Supporting Information

Enhanced autophagy induction *via* the mitochondrial delivery of methylated β-cyclodextrins-threaded polyrotaxanes by a MITO-Porter

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Materials and Method

Materials: 1,2-Dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE) was purchased from the NOF Corporation (Tokyo, Japan). Sphingomyelin (SM) was purchased from Avanti Polar lipids (Alabaster, AL, U.S.A.). Stearylated octaarginine (STR-R8) was obtained from KURABO Industries (Osaka, Japan). STR-S2 (stearylated-Dmt-D-Arg-FK-Dmt-D-Arg-FK-NH_2, Dmt = 2, 6-dimethyltyrosne) was obtained from the Toray Research Center (Tokyo, Japan). HeLa human cervix carcinoma cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Dulbecco's modified Eagle's medium (low glucose) (DMEM) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Sigma-Aldrich (St. Louis, MO). The phosphatase and protease inhibitors were purchased from Nacalai Tesque (Kyoto Japan). Radio-Immunoprecipitation Assay (RIPA) buffer was purchased from Wako (Osaka, Japan). 4×Laemmli sample buffer containing 10% of 2-mercaptoethanol and TGX FastCast acrylamide kit 12 were purchased from BIO-RAD (Hercules, CA, USA). MitoTracker Deep Red FM was obtained from Invitrogen Corporation (Carlsbad, CA). All other chemicals used were commercially available reagent-grade products.

Synthesis of methylated β-cyclodextrin-threaded acid-labile PRX (Me-PRX): P β-CD/Pluronic P103-based Acid-labile PRX composed of β-cyclodextrin *(*β-CD, Nihon Shokuhin Kako, Tokyo, Japan) as a cyclic molecule, PEG-*b*-PPG-*b*-PEG (Pluronic P103, Adeka, Tokyo, Japan:

$M_{n,PEG}$: 810 × 2, $M_{n,PPG}$: 3,250) as an axle polymer, and *N*-triphenylmethyl (*N*-Trt) groups as acid-cleavable stopper molecules was prepared as descebed in a previous report (K. Nishida, A. Tamura, N. Yui. *Macromolecules* **2016**, *49*, 6021-6030). The PRX (500 mg, 24.9 μmol of PRX, 0.32 mmol of threaded β-CDs) (β-CDs: 12.8, M_n: 20,100) was dissolved in dehydrated dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Osaka, Japan) (25 mL). Powdered NaOH (537 mg, 13.4 mmol, 2.0 molar equivalent to hydroxyl groups of threaded β-CDs) and methyl iodide (279 μL, 4.48 mmol, 0.67 molar equivalent to hydroxyl groups of threaded β-CDs) were added to this solution. The reaction mixture was stirred for 1 hr at room temperature. Then, the reaction mixture was poured into diethyl ether to precipitate the PRX. Then, the precipitate was dissolved in water and dialyzed against water (molecular weight cut-off of 3,500) (FastGene, Tokyo, Japan) for 2 days at 4 °C. The recovered solution was freeze-dried to yield Me-β-CD-threaded acid-labile PRX (Me-PRX) (398.2 mg, 72.0% yield). The numbers of threaded β-CDs and modified methyl groups of Me-PRX were determined by ^{1}H nuclear magnetic resonance (NMR) (Bruker Avance III 400 MHz spectrometer, Bruker BioSpin, Rheinstetten, Germany) in D_2O. The M_n of Me-PRX was calculated based on the number of threaded β-CDs and methyl groups. Size exclusion chromatography (SEC) of Me-PRX was carried out using an HLC-8120 system (Tosoh, Tokyo, Japan) equipped with a combination of TSKgel α-4000 and α-2500 columns (Tosoh). The system was eluted with DMSO containing 10 mM LiBr at a flow rate of 0.3 mL/min at 60 ºC. Moleculae weight was calculated using a calibration curve of standard PEG (Agilent Technologies, Wilmington, DE, USA). SEC (DMSO) M_n = 16,800, M_w/M_n = 1.08. ^{1}H NMR (400 MHz, D_2O) δ = 1.10 ppm (-CH_2-CH(C***H*****$_3$**)-O- of PPG), 3.0-4.0 (m, -C***H*****$_2$**C***H*****$_2$**O- of PEG, -C***H***(CH_3)-C***H*****$_2$**- of PPG, H2, H3, H4, H5, and H6 protons of β-CD, and -OC***H*****$_3$** of methyl group), 4.9-5.3 (m, H1 proton of β-CD), 7.21 (t, Trt group), 7.30 (t, Trt group), 7.42 (d, Trt group).

