Supplementary Material (ESI) for Medicinal Chemistry Communications

Supplementary Information

Preparation of Blood-brain barrier-permeable Paclitaxel-carrier Conjugate and its Chemotherapeutic Activity in the Mouse Glioblastoma Model

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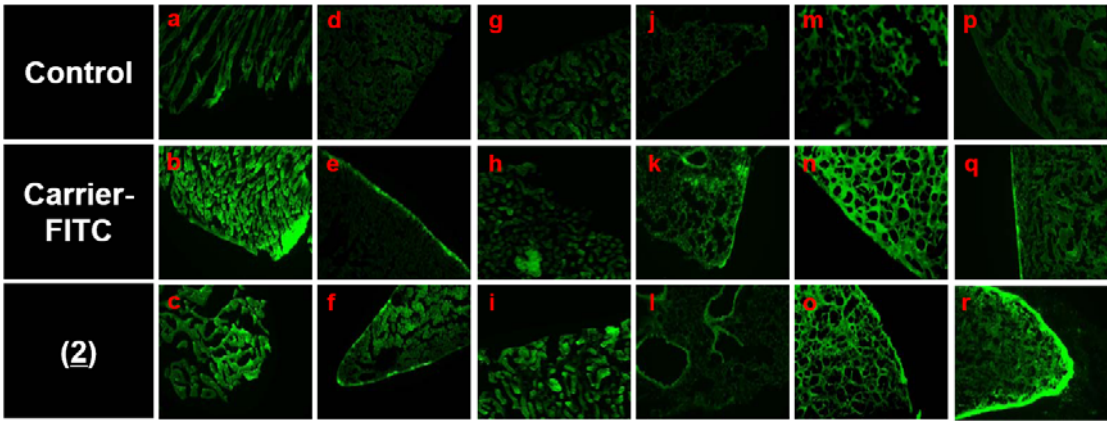

Fig. S1 Tissue distribution of (**2**) (HCl salt) in mouse. Fluorescence micrographs of heart muscle (**a-c**), liver (**d-f**), kidney (**g-i**), lung (**j-l**), brain (**m-o**), spleen (**p**–**r**) tissue sections isolated from mice 20 min after *ip* injection. Exposure time: heart (14000 ms), liver (300 ms), kidney (500 ms), lung (1200 ms), brain (15000ms), and spleen (500 ms); λmax = 488 nm (green fluorescence from FITC).

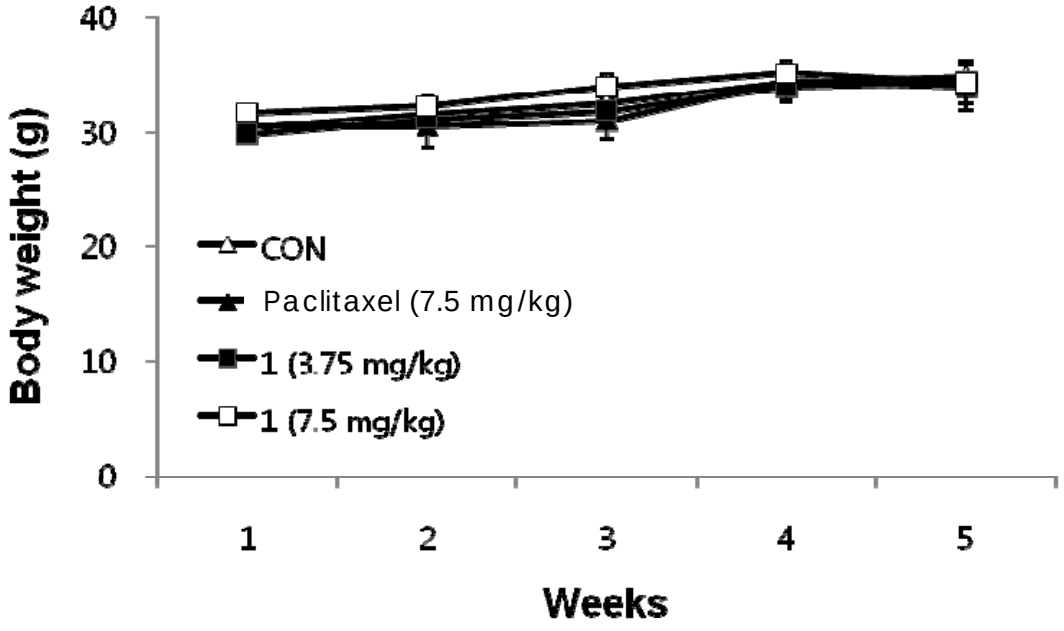

Fig. S2 The body weight changes in experimental groups.

