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Supporting Information

A Metastasis Suppressor Pt-Dendrimer Nanozyme for the Alleviation of Glioblastoma

Jong Min An,† Youngwon Ju,† Jeong Hee Kim, Hyein Lee, Yuna Jung, Jaehoon Kim, Yong Jun Kim, Joohoon Kim*, and Dokyoung Kim*

This PDF file includes:

1. Materials and Methods

General information

Chemical reagents were purchased from Aldrich (USA), TCI (Japan), Alfa Aesar (USA), and Acros Organics (USA). Commercially available reagents and anhydrous solvents were used without further purification. Amine-terminated sixth generation polyamidoamine dendrimer (G6-dendrimer, Cat #536717), K_2PtCl_4, $NaBH_4$, dimethyl sulfoxide (DMSO, anhydrous), hydrogen peroxide (H_2O_2), sodium acetate, acetic acid, 3,3',5,5'-tetramethylbenzidine (TMB), and cellulose dialysis sacks (M.W. cut-off 12,000, Cat #D6066) were purchased from MilliporeSigma, Inc. (USA). Cyanine-5 N-hydroxysuccinimide ester (Cy5-NHS, Cat #43020) was purchased from Lumiprobe, Co. (USA). 5(6)-carboxyfluorescein N-hydroxysuccinimide ester (fluorescein-NHS, Cat #C2210) was purchased from Thermo Fisher Scientific Inc. (USA). Hydrogen chloride (HCl) was purchased from Daejung, Inc. (Rep. of Korea). Deionized water (DI H_2O, Ultra370, Younglin Co., Rep. of Korea) was used to prepare aqueous solutions. Trackers (lysosome tracker red; Cat # L12492, mitochondria tracker deep-red; Cat #M22426, and endoplasmic reticulum tracker red; Cat #E34250, select-FX Alexa fluor 488 peroxisome labeling kit; Cat # S34201) were purchased from ThermoFisher (USA), and used for the monitoring of cellular sub-organelles. CCK-8 (cell counting kit 8 (Dojindo Molecular Tech. Inc., Japan) was used to confirm the cellular toxicity. The cell-based hydrogen peroxide assay kit (Cat #ab239701, abcam®, UK) was used to evaluate the intracellular H_2O_2. SPLInsert™ Hanging with polycarbonate membrane (PC, Cat # 35206, pore size 8.0 μm, SPL Life Sciences Co., Ltd., Rep. of Korea) and Corining® matrigel matrix (Lot #354234, Corining, USA) were used to evaluate the cellular invasion ability. SPLScar™ (Lot #201906, SPL Life Sciences Co., Ltd., Rep. of Korea) was used for the wound healing test. Dulbecco's modified Eagle's media (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Utah, USA) and used for the cell culture (U87MG, HEK293). Penicillin-streptomycin was purchased from Gibco Industries Inc. (Auckland, NZ) and used for the cell culture (U87MG, HEK293). Phosphate buffered saline (PBS) was purchased from Sigma-Aldrich (St Louis, MO, USA). Alexa Fluor™ 647 Phalloidin (Cat # A30107, Invitrogen™, USA) was used to perform the confocal fluorescence imaging of F-actin. Cell culture dishes (96-well plate, 24-well plate, 6-well plate, and 100-Ø dish) were purchased from SPL Life Science (Rep. of Korea). Confocal laser scanning microscope (CLSM) imaging was performed using LSM-800 instrument (Carl Zeiss, Germany). IncuCyte® live cell analysis system (IncuCyte zoom 2CLR, Sartorius AG, Germany) was used to observe the migration of U87MG cells.

Peroxidase-mimetic activity assay of Pt-dendrimer

The peroxidase-like activity of Pt-dendrimer was analyzed in a similar method to our previous report.[1] The Pt-dendrimer and H_2O_2 aqueous solutions were added into the nitrogen gas (N_2)-purged HAc-NaAc buffer (200 mM, pH 4.0) at 25 °C in the presence of TMB. The final concentrations of each reactant were 10 nM of Pt-dendrimer, 10 mM of H_2O_2, and 500 μM of TMB. All the reactions were monitored by measuring absorbance changes at 652 nm using a UV-vis spectrometer (Agilent 8453, Agilent Tech., USA).

Cell culture

Human glioblastoma cell line (U87 MG), human embryonic kidney cell line (HEK293), MDA-MB-231 cell line were obtained from Korean Cell Line Bank. Cells (U87MG and HEK293) were cultured in Dulbecco's modified Eagle's media (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Cat #SH30243.01, Hyclone, USA) and 1% penicillin-streptomycin (PS, Cat #15140122, Gibco, USA). The MDA-MB-231 cell line were cultured in RPMI-1640 (Hyclon, USA) supplement with 10% FBS and 1% PS. All cell lines were maintained in humidified air containing 5% CO_2 at 37 °C.

Human embryonic stem cells and astrocyte differentiation

hESC (WA09) was purchased from WiCell (Wisconsin, USA) and maintained with daily feeding of Essential 8 medium (Thermo Fisher, USA) on the laminin-511 coated 6-well plate (Nippi, Japan). [Steps to differentiate astrocytes] (i) hESCs were collected and dissociated into single cells using Accutase (Lot #AT-104, Sigma Aldrich, USA) to differentiate astrocytes. (ii) approximately 10^6 cells were seeded on geltrex coated a 24-well plate and incubated for 1 h at 37 °C. (iii) neurobasal medium containing FGF2 (20 ng/mL, Lot #4114-TC-01M, R&D and BMP4 (10 ng/mL, Lot #314-BP-050, R&D, USA) were treated to the cells with 50× B27 (Lot #12587001, Thermo Fisher, USA) and 100× N-2 (Lot #17502001, Thermo Fisher, USA), and cultured for thirty days following the reported protocol with minor modifications.[2] All the experiments using hESCs were performed under the institutional regulation of KHSIRB-20-489 (EA) using the KCDC reporting process.