Synthesis of HiLyte Fluor 488-labeled Me-PRX: Me-PRX (50 mg, 2.25 μmol) and 1,1'-carbonyldiimidazole (Sigma-Aldrich, Milwaukee, WI, USA) (4.68 mg, 28.8 μmol) were dissolved in dehydrated DMSO (5 mL) and the solution was stirred for 24 h at room temperature under a nitrogen atmosphere. HiLyte Fluor 488-amine (Anaspec, Fremont, CA, USA) (0.4 mg, 2.25 μmol) dissolved in an aliquot of dehydrated DMSO (100 μL) and triethylamine (Wako Pure Chemical Industries) (0.1 μL, 2.70 μmol) was then added to the reaction mixture, and the solution was stirred for an additional 24 hr at room temperature. The product was purified by dialysis against water (molecular weight cut-off of 3,500 Da) (FastGene) for 3 days at 4 °C. The recovered solutions were freeze-dried to yield HiLyte Fluor 488-modified Me-PRX (HiLyte Fluor 488-Me-PRX) (41.3 mg, 82.7% yield). The absorbance of HiLyte Fluor 488-Me-PRX in water was measured on a V-550 UV/VIS spectrophotometer (Jasco, Tokyo, Japan), and the number of HiLyte Fluor 488 modified on Me-PRX was determined to be 0.0462.

Construction of the MITO-Porter (Me-PRX): The MITO-Porter (Me-PRX) was prepared by the REV method as described below. Chloroform (500 μL) containing 2.75 μmol lipids [DOPE/SM = 9:2 (molar ratio)] and 500 μL of diisopropyl ether were added to a glass tube. And 500 μL of 10 mM HEPES buffer (pH 7.4) containing Me-PRX (4 mM, final concentration of β-CD) was then added, followed by sonication for 15 sec with a probe-type sonicator (SONIFIER Model 250D, BRANDSON, Kanagawa, Japan). After removing the organic solvent with a stream of N_2 gas, a liposome suspension was formed by sonication for 30 sec in a bath-type sonicator (AU-25C, Aiwa Company, Tokyo, Japan). The STR-R8 or STR-S2 solutions (10 mol% of total lipids) were added to the liposome suspension to produce MITO-Porter (Me-PRX).

Characterization of Construction of the MITO-Porter (Me-PRX): Particle diameter and Poly dispersity index (PDI), an indicator of the particle-size distribution, were measured using a dynamic light scattering (DLS) method (Zetasizer Nano ZS; Malvern Instruments,

Worcestershire, U.K.). Samples were prepared in 10 mM HEPES buffer at 25°C and the values of particle diameters are shown. The ζ-potentials of the samples were also determined in 10 mM HEPES buffer at 25°C using a Zetasizer Nano ZS.

Estimation of encapsulation efficiency of Me-PRX in MITO-Porter: To investigate the recovery of the Me-PRX, MITO-Porter (Me-PRX) contains fluorescent labelled Me-PRX (Me-PRX: HiLyte Fluor 488-Me-PRX = 9 : 1, molar ratio) in the aqueous phase of the carriers. To investigate the recovery of the lipids, the carriers were labeled with DiI (0.1 mol% of the total lipids). The MITO-Porter (Me-PRX) was separated from the unencapsulated Me-PRX by ultracentrifugation (74,000 g, 20 min, 4ºC) using HIMAC CS 150GX (Hitachi Koki Co., Ltd., Tokyo, Japan). After removing the supernatant, the pellet fraction was suspended in 10 mM HEPES buffer (pH 7.4). The amounts of Me-PRX and the amounts of lipid were determined by measuring the fluorescent intensities of the HiLyte Fluor 488-Me-PRX (excitation at 497 nm and emission at 525 nm) and the DiI (excitation at 549 nm and emission at 565 nm) after treatment with SDS (1%, final concentration). The encapsulation efficiency was calculated using equation shown below:

Encapsulation efficiency (%) = (recovered Me-PRX / recovered lipid) / (applied Me-PRX / applied lipid) ×100.

