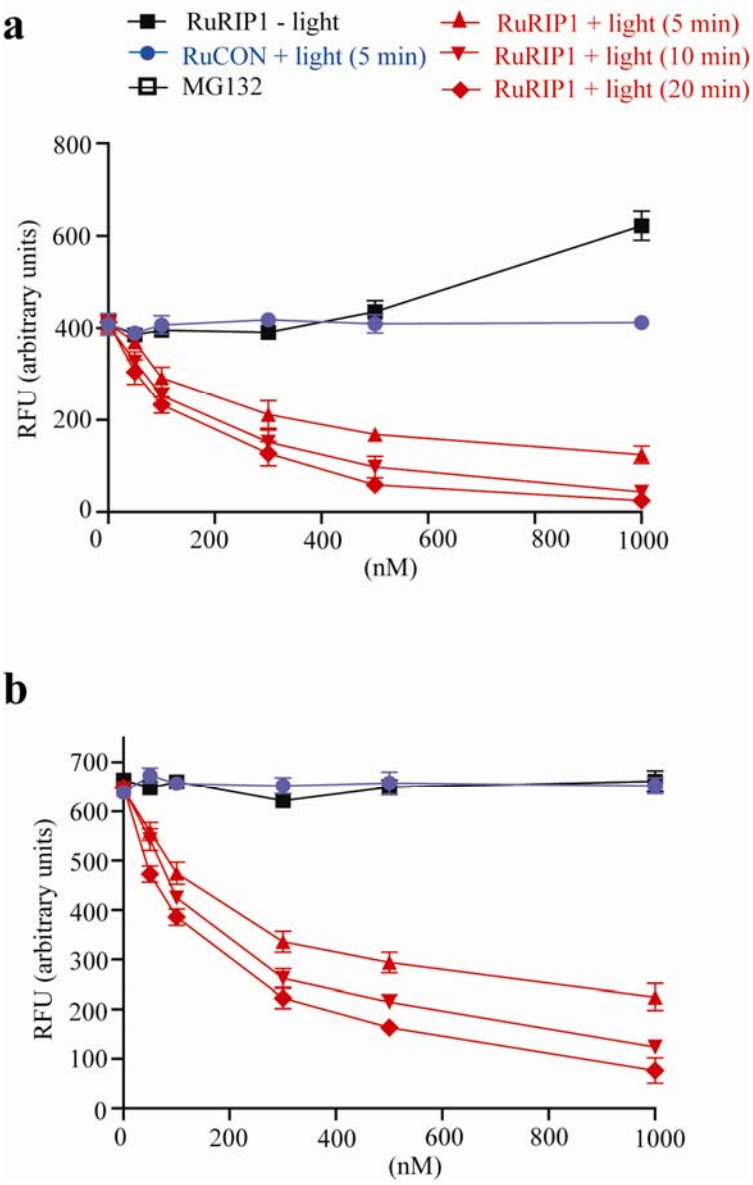
Supplementary Figure 7. Effect of RuGU40C on the cell viability of PAE/KDR cells. Cells were incubated with indicated concentration of RuGU40C and irradiated or incubated in the dark (10 min). After 2 days, the cell viability was assessed by CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) and expressed as a percentage of DMSO (-light) control.