Cellular uptake analysis

Approximately 2×10^4 cells (U87MG) were seeded on 35-mm glass bottom confocal dishes (Cat #200350, SPL Life Science, Rep. of Korea) and incubated for 24 h at 37 °C in 5% CO_2 incubator. At the 80% confluency, media was changed to fresh media containing 10% FBS. The cells were treated with the FITC-conjugated Pt-dendrimer and Cy5-conjugated Pt-dendrimer (0.1–0.5 μM) and incubated for 12 h at 37 °C in 5% CO_2 incubator. After the incubation, the cells were washed three times with 1× PBS (pH 7.4) and treated with 4% formaldehyde with incubation for 6 min at 25 °C. Next, the cells were washed twice with 1× PBS (pH 7.4), and the prepared samples were imaged using CLSM. Wavelength channel was used for CLSM images [FITC-conjugated Pt-dendrimer (excitation wavelength: 488 nm, emission channel: 490–700 nm), Cy5-conjugated Pt-dendrimer (excitation wavelength: 640 nm, emission channel: 645–700 nm)].

Cellular sub-organelles co-localization analysis

Approximately 1×10^5 cells (HeLa) were seeded on 35-mm glass bottom confocal dishes and incubated for 24 h under the same cell culture conditions as above. At the 80% confluency, cells were treated with the FITC-conjugated Pt-dendrimer or Cy5-conjugated Pt-dendrimer (0.2 μM), and incubated for 12 h at 37 °C. After the incubation, cells were washed three times with 1× PBS (pH 7.4). For the cellular sub-organelles (mitochondria, endoplasmic reticulum, lysosome, and peroxisome) co-localization analysis, the cells were treated with Lysosome Tracker Red (2000×, 1 μL), Mitochondria-Tracker Deep-Red (2000×, 1 μL), and ER-Tracker Red (1000×, 2 μL), respectively, and incubated within serum free media for 20 min at 37 °C in 5% CO_2 incubator. Next, the cells were washed three times with 1× PBS (pH 7.4), and then treated with 4% formaldehyde with incubation for 7 min at 25 °C. The resulting cells were washed twice with 1× PBS (pH 7.4) and the prepared samples were imaged using CLSM. In case of the peroxisome tracing, an anti-peroxidsomal membrane protein 70 (PMP 70) rabbit IgG (250 μg/mL solution in PBS containing 2 mM sodium azide) fraction was used. Pt-dendrimer (0.2 μM) were pre-treated into U87MG cells, and incubated for 12 h at 37 °C. After that, the cells were fixed with a 4% fixative solution (methanol-free formaldehyde) with incubation for 15 min at 37 °C, and then washed three times with 1× PBS (pH 7.4). To permeabilize the cells, 0.2% (*w*/*v*) Triton X-100 was used with incubation for 5 min at 25 °C. Next, the cells were washed twice with 1× PBS (pH 7.4), and the fixed cells were blocked using 10% heat-inactivated normal goat serum (NGS) with incubation for 30 min at 25 °C. Primary antibody (anti-PMP 70) (1000× dilution, 2 μL) were incubated with blocked cells for 2 h at 25 °C. After the washing (3 times using 1× PBS), secondary antibody labeled Alexa Fluoro 488 (10,000× dilution, 0.2 μL) were incubated with the cells in 1× PBS. Next, the cells were rinsed with 1× PBS (3 times) to remove the remaining antibody. The prepared samples were imaged using CLSM (Emission band for the Cy5-conjugated Pt-dendrimer channel under excitation at 640 nm was 645-700 nm, emission band for the antibody channel under excitation at 488 nm was 517–617 nm).

F-actin image

Approximately 2×10^4 cells (U87MG) were seeded on a 35 mm confocal dish (Cat #200350, SPL Life Science, Rep. of Korea). The FITC-conjugated Pt-dendrimer (0.2 μM) was treated into the cell culture media after the cells were attached on the dish completely. After 12 h incubation at 37 °C in 5% CO_2 incubator, the cells were rinsed three times using 1× PBS (pH 7.4) and fixed by treatment of 4% PFA for 10 min incubation in dark room at 25 °C. The permeabilized process was performed using 0.2% Triton X-100 (incubation for 3 min at cold condition; 4 °C). After the rinsing with 1× PBS, the cells were treated with Alexa Fluoro Plus™ 647 Phalloidin (40× stock solution in methanol) (50 μL) and incubated for 20 min in dark room at 25 °C. Next, the cells were rinsed three times with 1× PBS to remove remnants, and the prepared cells were imaged using the CLSM (Emission band for the FITC-conjugated Pt-dendrimer channel under excitation at 488 nm was 495–519 nm, emission band for the antibody channel under excitation at 640 nm was 645–700 nm)

mRNA expression level analysis

RNA was extracted from the cultured U87MG cells using RNA quizol (Lot #79306, Qiagen, Germany) with/without treatment of Pt-dendrimer (0.2 μM). cDNA libraries were synthesized using cDNA synthesis kit with random primer (Lot #4368813, Applied Biosystem, USA). To analyze mRNA expression, each cDNAs was amplified using a KAPA SYBR FAST (Lot #KK4605, Sigma Aldrich, USA) and a step-one plus real time PCR instrument (Applied Biosystem, USA).

Cellular motion analysis

Approximately 1×10^3 cells (U87MG) were seeded on flat bottom 96-well plate (Cat #30096, SPL Life Science, Rep. of Korea). After 24 h incubation at 37 °C in 5% CO_2 incubator, cells were treated with Pt-dendrimer (0.2 μM). Cellular motion was monitored for 12 h using the IncuCyte® live cell analysis system (IncuCyte zoom 2CLR, Sartorius AG, Germany).