Evaluation of autophagy induction by western-blotting: HeLa cells (5×10^4 cells/well) were incubated on a 12-well plate (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) for 24 hr (5% CO_2, 37ºC). After washing the cells with phosphate buffered saline (PBS (-)), the DMEM (FBS+) containing samples was added to the cell suspension, followed by incubation for 16 hr (5% CO_2, 37ºC). After washing the cells with PBS (-), the cells were lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors. The lysate was centrifuged (20,630 g, 10 min, 4ºC) and the supernatant collected, followed by western blotting.

Western-blotting: The supernatant of cell lysate was mixed with 4×Laemmli sample buffer and incubated at 95°C for 5 min. The resulting supernatants (1.5 μg proteins) were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was prepared using TGX FastCast acrylamide kit 12. After electrophoresis, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (0.2 μm pore size, Bio-Rad) using the Trans-Blot Turbo transfer system (Bio-Rad), and the membranes were then blocked by shaking for 1 hr at room temperature in Tris buffered saline (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) with 0.1% Tween-20 (TBS-T) containing 5% nonfat dry milk. The membrane was then shaken in a 1% nonfat dry milk solution containing the primary antibodies: rabbit anti-LC3B polyclonal antibody derived from rabbit (Novus Biologicals, Littleton, CO, U.S.A.) (1/1,000 dilution, 4°C, overnight) to detect the LC3 protein (an autophagy marker) and a mouse anti-beta Actin monoclonal antibody derived from mice (Abcam plc, Cambridge, UK) (1/5,000 dilution, 4°C, 3 hr) to detect the β-actin (internal control). After washing the membrane at three times with TBS-T, the membrane was shaken in TBS-T with 1% nonfat dry milk containing the secondary peroxidase-conjugated antibodies: Anti-IgG (H+L chain) (Rabbit) pAb-HRP (Medical and Biological Laboratories, Nagoya, Japan) (1/10,000 dilution, room temperature, 1 hr) or ECL Anti-Mouse IgG, Horseradish Peroxidase-Linked Species-Specific Whole Antibody (GE Healthcare, Little Chalfont, UK) (1/10,000 dilution, room temperature, 1 hr). After washing three times with TBS-T, blots were developed with ImmunoStar Zeta (Wako), and immunoreactive bands were visualized using the ImageQuant LAS 4000 mini (GE Healthcare UK), and immunereactive bands were visualized using the Image J (Image Processing and Analysis in Java). We quantified the relative LC3-II /β-actin protein expression levels based on the intensity of the bands corresponding to the protein.

Quantification of intracellular triphenylmethanol by GC-MS: HeLa cells (1×10^5 cells/well) were incubated on a 16-well plate (Thermo Fisher Scientific Inc.) for 24 hr (5% CO_2, 37°C).

After washing the cells with PBS (-), the DMEM (FBS+) containing samples was added to the cell suspension, followed by incubation for 16 hr (5% CO_2, 37ºC). After washing the cells with PBS (-), the cells were mixed with cholesterol-d_7 (10 μL, 1 mg/mL in pyridine) (Avanti Polar Lipids) as an internal standard and homogenized in PBS (1 mL). Homogenized samples were extracted with a mixture of chloroform and methanol (chloroform:methanol = 1:2; 3.75 mL), followed by extracted with chloroform (2.5 mL). The organic phase was combined and evaporated to dryness via nitrogen flow on an MGS-2200 instrument (Eyela, Tokyo, Japan) at 40 °C. Dried residues were dissolved in dehydrated pyridine (150 μL) and derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA, Sigma-Aldrich) (50 μL) for 30 min at 60 °C. The concentration of triphenylmethanol (Trt-OH) liberated from the acid-labile Me-PRX was quantified by GC-MS measurement on a GCMS-QP2020 instrument (Shimadzu, Tokyo, Japan) equipped with an AOC-20i auto injector (Shimadzu) and an MXT-1 cross-linked poly(dimethylsiloxane) capillary column (30 m × 0.25 mm I.D., 0.25 μm phase thickness) (Restek, Bellefonte, PA, USA). Ultra-high-purity helium (> 99.999%) was used as a carrier gas. Sample solutions (2 μL) were injected in splitless mode. The oven temperature was initially held at 100 °C for 1 min (0 to 1 min), increased to 300 °C at a rate of 20 °C/min (1 to 21 min), increased further to 330 °C at a rate of 10 °C/min (21 to 24 min), and finally held at 330°C for 46 min (24 to 60 min). For quantitative analysis, measurements were performed with a selected-ion monitoring (SIM) mode. The ions used for the determination ions were m/z = 77, 105, and 260 and the quantification was m/z = 183.