Experimental section

General Synthetic Chemistry Methods. All non-hydrolytic reactions were carried out in oven-dried glassware under an inert atmosphere of dry argon or nitrogen. All commercial chemicals were used as received except for solvents, which were purified and dried by standard methods prior to use. Analytical TLC was performed on a Merck 60 F254 silica gel plate (0.25mm thickness), analytical reverse-phase TLC on a Merck RP-8 F254s, and visualization was done with UV light (254nm and 365nm), or by spraying with a 5% solution of phosphomolybdic acid or ninhydrine solution followed by charring with a heat gun. Column chromatography was performed on Merck 60 silica gel (70-230 or 230-400 mesh), and MPLC was performed on Fluka 100 C_8-reversed phase silica gel. NMR spectra were recorded on a Bruker DPX 300 (^{1}H-NMR at 300MHz; ^{13}C-NMR at 75 MHz) and Bruker DRX 500 (^{1}H-NMR at 500MHz; ^{13}C-NMR at 125MHz) spectrometers. Tetramethysilane was used as reference for ^{1}H NMR, and the chemical shift were reported in □ ppm and the coupling constant in Hz. Analytical HPLC was performed by Agilent 1100-HPLC Chemstation with an analytical column ZORBAX SB-C8 (5μm, 4.6mm ID x 25cm). Low resolution mass spectra were determined on a Micromass PLATFORM ll (EI and FAB). High resolution mass spectra were determined on a JMS-700, and MALDI-TOF mass spectra on a Voyager-DE STR system at the Korea Basic Science Support Center. The standard extractive work-up procedure consisted of pouring into a large amount of water, extracting with organic solvent indicated, washing the combined extracts successively with water and brine, drying the extract over anhydrous Na_2SO_4 or $MgSO_4$, and evaporating the solvent.

Preparation of the paclitaxel-carrier conjugate 1: A solution of **3a** (170mg, 0.052mmol), 2'-succinyl-paclitaxel (58mg, 0.067mmol), EDC (25mg, 0.13 mmol) and DMAP (3.01 mg, 0.025mmol) in CH_2Cl_2 (4ml) was stirred at rt for 2 days under N_2, and extractively worked up with EtOAc. The extract was washed several times with saturated $NaHCO_3$, water and brine, and was dried and concentrated to give the crude product, which was purified by column chromatography on silica gel to afford the coupled product (147 mg, 69%) as a white foamy solid: R_f: 0.44 (CH_2Cl_2: MeOH = 10:1); ^{1}H NMR ($CDCl_3$) 1.13-1.89 (m, 193H), 2.03-2.52 (m, 48H), 3.42-3.46 (m, 16H), 3.66-3.69 (m, 5H), 4.10-4.22 (m, 4H), 5.28-4.42 (m, 5H), 5.66 (m, 3H), 5.90 (m, 1H), 6.29 (m, 2H), 7.26-7.61 (m, 26H), 7.78 (m, 2H), 8.12 (m, 2H), 8.52 (brs, 8H), 11.49 (brs, 8H); ^{13}C NMR ($CDCl_3$) 13.96, 14.56, 20.55, 21.19, 25.03, 25.22, 25.66, 26.39, 27.42, 28.42, 28.68, 30.04, 34.38, 39.85, 43.57, 51.87, 52.57, 53.84, 54.06, 60.74, 63.60, 69.47, 72.38, 75.95, 79.50, 83.27, 84.81, 127.59, 127.68, 128.27, 128.53, 128.76, 128.95, 129.38, 130.59, 134.05, 143.17, 143.59, 143.95, 150.08, 153.45, 156.47, 163.96, 171.49, 173.88, 174.44, 204.17.