2. Supporting Figures

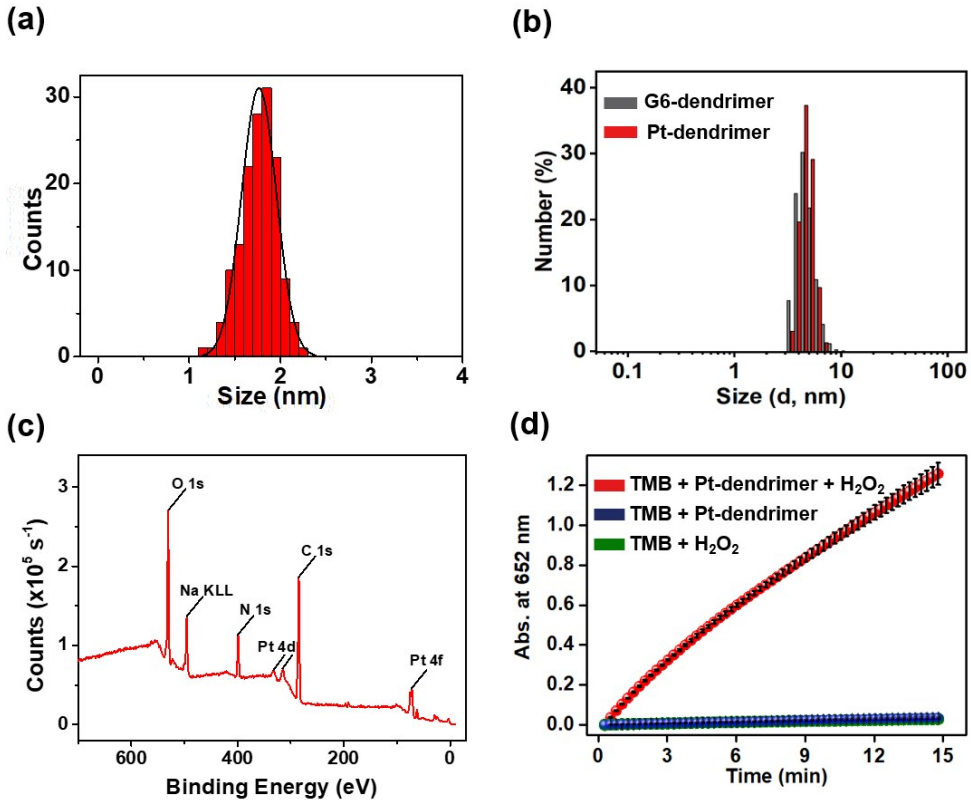

Fig. S1. Characterization of Pt-dendrimer. (a) Size-distribution histogram of Pt-dendrimer from transmission electron microscopy (TEM) images. (b) Mean hydrodynamic diameter distribution of G6-dendrimer (10 μM) and Pt-dendrimer (10 μM), which were measured by Dynamic Light Scattering (DLS) in DI H_2O. (c) X-ray photoelectron spectroscopy (XPS) spectrum of Pt-dendrimer. (d) Time-dependent (0–15 min) absorbance changes at 652 nm of 500 μM tetramethylbenzidine (TMB) solutions [N_2-purged 200 mM HAc-NaAc buffer (pH 4.0) containing 10 mM H_2O_2 or 10 nM Pt-dendrimer]

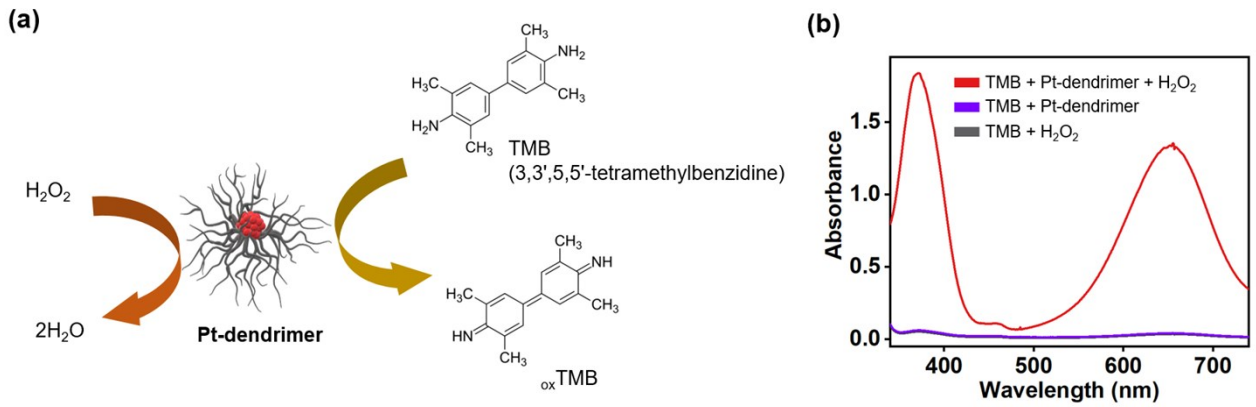

Fig. S2. (a) Schematic illustration of TMB assays of H_2O_2 in the presence of Pt-dendrimer. (b) UV-Vis absorption spectra of TMB solutions (N_2-purged 200 mM HAc-NaAc buffer, pH 4.0) containing (red line) H_2O_2 andPt-dendrimer, (purple line) Pt-dendrimer, or (grey line, overlapped with the purple line) H_2O_2. Concentration: 500 μM TMB, 10 mM H_2O_2, and 10 nM Pt-dendrimer. Reaction time: 15 min.

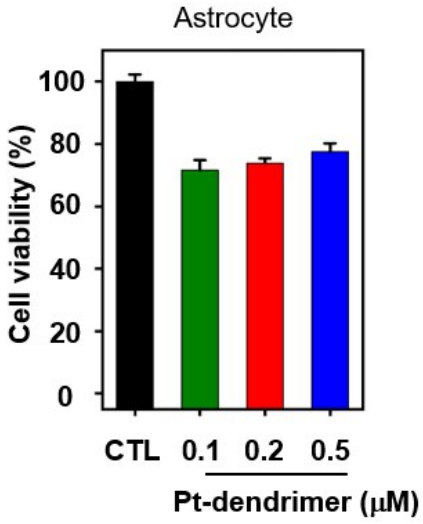

Fig. S3. Cell viability (%) of astrocyte after treatment of Pt-dendrimer with incubation for 24 h at 37 °C. Error bar: mean ± standard deviation (S.D.) with an unpaired t-test.