Observation of autophagosomes formation using CLSM: HeLa cells (1×10^5 cells/dish) were seeded on a 3.5 cm glass-base dish (Iwaki; ASAHI GLASS Company, Ltd., Tokyo, Japan) at one day before being assayed and incubated in DMEM, which contained 10% FBS, under an atmosphere of 5% CO_2/air at 37ºC. The cells were washed with DMEM containing 10% FBS, and then incubated with carriers containing Me-PRX or naked Me-PRX suspended in the

medium for 16 hr under an atmosphere of 5% CO_2/air at 37ºC. The cells were incubated in 1 x Assay buffer with 5% FBS containing Cyto-ID Green Detection Reagent (Cosmo Bio Co., Ltd., Tokyo, Japan) for staining autophagoseomes and Hoechst 33342 for staining the nucleus for 30 min under an atmosphere of 5% CO_2/air at 37ºC, after washing cells with 1 x Assay buffer with 5% FBS twice. After washing the cells in 1 x Assay buffer the cells were incubated in 4% formaldehyde for 20 min. The cells were then washed in 1 x Assay buffer three times, followed by observation by CLSM (FV10i-LIV; Olympus Corporation, Tokyo, Japan). The cells were excited with a 405 nm light for detecting Hoechst 33342 and a 473 nm light for detecting Cyto-ID Green Detection Reagent using a LD laser. Images were obtained using a FV10i-LIV equipped with a water immersion objective lens (UPlanSApo 60x/NA. 1.2) and a diachronic mirror (DM 405/473/559/635). The two fluorescence detection channels (Chs) were set to the following filters: Ch1: BP 420-460 (cyan pseudo color) for Hoechst 33342 and Ch2: BP 490-540 (green color) for Cyto-ID Green Detection Reagent.

Intracellular observation of MITO-Porter (Me-PRX) using CLSM: HeLa cells (1×10^5 cells/well) were incubated on a 3.5 cm glass-base dish (Iwaki) for 24 hr (5% CO_2, 37ºC). The DMEM (FBS+) containing MITO-Porter (Me-PRX) (20 μM, final concentration of β-CD) labeled with DiI (0.1 mol% of the total lipids) was added to the cells after washing the cells in DMEM (FBS+), followed by incubation for 1 hr (5% CO_2, 37ºC). The medium was replaced with fresh DMEM (FBS+) and the cells were incubated for 100 min (5% CO_2, 37ºC). After a 100 min period of incubation, the medium was replaced with the DMEM (FBS+) containing MitoTracker Deep Red (100 nM, final concentration) and the cells were incubated for 20 min (5% CO_2, 37ºC). After washing the cells in DMEM (FBS+), they were observed by CLSM (FV10i-LIV). The cells were excited with a 559 nm light for detecting DiI and a 635 nm light for detecting MitoTracker Deep Red using a LD laser. Images were obtained using a FV10i-LIV equipped with a water immersion objective lens (UPlanSApo 60x/NA. 1.2) and a

diachronic mirror (DM 405/473/559/635). The two fluorescence detections Chs were set to the following filters: Ch1: BP 570-620 (green pseudo color) for DiI and Ch2: BP 660-710 (red color) for MitoTracker Deep Red.

Statistical analysis: Data are expressed as the mean ± S.D. for the indicated number of experiments. For multiple comparisons, one way ANOVA followed by Student Newman Keuls (SNK) test was performed. Levels of $p < 0.05$ were considered to be significant.