To a solution of the coupled product (150 mg, 0.035 mmol) in EtOAc (1 ml) at rt, was added the HCl (gas) saturated solution of EtOAc (4 ml). After stirring for 24 h, the solution was concentrated, and the residue was washed with a mixture of diethyl ether and MeOH (20:1), dried and purified by preparative HPLC on reverse phase C18 silica gel column (VYDAC 238EV510) (H_2O:CH_3CN = 70:30). The purified product was dissolved in de-ionized water, filtered through PTFE syringe filter, and lyophilized to give **1** (62 mg, 65%) as a white foamy solid (HCl salt): ^{1}H NMR (CD_3OD) 1.30-1.83 (m, 48H), 2.03-2.13 (m, 24H), 2.36-2.97 (m, 40H), 3.17-3.25 (m, 16H, partially overlapped with CD_3OD peak), 3.46-3.55 (m, 2H), 3.88-3.92 (m, 4H), 4.16-4.41 (m, 4H), 4.73-4.67 (m, 4H), 5.00-5.90 (m, 5H), 6.52 (m, 2H), 7.64-7.32 (m, 11H), 7.88 (m, 2H), 8.01 (m, 2H); MALDI-TOF-MS: m/z calcd. for $C_{113}H_{184}N_{29}O_{26}$ 2364.40; found 2364.29 $[M+H]^+$; Analytical HPLC (ZORBAX SB-C8): RT = 2.29 min (flow rate: 1 ml/min; UV 220 nm; H_2O:CH_3CN = 60:40), purity 91+ %.

1-*O*-{(benzyloxycarbonyl)-amino-hexanoyl}-2,3,4,5-tetra-*O*-{bis-[3-(*N*,*N'*-di-Boc-*N''*-aminopropylguanidine)-amino]-hexanoyl}-D-Sorbitol (4): Off white, foamy solid (110 mg, 62 %); R_f 0.35 (CH_2Cl_2: MeOH= 9:1); ^{1}H-NMR ($CDCl_3$): δ 1.25-1.72 (m, 190H), 2.32-2.50 (m, 26H), 3.32 (br.s., 4H), 3.44-3.53 (m, 24H), 4.09-4.36 (m, 4H), 4.81 (br.s., 12H), 5.01 (s, 2H), 5.21 (br.s., 2H), 7.34 (br.s., 5H), 8.51 (br.s., 8H), 11.48 ppm (br.s., 8H); ^{13}C-NMR ($CDCl_3$): δ 24.83, 25.01, 26.48, 27.36, 28.43, 28.70, 29.96, 30.09, 34.31, 39.82, 41.22, 51.19, 51.68, 52.47, 53.94, 66.90, 79.60, 83.37, 102.10, 116.14, 128.45, 128.88, 137.09, 153.47, 156.53, 156.89, 163.94, 170.90, 171.46, 173.46 ppm; MALDI-TOF-MS: m/z calcd. for $C_{156}H_{276}N_{29}O_{45}$ 3278.03, found 3278.30 $[M+H]^+$.

Preparation of the paclitaxel-carrier conjugate (5): Off white, foamy solid (67 mg, 73 %); R_f 0.37 (CH_2Cl_2: MeOH= 9:1); ^{1}H-NMR ($CDCl_3$): δ 1.28-1.67 (m, 190H), 1.89-2.04 (m, 18H), 2.21-2.45 (m, 32H), 2.63-2.81 (12H), 3.11-3.48 (m, 18H), 3.82 (br.s., 2H), 4.13-4.41 (m, 8H), 4.82-5.23 (m, 5H), 5.57-5.82 (m, 3H), 6.03 (br.s., 1H), 6.11-6.32 (m, 2H), 7.26-7.50 (m, 18H), 8.22 (br.s., 2H), 8.50 (br.s., 8H), 11.49 ppm (br.s., 8H); ^{13}C-NMR ($CDCl_3$): δ 23.49, 23.88, 25.19, 27.50, 27.87, 28.46, 28.71, 29.10, 29.29, 30.29, 33.85, 34.75, 39.77, 43.15, 52.90, 54.39, 66.17, 69.22, 73.36, 74.76, 79.14, 80.82, 83.73, 84.35, 127.76, 128.27, 128.89, 129.14, 130.88, 133.87, 153.29, 155.46, 165.11, 172.54, 172.92, 173.31 ppm; MALDI-TOF-MS: m/z calcd. for $C_{206}H_{326}N_{30}O_{61}Na$ 4221.95, found 4220.89 $[M+Na]^+$.