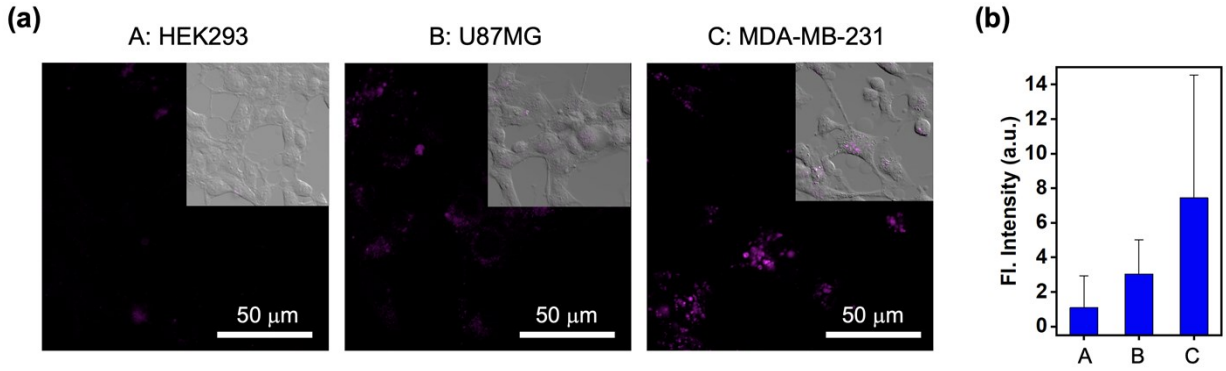

Fig. S4. (a) CLSM images of HEK293, U87MG, and MDA-MB-231 cells after treatment of the H_2O_2-imaging agent (analysis kit, incubation time: 30 min, 37 °C). Scale bar: 100 μm. (b) The fluorescence intensity plot from the images in panel (a). The fluorescence intensity was obtained using the Image-J program drawing the entire cell. The images were obtained with excitation at 561 nm (4.00% laser power) and emission channel at 563–700 nm (LSM800 GaAsP detector), and a pseudo purple color was introduced to enhance visualization. Error bar: mean ± standard deviation (S.D.). A: HEK293, B: U87MG, C: MDA-MB-231.

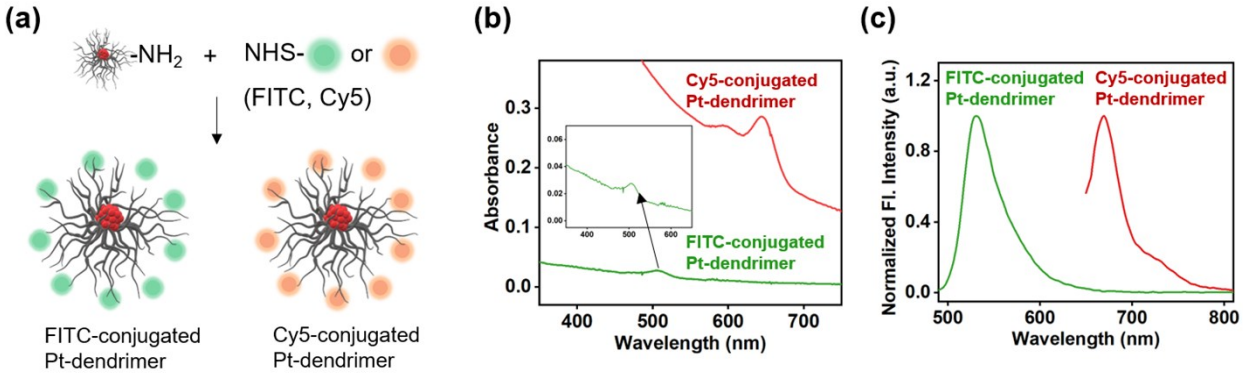

Fig. S5. Synthesis of FITC-conjugated Pt-dendrimer and Cy5-conjugated Pt-dendrimer. (a) Synthetic scheme of the FITC-conjugated Pt-dendrimer and Cy5-conjugated Pt-dendrimer. (b) Absorption spectra of the FITC-conjugated Pt-dendrimer (1 μM) and Cy5-conjugated Pt-dendrimer (10 μM). Optical path length: 2 mm. Blank solution: DI H_2O. (c) Normalized emission spectra of the FITC-conjugated Pt-dendrimer (10 μM) and Cy5-conjugated Pt-dendrimer (10 μM) in DI H_2O solvent with excitation at 470 nm and 646 nm respectively.

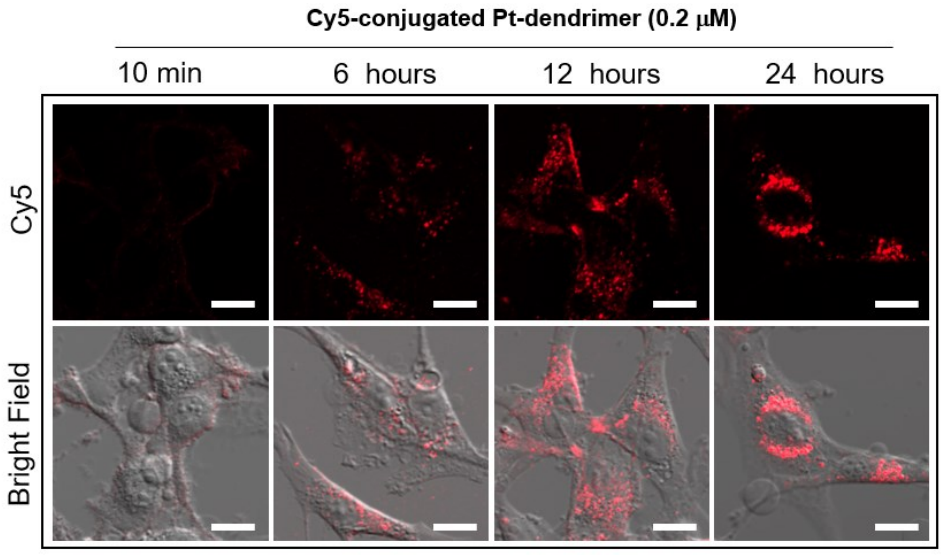

Fig. S6. Time-dependent (10 min–24 h) Confocal Laser Scanning Microscopy (CLSM) images of U87MG cells after treated with the Cy5-conjugated Pt-dendrimer (0.2 μM). Excitation wavelength: 640 nm. Detection channel: 645–700 nm. Scale bar: 20 μm.

