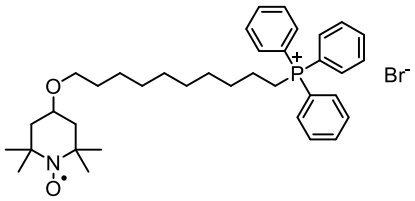
Supplemental material

Lipophilic triphenylphosphonium derivatives enhance radiation-induced cell killing via inhibition of mitochondrial energy metabolism in tumor cells

S1. Supplemental methods

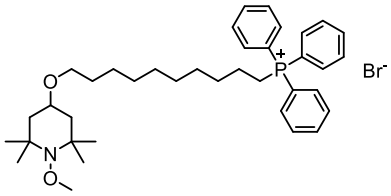
S1.1. Synthetic procedures for the tested compounds.

Mito-$(CH_2)_{10}$-Tempol (M10T)



TEMPOL (3.44 g, 20 mmol) was added to a solution of NaH (2.40 g, 60 % in oil) in DMF (15 mL). After stirring for 30 min at RT, 1,10-dibromodecane (30.0 g, 100 mmol) was added to the solution, which was stirred overnight at RT. The reaction was quenched by the addition of water and extracted with diethyl ether. Combined organic layers were washed with brine and dried over Na_2SO_4. Solvent was removed in a rotary evaporator. The crude was purified with column chromatography (hexane : ethyl acetate = 9:1) to yield 4-((10-bromodecyl)oxy)-2,2,6,6-tetramethylpiperidin-1-oxyl (4.90 g, 63 %). 4-((10-Bromodecyl)oxy)-2,2,6,6-tetramethylpiperidin-1-oxyl (4.90 g, 12.5 mmol) and PPh3 (3.28 g, 12.5 mmol) were dissolved in benzene, and the mixture was refluxed for 2 days. Solvent was removed in a rotary evaporator. The crude was purified with column chromatography ($CHCl_3$: MeOH = 50:1) and trituration by ether to obtain the titled compound (3.96 g, 48%). HRMS (ESI) m/z calcd for $C_{37}H_{52}NO_2P$ $[M\text{-}Br]^+$ 573.3730, found 573.3703.

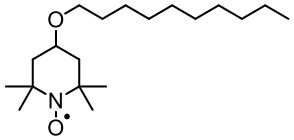
Mito-$(CH_2)_{10}$-Tempol-Me (M10T-Me)



To a stirred mixture of M10T (2.91 g, 4.5 mmol) and $FeSO_4 \cdot 7H_2O$ (1.36 g, 4.90 mmol) in DMSO (15 mL), H_2O_2 (0.660 mL, 30 %) was added dropwise to maintain the

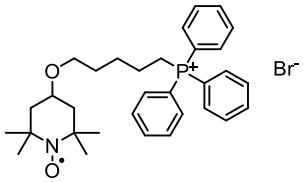
temperature at 15–20°C. After stirring for 30 min, H_2O was added and extracted with $CHCl_3$. The combined organic layers were dried over Na_2SO_4 and solvent was removed in a rotary evaporator. The crude was purified by column chromatography ($CHCl_3$: MeOH= 99:1) to obtain the titled compound (1.79 g, 60%). ^{1}H-NMR (400 MHz; CD_3Cl) δ (ppm): 1.12 (6H, s), 1.20–1.28 (16H, s), 1.50 (2H, t, *J* = 7.3 Hz), 1.60 (11H, m), 3.38 (2H, t, *J* = 6.8Hz), 3.60 (3H, s), 7.68–7.72 (6H, m), 7.77–7.81 (3H, m) 7.85–7.88 (6H, m); HRMS (ESI) m/z calcd for $C_{38}H_{55}NO_2P$ $[M\text{-}Br]^+$ 588.3965, found 588.3956.

$C_{10}H_{21}$-Tempol (10T)



TEMPOL (3.44 g, 20 mmol) was added to a solution of NaH (2.40 g, 60% in oil) in DMF (15 mL). After stirring for 30 min at RT, 1-bromodecane (20.7 mL, 100 mmol) was added dropwise to the solution and stirred overnight at RT. The reaction was quenched by addition of water, and extracted with diethyl ether. The combined organic layers were washed with brine and dried over Na_2SO_4. Solvent was removed in a rotary evaporator. The crude was purified with column chromatography (hexane : ethyl acetate = 95:5) to obtain the titled compound (5.78 g, 91%). HRMS (ESI) m/z calcd for $C_{19}H_{38}NO_2$ $[M\text{-}Na]^+$ 335.2795, found 335.2787.

Mito-$(CH_2)_5$-Tempol (M5T)



M5T was synthesized according to the procedure for M10T with 1,5-dibromopentane instead of 1,10-dibromodecane. Yield: 12 %. HRMS (ESI) m/z calcd for $C_{31}H_{40}NO_2P$ $[M\text{-}Br]^+$ 489.2791, found 489.2800.