Preparation of the Cbz-removed conjugate (6): Off white, sticky solid (54 mg, 76 %); R_f 0.19 (CH_2Cl_2: MeOH= 9:1); ^{1}H-NMR ($CDCl_3$): δ 1.25-1.69 (m, 190H), 2.28-2.40 (m, 38H), 2.71-

3.56 (m, 36H), 4.10-4.40 (m, 8H), 4.88 (br.s., 2H), 8.36 (br.s., 8H), 11.49 ppm (brs., 8H); MALDI-TOF-MS: m/z calcd. for $C_{198}H_{321}N_{30}O_{59}$ 4065.8350, found 4066.27 $[M+H]^+$.

FITC attachment to the conjugate (7): Light yellowish, sticky solid (32 mg, 56 %); R_f 0.30 (CH_2Cl_2: MeOH= 9:1); ^{1}H-NMR ($CDCl_3$): δ 1.21-1.78 (m, 204H), 2.02-2.38 (m, 48H), 3.02-3.41 (m, 20H, overlapped with CD_3OD peak at 3.31), 3.44-4.19 (m, 6H), 4.51-4.63 (m, 4H), 5.11-5.36 (m, 3H), 5.68-5.82 (m, 2H), 6.33 (br.s., 1H), 6.66-6.81 (m, 6H), 7.26-7.73 (m, 16H), 7.98-8.31 (m, 2H); MALDI-TOF-MS: m/z calcd. for $C_{219}H_{332}N_{31}O_{64}S$ 4455.21, found 4455.89 $[M+H]^+$.

HCl salt of the paclitaxel-carrier conjugate with FITC (2): Light yellowish, foamy solid (11.2 mg ,74 %); UV (H_2O): λmax (ε): 499 nm (17600 cm^{-1} M^{-1}); ^{1}H-NMR (CD_3OD): δ 1.26-1.67 (m, 66H), 1.93-2.36 (m, 44H), 3.07-3.32 (m, 24H, marged with CD_3OD peak at 3.31), 3.43-4.21 (m, 8H), 4.55-4.61 (m, 2H), 5.21-5.33 (m, 3H), 5.76-5.82 (m, 2H), 6.31 (br.s., 1H), 6.62-6.79 (m, 6H), 7.21-7.53 (m, 15H), 7.94-8.33 ppm (m, 3H); MALDI-TOF-MS: m/z calcd. for $C_{139}H_{203}N_{31}O_{32}Na$ 2875.35, found 2875.43 $[M+Na]^+$; Analytical HPLC (ZORBAX SB-C8): R_t = 3.19 min (Flow rate = 1ml/min, UV 220 nm, CH_3CN: H_2O = 40:60); purity 95% +.

Tissue Distribution Study. The transporter **2** (HCl salts, 115.6 mg/Kg) was dissolved in sterile distilled water (500 μL) and injected into 8-week-old mice (C57BL/6) intraperitoneally (*i.p.*). The treated mice were perfused after 20 minutes with 4 % paraformaldehyde in PBS (pH 7.4). The organs were incubated overnight in 0.5 M sucrose in PBS. Placed in cryoprotectant, they were cut into 15-μm sections with cryostat, and transferred to coated glass slides. After drying at 37 °C, the sections were washed with PBS and treated with 0.3 % Triton X-100 for 15 minutes at room temperature and analyzed under an Axioplan2 fluorescence imaging microscope.

Cell culture and Reagents. U-87MG human GBM cells (ATCC) were grown in EMEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL).