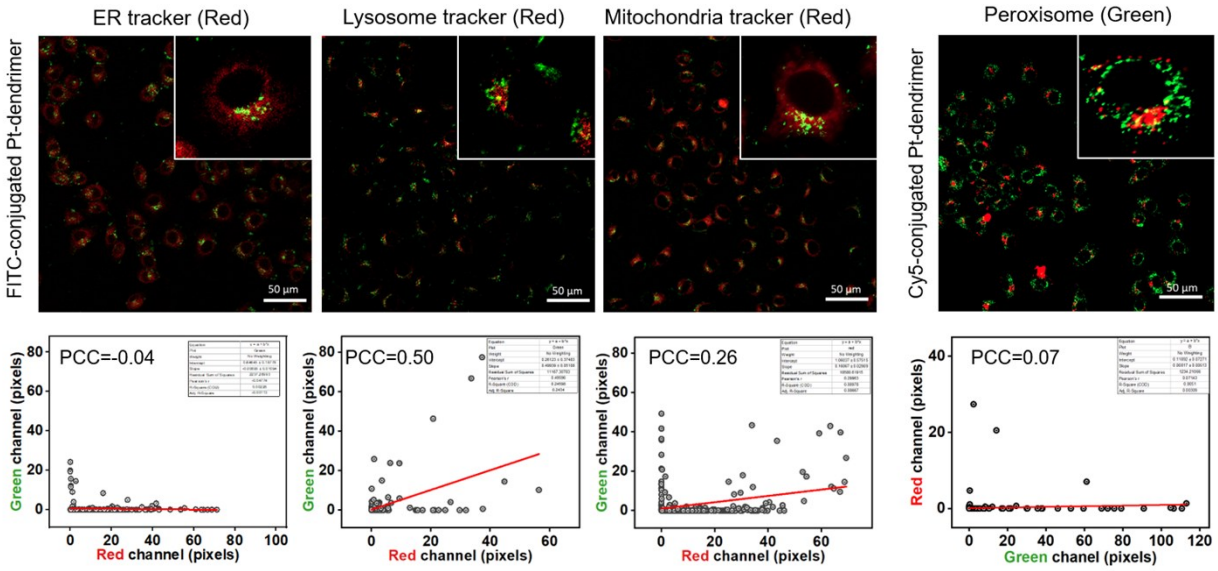

Fig. S7. Confocal Laser Scanning Microscopy (CLSM) images of HeLa cells after treatment of dye-conjugated Pt-dendrimer (FITC-conjugated Pt-dendrimer; 0.2 μM, Cy5-conjugated Pt-dendrimer; 0.2 μM) for 12 h at 37 °C and then sub-organelle trackers (ER, lysosome, mitochondria, peroxisome) with incubation for 20 min at 37 °C. The Pearson Correlation Coefficient (PCC) value was calculated using image-J program. Green fluorescence images: excitation at 488 nm, emission channel at 495–519 nm. Red fluorescence images: excitation at 640 nm, emission channel at 645–700 nm.