S1.2. Measurement of oxygen consumption rate by electron spin resonance (ESR) spectroscopy

The peak-to-peak linewidth of the ESR spectrum of lithium 5,9,14,18,23,27,32,36-octa-n-butoxy-2,3-naphthalocyanine(LiNc-BuO) demonstrates a linear response to the partial pressure of oxygen (pO_2), and has been used extensively to

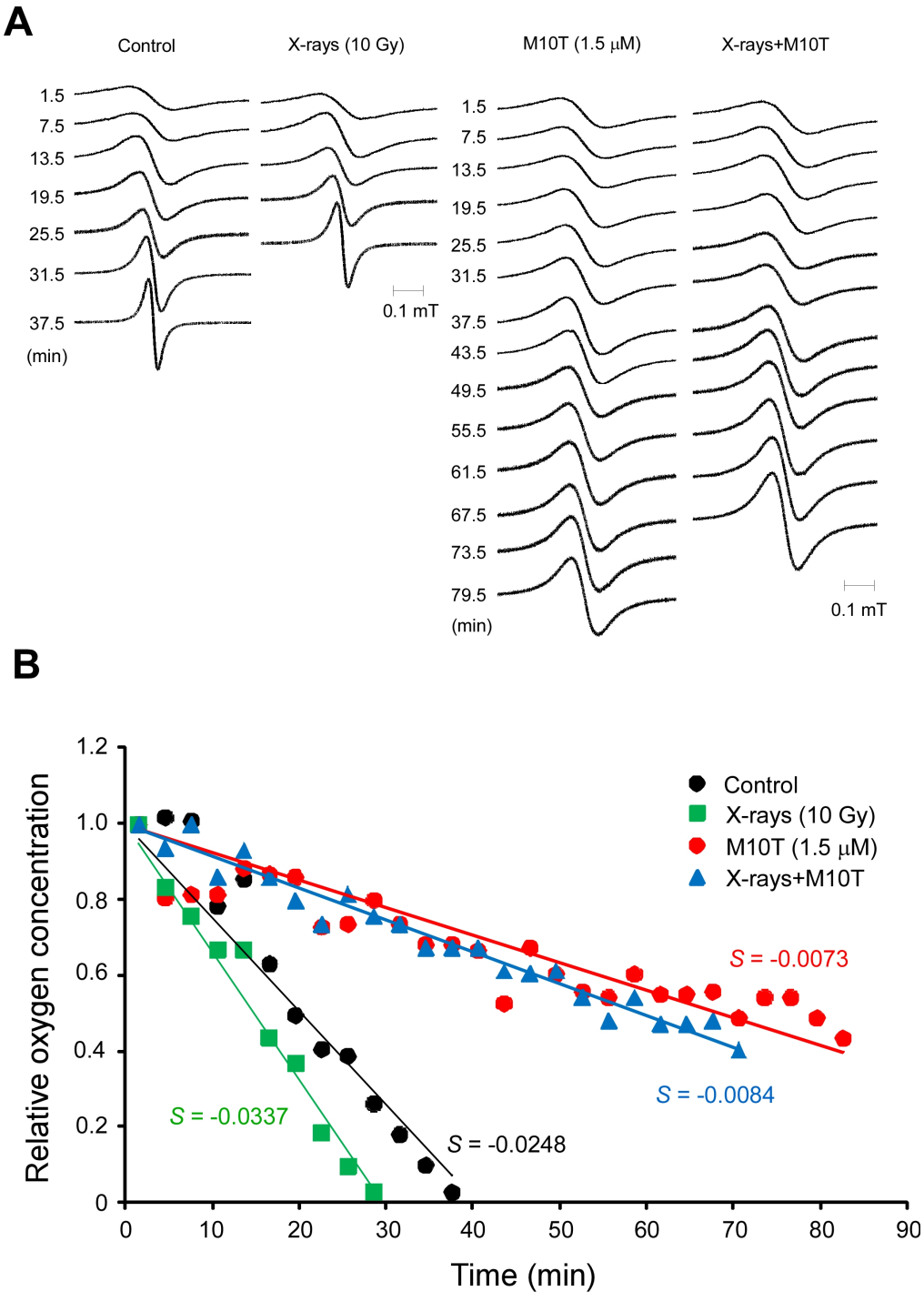
measure oxygen consumption *in vitro* [1, 2]. LiNc-BuO has been described previously [3]. Cells were trypsinized, washed, and 5×10^5 cells were suspended in 100 μL of serum-free medium containing 0.2 mg LiNc-BuO and 2% dextran before being drawn into a glass capillary tube. The tube was sealed at both ends and subjected to X-band electron spin resonance spectroscopy (JEOL RE; JEOL, Tokyo, Japan) every 2 min. The cavity was maintained at 37°C using a temperature controller (ES-DVT4; JEOL). ESR conditions were as follows: microwave power 10 mW, modulation frequency 100 kHz, field modulation amplitude 63 μT, and scan range 0.5 mT. The spectral linewidths were analyzed using a Win-Rad radical analyzer system (Radical Research, Tokyo, Japan), and converted into pO_2 values according to the following equation described by Fujii et al [1]:

pO_2 (mm Hg) = (LW × 10-340)/10.33,

where LW is the ESR line width (μT).