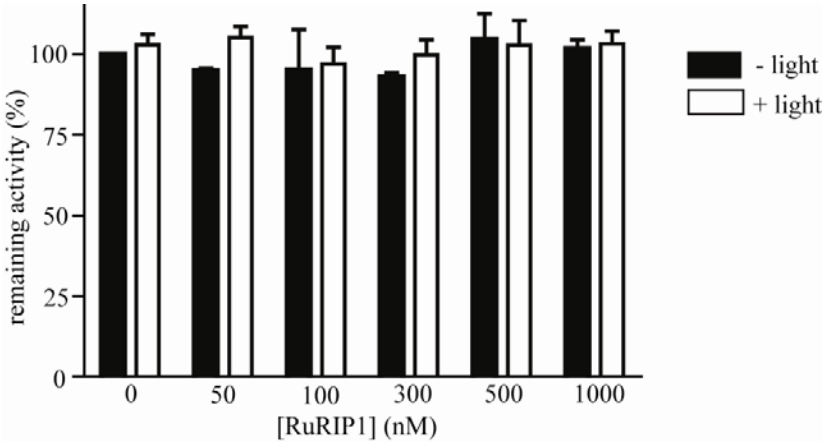
Supplementary Figure 8. Competitive binding assay of RuGU40C4. Displacement of fluorescein conjugate of GU40C4 [3] on immobilized VEGFR2 extracellular domain (ECD) by increased concentration of RuGU40C4 was assessed by measuring fluorescence emission at 520 nm.



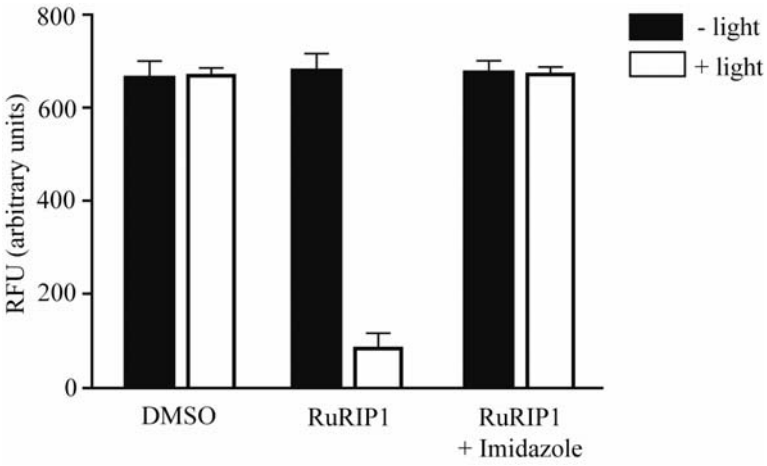
Supplementary Figure 9. The Effect of RIP1 on chymotrypsin-like peptidase activity of the 26S proteasome in HeLa cells was assessed by measuring luminescence generated by substrate (Suc-LLVY-aminoluciferin) cleavage.



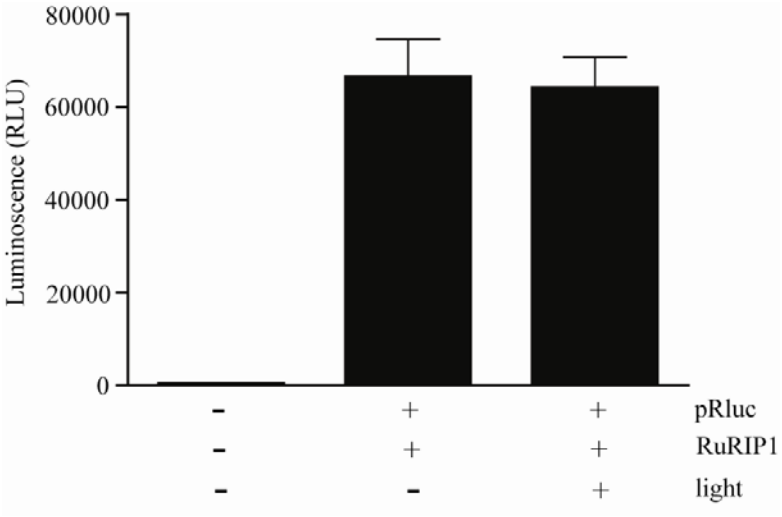
Supplementary Figure 10. Trypsin-like (a) or caspase-like (b) peptidase activity of purified 26S proteasome was measured in the presence of RuRIP1 with or without irradiation by monitoring the cleavage of fluorogenic substrate Cbz-ARR-AMC and Cbz-LLE-AMC, respectively.