Supplementary Figure

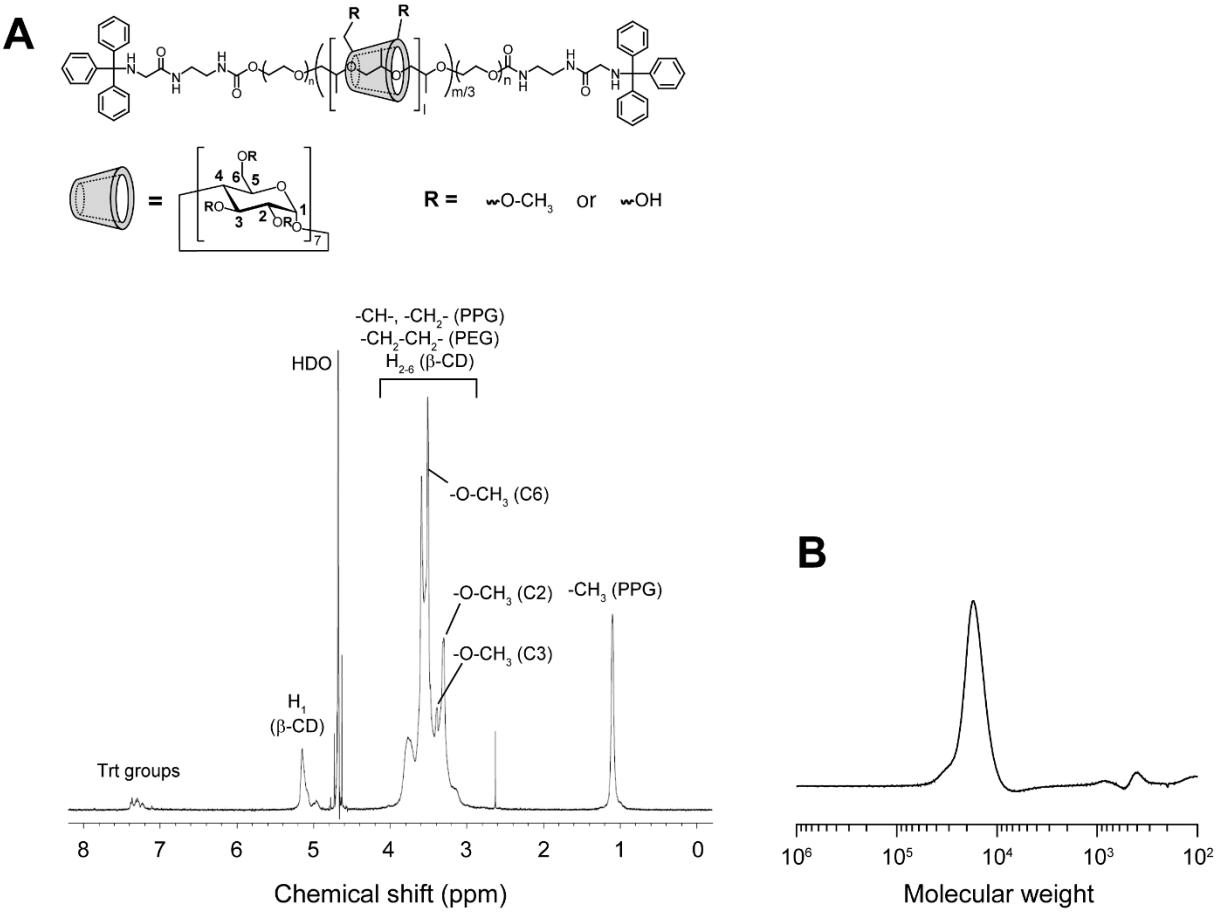
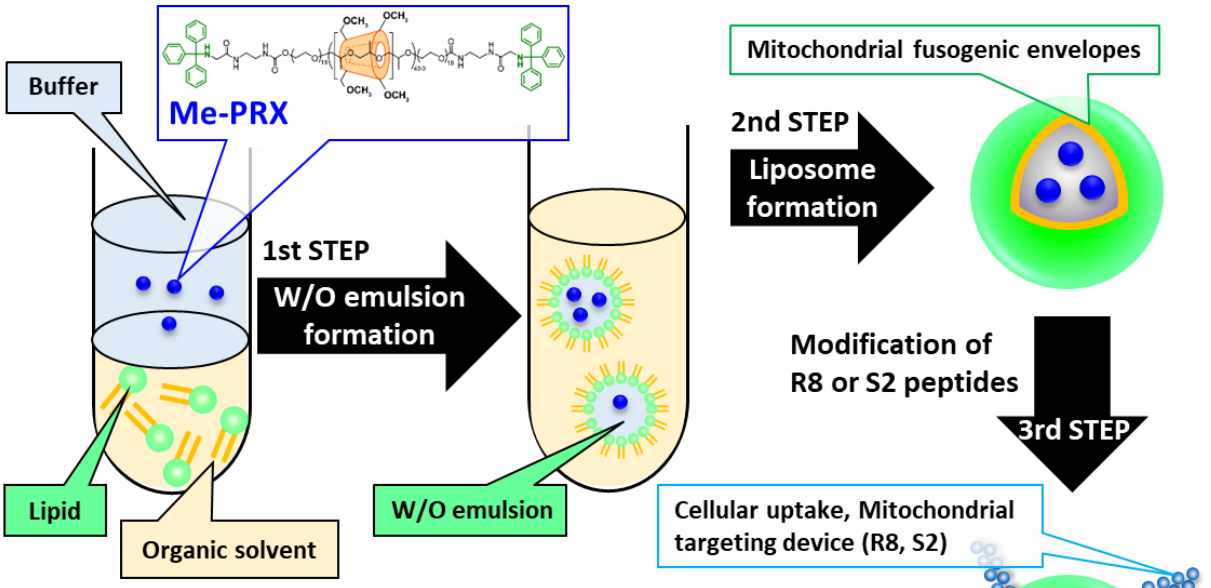