GBM orthotopic animal model. Animal experiments were approved by the appropriate Institutional Review Boards of the Samsung Medical Center (Seoul, Korea) and conducted in accord with the 'National Institute of Health Guide for the Care and Use of Laboratory Animals' (NIH publication No. 80-23, revised in 1996). For orthotopic GBM model, anesthetized 6-week-old male Balb/c-nu (for U-87MG human GBM cells) mice were secured in a rodent stereotactic frame. A hollow guide screw was implanted into a small drill hole made at 2 mm

left and 1 mm anterior to the bregma, and 2 x 10^5 U-87MG cells in 5 μL HBSS were injected through this guide screw into the white matter at a depth of 2 mm [anterior/posterior (AP) +0.5 mm, medial/lateral (ML) +1.7 mm, dorsal/ventral (DV) −3.2 mm].

Drug administration. To determine anti-tumor activities of paclitaxel-carrier conjugate, tumor cells were orthotopically implanted and the mice were randomized into four groups (n=7 per group) two weeks after tumor implantation : PBS (control group), 7.5 mg/kg of paclitaxel, and 3.75 and 7.5 mg/kg of paclitaxel-carrier conjugate. Paclitaxel and paclitaxel-carrier conjugate were administered per oral administered two times per week for 2 weeks.

Harvesting of specimens. The mice were sacrificed on day 28, and the brains were removed and sectioned axially. One section was fixed in 10% buffered formalin and embedded in paraffin, and the other was embedded in OCT compound (Miles, Inc.), frozen rapidly in liquid nitrogen, and stored at -70˚C. The tumor volume was calculated by measuring the section with the largest tumor portion and applying the formula: $(\text{width})^2$ x length x 0.5.

Immunohistochemistry. Immunohistochemistry against PCNA and CD31 was performed as described previously (1). The mouse anti-PCNA (PC10, Dako, USA) and mouse anti-CD31/PECAM-1 (BD Pharmingen, NJ, USA) antibody were utilized. TUNEL assay was performed by the DeadEnd fluorometric TUNEL system (Promega, USA).

Quantification of immunostaining. For the quantification of immunostaining for PCNA and TUNEL assay, the number of stained cells was counted in ten random fields at x400 magnification. For the CD31 and ED1, the number of stained cells was counted in ten random fields at x200 magnification.

Statistical analyses. Statistical comparisons between groups were performed using Student's t-test, one-way ANOVA or multiple comparison tests. $P < 0.05$ was considered statistically significant.

Toxicity test (MTT assay). HeLa, COS-7, and RAW264.7 cells were cultured in each 96-well microplate respectively with Dulbecco's modification of Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) in an initial density of $5x10^3$ cells per well. They were maintained at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. After 24 h, serial dilutions of paclitaxel and compound **1** at concentrations of 0.1, 1, 10, and 100 μM were added to the culture wells to replace the original culture medium, with a final volume of 100 μL. Following

48 hr of continuous exposure to the compounds, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[1] In this assay, cells that properly metabolize the MTT dye undergo a visible color change, while cells that are incapable of metabolizing the dye remain colorless. The culture medium was removed and replaced by 100 μL of the new culture medium containing 10% MTT reagent. The cells were then incubated for 4h at 37 °C to allow the formation of formazan dye. The medium containing 10% MTT reagent was replaced by DMSO, which was used to solubilize the formazan crystals. After aging for 10 min followed by centrifugation for 10 min, 80 μL from each well was transferred to a new plate for spectrophotometric measurements. The quantification of cell viability was performed using optical absorbance (540/690 nm) and an ELISA plate reader.

	Cell Viability of COS-7 (%)				
	0	0.1 uM	1 uM	10 uM	100 uM
Compound **1**	100.0	96.2	92.4	94.5	20.6
Paclitaxel	100.0	65.4	66.5	71.9	41.5

	Cell Viability of HeLa (%)				
	0	0.1 uM	1 uM	10 uM	100 uM
Compound **1**	100.0	87.4	78.0	83.1	3.8
Paclitaxel	100.0	76.3	86.3	96.1	56.5

	Cell Viability of RAW264.7 (%)				
	0	0.1 uM	1 uM	10 uM	100 uM
Compound **1**	100.0	117.0	111.1	53.4	2.6
Paclitaxel	100.0	39.3	39.2	49.9	13.3

Table S1 Cell viability of paclitaxel and paclitaxel-carrier conjugate (**1**) in three cell lines (COS-7, HeLa, and RAW264.7)

Reference

1. Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. *Cancer Res.* **1987**, *47*, 936-942.