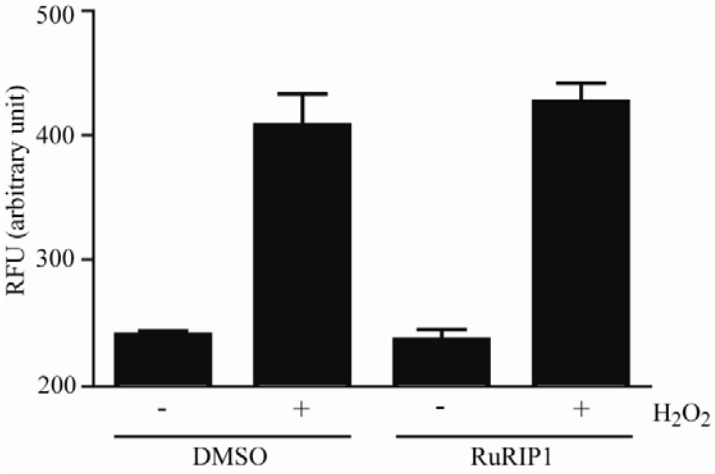
Supplementary Figure 11. Effect of RuRIP1 on recombinant Luciferase activity. Activity of recombinant Luciferase with indicated concentration of RuRIP1 was measured after irradiation or dark incubation (20 min).



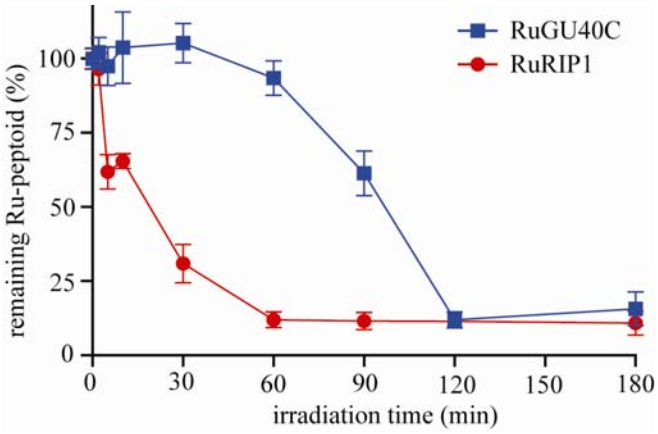
Supplementary Figure 12. Effect of imidazole on the RuRIP1-mediated light-triggered inactivation of 26S proteasome. Caspase-like peptidase activity of purified 26S proteasome was measured in the presence of RuRIP1 (1 μM) with or without imidazole (150 mM) after irradiation or dark incubation (20 min) by monitoring the cleavage of fluorogenic substrate Cbz-LLE-AMC.



Supplementary Figure 13. Effect of RuRIP1 on activity of *Renilla* Luciferase activity in HeLa cells. HeLa cells (1×10^4 cells / well) in 96-well plates were transfected with pRLuc which is a plasmid encoding *Renilla* Luciferase controlled by a constitutively active promoter. Cells were treated with RuRIP1 (1 μM) as indicated and irradiated or incubated in the dark (30 min). After lysis, *Renilla* Luciferase activity was measured.



Supplementary Figure 14. ROS detection assay. HeLa cells were incubated with DMSO or RuRIP1 as indicated in the presence of Carboxy-H_2DCFDA. After irradiation and addition of H_2O_2, fluorescence emission was measured.



Supplementary Figure 15. Relative amount of RuGU40C and RuRIP1 was monitored after irradiation with indicated times using MALDI-TOF MS.

Supplementary References

1. H. J. Olivos, P. G. Alluri, M. M. Reddy, D. Saloney, T. Kodadek. *Org. Lett*. ***4***, 4057 (2002).
2. J. W. Lee, S. I. Jun, K. Kim. *Tetrahedron Lett*. ***42***, 2709 (2001).
3. D. G. Udugamasooriya, S. P. Dineen, R. A. Brekken, T. Kodadek. *J. Am. Chem. Soc*. **130**, 5744 (2008)