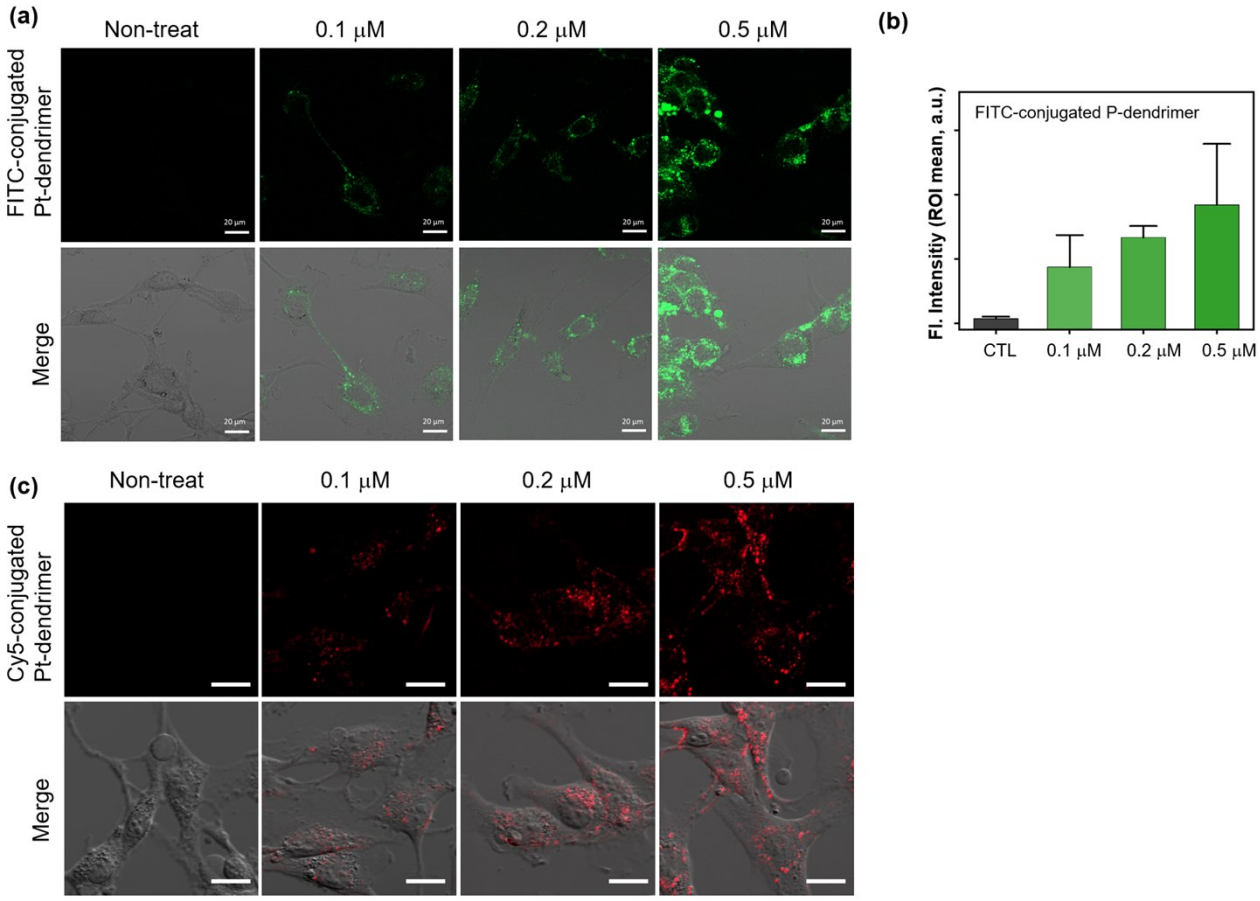

Fig. S8. Concentration-dependent (0.1–0.5 μM) Confocal Laser Scanning Microscopy (CLSM) images of U87MG cells after treatment of dye-conjugated Pt-dendrimer (FITC-conjugated Pt-dendrimer, Cy5-conjugated Pt-dendrimer). (a) CLSM images of U87MG cells after treatment of the FITC-conjugated Pt-dendrimer with incubation for 12 h at 37 °C. Scale bar: 20 μm. (b) Fluorescent intensity plot from panel (a). The intensity was obtained using the Image-J program drawing the entire cell region. (c) CLSM images of U87MG cells after treatment of the Cy5-conjugated Pt-dendrimer with incubation for 12 h at 37 °C. Scale bar: 20 μm.

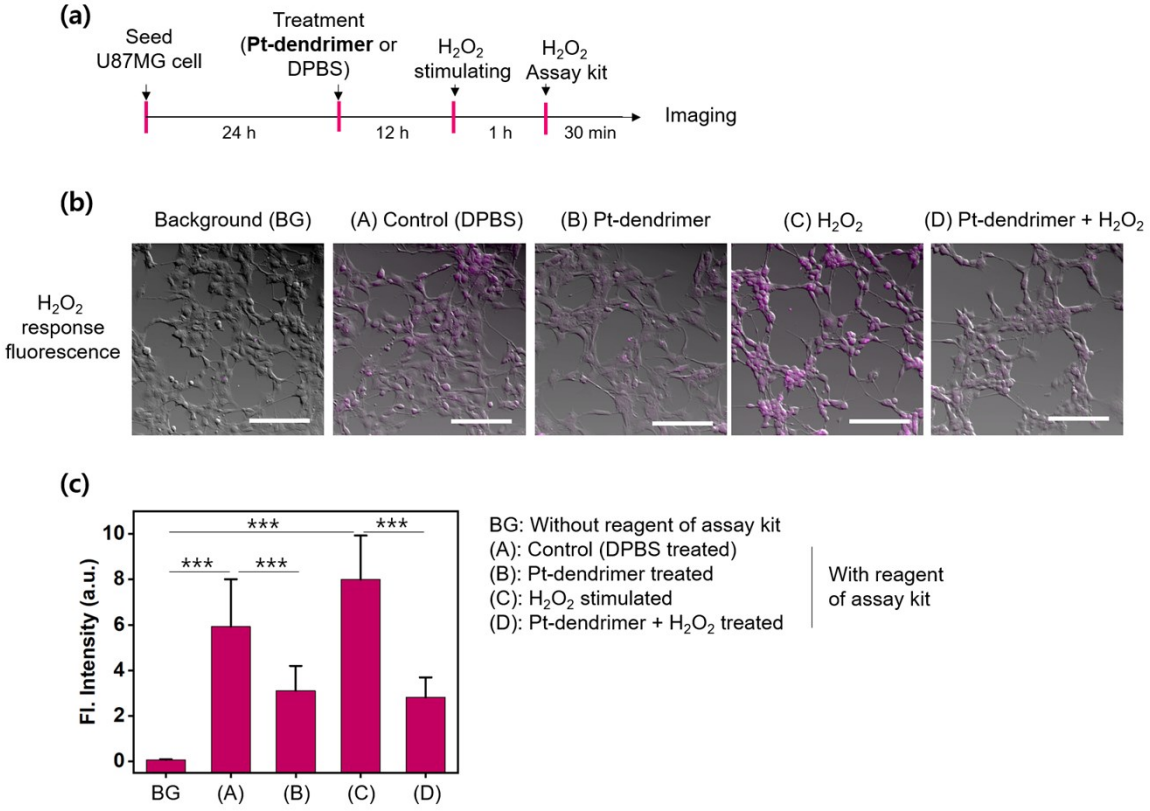

Fig. S9. Visualization of the intracellular H_2O_2 level in U87MG cells after treatment of Pt-dendrimer. (a) The experimental flow to observe the H_2O_2 level after treatment of the Pt-dendrimer (0.2 μM), DPBS (negative control), H_2O_2 (stimulated positive control) with incubation for 12 h at 37 °C. (b) CLSM images of U87MG cells using the H_2O_2 analysis kit (incubation time: 30 min) with pre-treatment of Pt-dendrimer (0.2 μM, 12 h incubation, 37 °C) and H_2O_2 (500 μM, 1 h incubation, 37 °C). Scale bar: 100 μm. (c) The fluorescence intensity plot from the images in panel (b). The fluorescence intensity was obtained using the Image-J program drawing the entire cell. The error bar: mean ± standard deviation (S.D.) with one-way ANOVA analysis of variance by Tukey's multiple comparison, ***$p < 0.001$. BG: background. (A): control. (B): Pt-dendrimer-treated group. (C): H_2O_2-stimulated group. (D): Pt-dendrimer pre-treated and H_2O_2-stimulated group.