Figure S1. (A) Chemical structure and ^{1}H NMR spectrum of Me-PRX, where where *n*, *m*, and *l* denote the polymerization degree of the PEG segments, the polymerization degree of the PPG segment, and the number of threaded β-CDs, respectively. ^{1}H NMR spectrum of Me-PRX was recorded in D_2O. (B) SEC chart of Me-PRX in DMSO containing 10 mM LiBr at 60 ºC. The molecular weight was calibrated with standard PEGs.



	Diameter (nm)	PdI	ζ-potential (mV)
R8-MITO-Porter (Me-PRX)	121 ± 11	0.20 ± 0.04	40 ± 5
Empty R8-MITO-Porter	100 ± 4	0.19 ± 0.02	35 ± 4
S2-MITO-Porter (Me-PRX)	106 ± 4	0.23 ± 0.02	37 ± 3
Empty S2-MITO-Porter	105 ± 6	0.23 ± 0.01	43 ± 2

Figure S2. A schematic diagram for the preparation of the MITO-Porter containing Me-PRX by the reverse phase evaporation vesicle method [1, 2] is shown. The preparation was performed in the following steps: 1) W/O emulsion formation by sonication, 2) liposome formation by removing the organic solvent followed by sonication, 3) modification of R8 or S2 to lipid envelopes. Physicochemical properties of MITO-Porter (Me-PRX) and empty MITO-Porter are shown. Data are represented as the mean ± S.D. (n = 5).

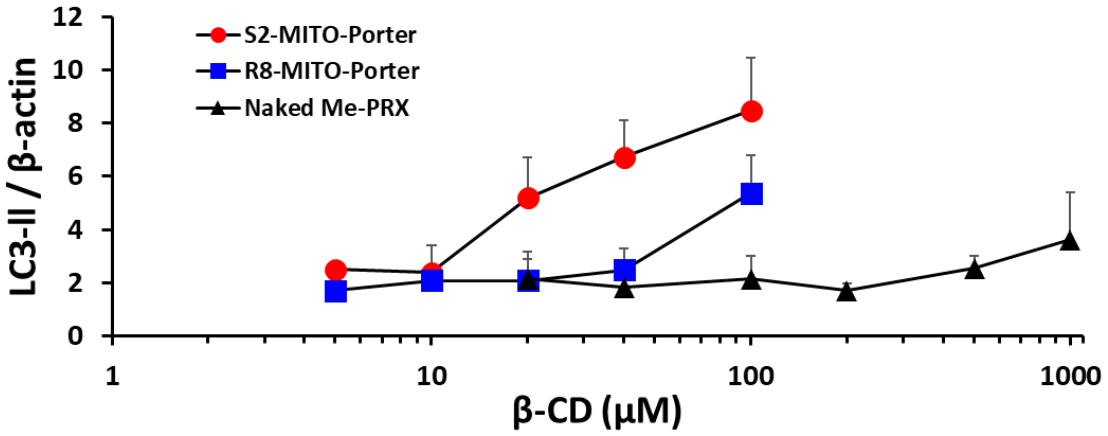


Figure S3 Quantification of LC3-II expression levels. The LC3-ll / β-actin values in various β-CD concentration of S2-MITO-Porter (Me-PRX), R8-MITO-Porter (Me-PRX), naked Me-PRX are shown. Data are represented by the mean ± SD (n = 5).