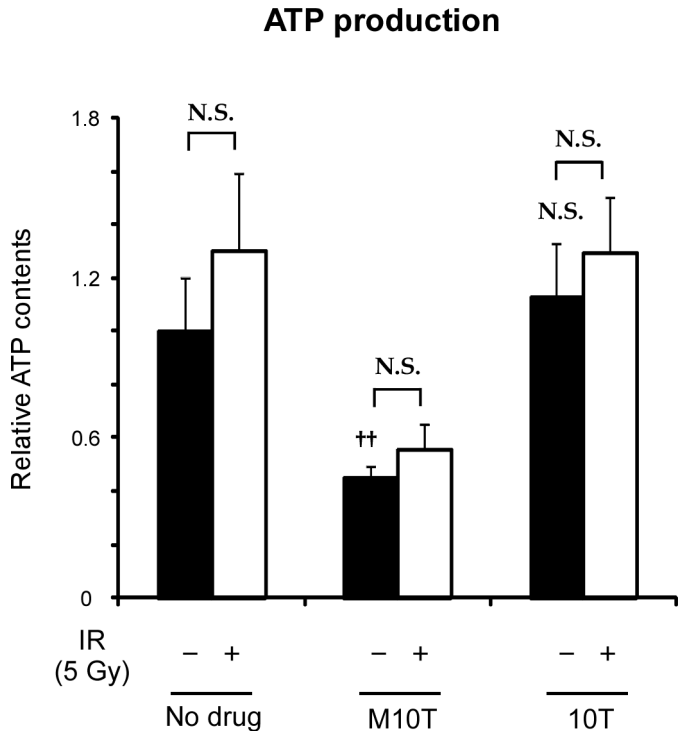
S2. Supplemental results

S2.1. X-irradiation increased cellular respiration, whereas M10T treatment abrogated this radiation effect and basal oxygen consumption completely in HeLa cells



Supplemental Fig. 1. Oxygen consumption in HeLa cells after X-irradiation and/or M10T treatment was measured by ESR oxymetry. (A) Representative ESR spectra obtained from control, 10 Gy of X-irradiation, 1.5 μM M10T, and the combination of X-irradiation and M10T. (B) Changes in pO_2 of medium containing HeLa cells over time after each treatment. *Black circle*, control; *Green square*, 10 Gy of X-irradiation alone; *red circle*, 1.5 μM M10T alone; *blue triangle*, X-irradiation and M10T. The *S* values represent the slopes of lines fitted to each dataset.

S2.2. Low-dose (5 Gy) X-irradiation showed a tendency to increase ATP production in HeLa cells. Combination treatment with M10T completely abrogated this increase in ATP production.



Supplemental Fig. 2. The effect of combination treatment with Mito-tempol derivatives and X-irradiation on cellular ATP production. Twenty-four hours after treatment with M10T or 10T (1.5 μM) and subsequent X-irradiation (10 Gy), HeLa cells were collected. ATP production in cells after treatment was assessed by an ATP-dependent luminescence assay. Data are shown as relative values normalized to untreated controls. Data are expressed as means ± S.D. for three experiments. $^{\dagger\dagger}p < 0.01$, significant difference vs. no treatment control. N.S., not significant.

References

[1] H. Fujii, K. Sakata, Y. Katsumata, R. Sato, M. Kinouchi, M. Someya, S. Masunaga, M. Hareyama, H.M. Swartz, H. Hirata, Tissue oxygenation in a murine SCC VII tumor after X-ray irradiation as determined by EPR spectroscopy, Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology, 86 (2008) 354-360.

[2] T. Yamamori, H. Yasui, M. Yamazumi, Y. Wada, Y. Nakamura, H. Nakamura, O. Inanami, Ionizing radiation induces mitochondrial reactive oxygen species production accompanied by upregulation of mitochondrial electron transport chain function and mitochondrial content under control of the cell cycle checkpoint, Free radical biology & medicine, 53 (2012) 260-270.

[3] R.P. Pandian, N.L. Parinandi, G. Ilangovan, J.L. Zweier, P. Kuppusamy, Novel particulate spin probe for targeted determination of oxygen in cells and tissues, Free radical biology & medicine, 35 (2003) 1138-1148.