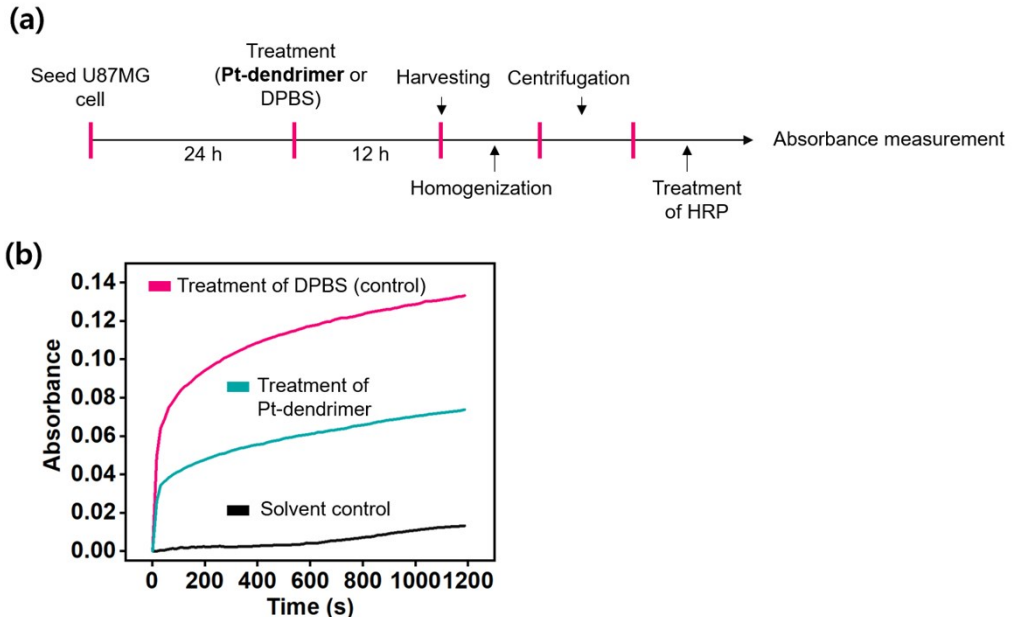

Fig. S10. The H_2O_2 level analysis within U87MG cell lysis media after treatment of Pt-dendrimer. (a) The experimental flow to observe the H_2O_2 level within cell lysis media after treatment of Pt-dendrimer (0.2 μM) with incubation for 12 h at 37 °C. (b) Time-dependent (0–1200 sec) absorption intensity plot of TMB (500 μM) (at 652 nm) with horseradish peroxidase (HRP, 50 ng/mL) with Pt-dendrimer-treated (0.2 μM) or DPBS-treated cell solution. The value was averaged from independent duplicated experiments. Statistics were obtained by repeated-measured (RM) one-way ANOVA, ****P < 0.0001 (DPBS-treated vs Pt-dendrimer-treated), ****P < 0.0001 (Pt-dendrimer-treated vs solvent control).

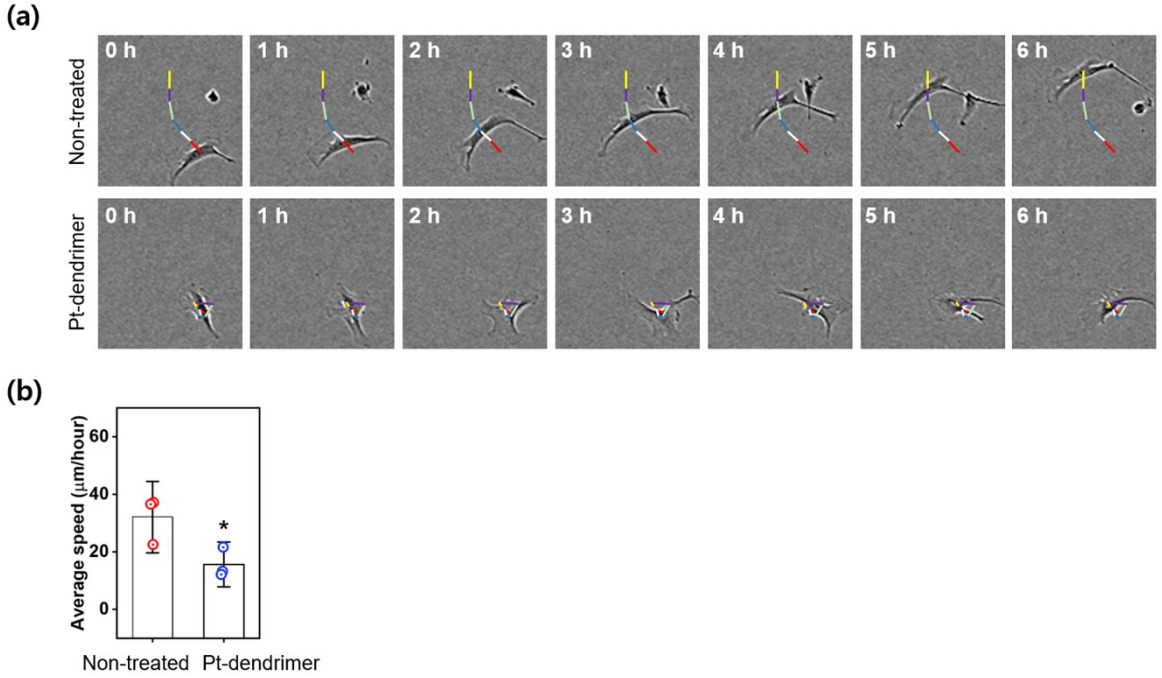

Fig. S11. (a) Time-dependent (0–6 h) live images of single U87MG cell after treatment of Pt-dendrimer (0.2 μM) using IncuCyte®Live cell analysis instrument and Image-J program. Cell movement: from red to yellow bar. (b) Average speed plot of U87MG with/without Pt-dendrimer (0.2 μM). The data originated from (a) and was collected using the Image J program. The error bar represents mean ± S.D. with an unpaired t-test, *P < 0.05; non-treated vs Pt-dendrimer.