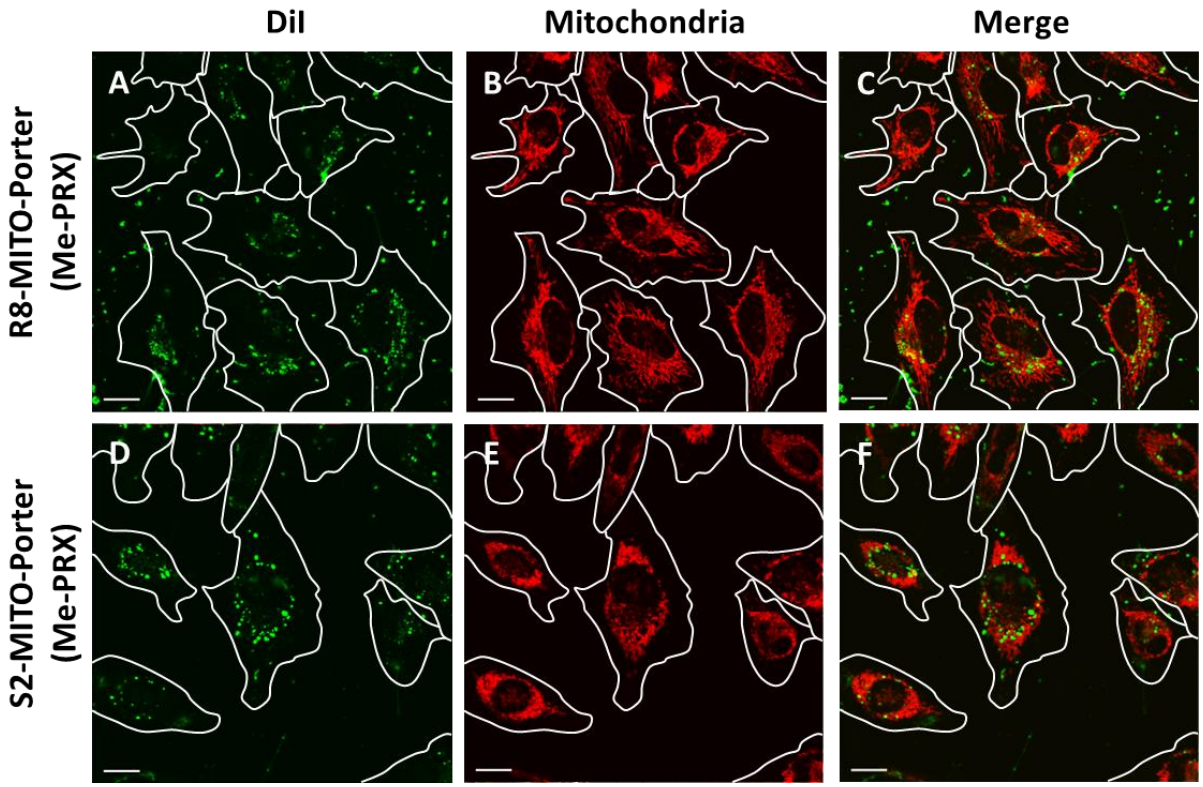


Figure S4 Intracellular observation of MITO-Porter (Me-PRX) in serum free medium. R8-MITO-Porter (Me-PRX) (A, B, C) and S2-MITO-Porter (Me-PRX) (D, E, F) were added to HeLa cells in serum free medium and intracellular localization of the carriers after 1 h incubation was observed using CLSM. Mitochondria were stained with MitoTracker Deep Red (red) prior to intracellular observation. Carriers labeled with DiI (green) are observed as yellow signals in merged images by colocalization with mitochondria (red). Scale bars, 20 μm.

References

1. J. Abe, Y. Yamada and H. Harashima, *J Pharm Sci*, 2016, **105**, 734-740.
2. Y. Yamada, L. Burger, E. Kawamura and H. Harashima, *Biological & Pharmaceutical Bulletin*, 2017, **40**, 2183-2190.