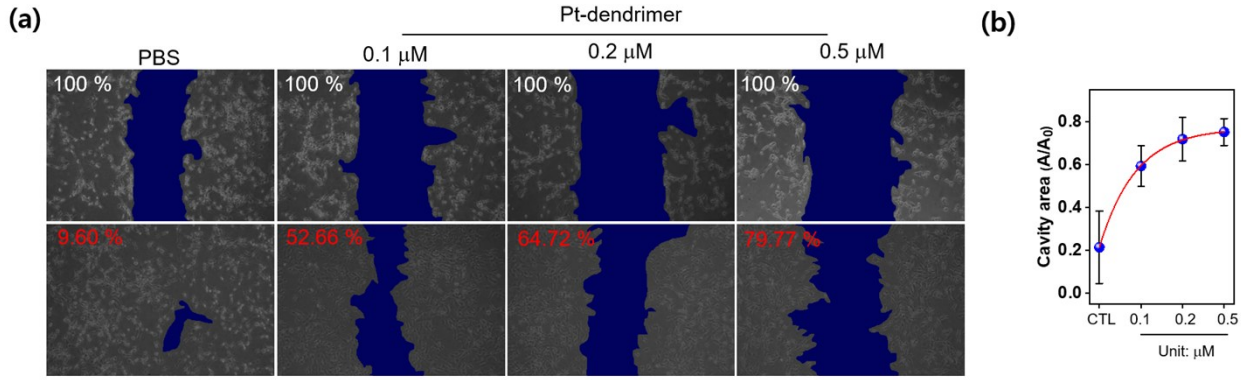

Fig. S12. Concentration-dependent cellular wound healing assay. (a) Images of U87MG cells after treatment of Pt-dendrimer (0.1–0.5 μM) with incubation for 12 h at 37 °C. Top: images of as-prepared wound healing models. Bottom: images of 12 h incubation with Pt-dendrimer. Image analysis was performed with Image-J program. Percent: means area represented in blue. (b) The cavity area plot after treatment of Pt-dendrimer (0.1–0.5 μM). A_0: Initial area (upper images within panel (a)). A: area after treatment of Pt-dendrimer with 12 h incubation (bottom images within panel (a)). The data value is the mean ± standard deviation (S.D.) from independent triplet experiments.

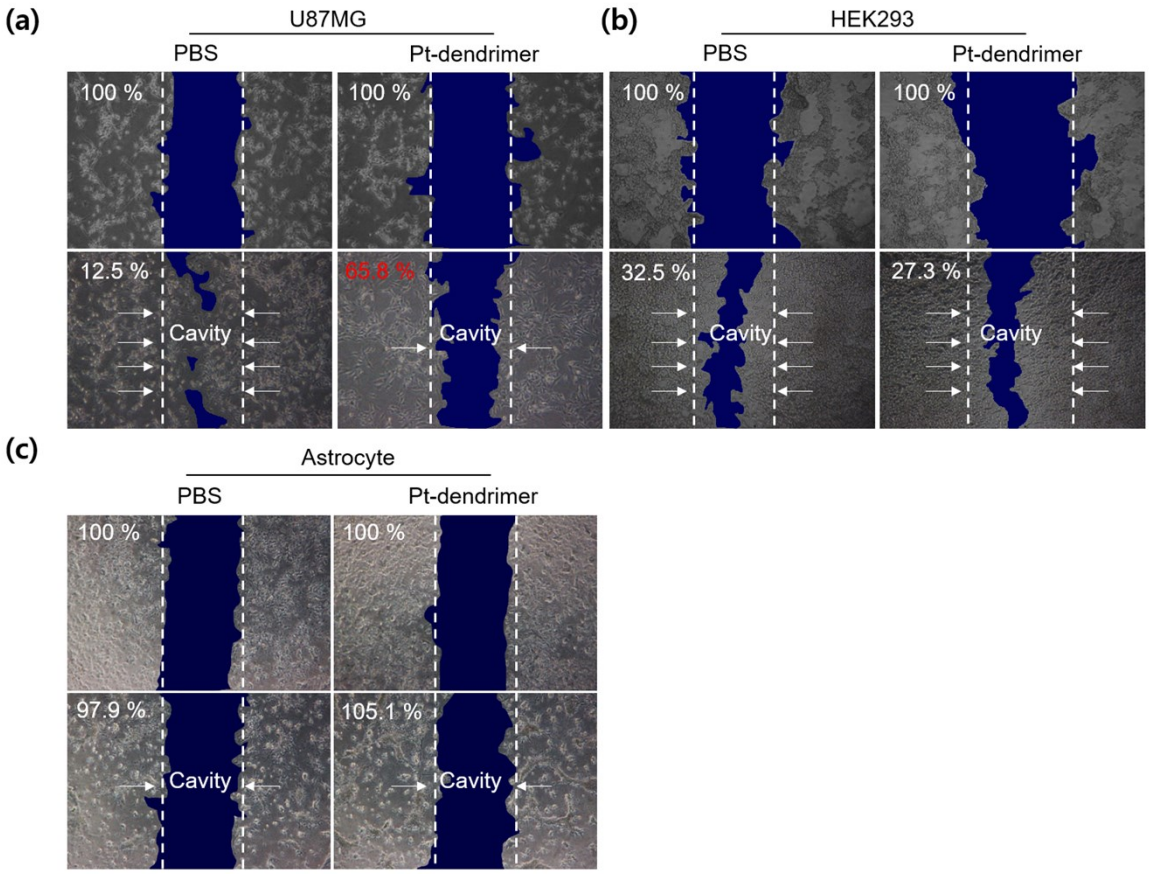

Fig. S13. Cellular wound healing assay in different cell lines. (a) U87MG, (b) HEK293, and (c) astrocyte cell images after treatment of Pt-dendrimer (0.2 μM) with incubation for 12 h at 37 °C. Image analysis was performed with Image-J program. Percent: means area represented in blue.

3. Supporting Tables

Table S1. The sequence of mRNA-related epithelial mesenchymal transition (EMT).

mRNA	**Sequence**	
SLUG	Forward	CAGACCCTGGTTGCTTCAAG
	Reverse	GAGCCCTCAGATTTGACCTG
TWIST1	Forward	GGCACCATCCTCACACCTCT
	Reverse	GCTGATTGGCACGACCTC
ETS1	Forward	CTGCGCCCTGGGTAAAGA
	Reverse	CCCATAAGATGTCCCCAACAA
TWIST2	Forward	GCAAGAAGTCGAGCGAAGAT
	Reverse	GCTCTGCAGCTCCTCGAA
MMP2	Forward	AGGAGGAGAAGGCTGTGTTC
	Reverse	CTCCAGTTAAAGGCGGCATC
ZEB2	Forward	AATGCACAGAGTGTGGCAAGGC
	Reverse	CTGCTGATGTGCGAACTGTAGG
SNAIL1	Forward	TGCCCTCAAGATGCACATCCGA
	Reverse	GGGACAGGAGAAGGGCTTCTC

4. References

1. Y. Ju and J. Kim, *Chemical Communications*, 2015, **51**, 13752-13755.
2. S. Kleiderman, S. Gutbier, K. Ugur Tufekci, F. Ortega, J. V. Sá, A. P. Teixeira, C. Brito, E. Glaab, B. Berninger, P. M. Alves and M. Leist, *Stem cells*, 2016, **34**, 2861-2874.