Supporting Information

Experimental Section

Materials

Paclitaxel (PTX) was bought from Lc Laboratories (Woburn, MA). TPGS was bought from Eastman (Anglesey, U.K.). Rhodamine 123 and β-tubulin monoclonal antibodies were purchased from Sigma-Aldrich (St. Louis, MO). CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assays (MTS) were manufactured by the Promega Corporation (Madison, WI). ^{3}H-PTX was obtained from PerkinElmer Life Sciences. Pierce BCA assay kits and Pierce ECL Western Blotting Substrate kits were from Thermo Scientific Inc. (Rockford, IL). The mouse monoclonal antibody for p53 with HRP and the secondary antibody, anti-mouse or anti-rabbit IgG with HRP were products of Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Tumor cell lines and cell culture

The resistant human, lung cancer cell line, H460/TaxR, was obtained from the National Cancer Institute. H460/TaxR cells were maintained in RPMI-1640 medium supplemented with 10% heated fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). Resistant human ovarian cancer cell lines, NCI/ADR-RES, was provided by Dr. Russell Mumper (UNC-Chapel Hill, Chapel Hill, NC). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX™ medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). KB-3-1 cells were obtained from American Type Culture Collection. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA).

Cytotoxicity on Resistant Cancer Cells

To evaluate the cytotoxicity of PTX/TPGS or PTX/5-FU-TPGS, an MTS ([3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed on H460/TaxR cells. Cells were seeded into 96-well plates at a concentration of $1x10^4$ cells per well in a volume of 200 µL per well. After 24 h, various formulations containing 5 µM PTX were added, including free 5-FU, PTX, PTX/TPGS NPs and multifunctional nanoparticles (MFNPs) of PTX/5-FU-TPGS. Free PTX was prepared by dissolving PTX in DMSO. Following a 48 h period of incubation, the medium was removed and 100 µL of fresh medium and 20 µL of the combined MTS/PMS solution was added into each well of the 96-well assay plates. The plates were incubated for an additional 2 h at 37°C in a humidified, 5% CO_2 atmosphere. The absorbance values were read using a Bio-Rad microplate imaging system (Hercules, CA) at a wavelength of 490 nm. Cell viability was calculated using the following formula: (viability %) (A490 nm for the treated cells/A490 nm for the control cells) × 100, where A490 nm is the absorbance.

Cellular uptake of ^{3}H-PTX in H460/TaxR cells

H460/TaxR cells were seeded in 24-well plates at a density of $1x10^5$ cells per well and incubated overnight. Confluent cell monolayers were treated with PTX, PTX/F127 NPs, PTX/TPGS NPs and MFNPs (5 µM PTX containing 32 nCi ^{3}H-PTX per well) and incubated at 37°C for 5 h. After treatment, cells were washed three times with ice-cold PBS and lysed. The radioactivity in cell lysates was then quantified with a liquid scintillation analyzer (TRI-CARB, Packard Bioscience Company, Waltham, MA). The uptake of ^{3}H-PTX was normalized for protein content, which was assayed by using the Pierce BCA assay kit.

Measuring Rhodamine 123 (Rh 123) accumulation with flow cytometry

The effects of TPGS or 5-FU-TPGS on the cellular accumulations of RH 123 were measured using flow cytometry. Approximately $2.5x10^5$ H460/TaxR cells were incubated in 12-well plates and allowed to attach overnight. The cells were then treated with 15 μM of TPGS or 5-FU-TPGS at 37 °C for 3 h. Then, 5 μM of Rh 123 was added and incubated for another 1 h. Following this incubation, the cells were collected and washed twice with ice-cold PBS buffer. Finally, the cells were re-suspended in PBS buffer for flow cytometric analysis (BD Biosciences, San Jose, CA).

Microtubule assembly assay

Microtubule assembly assays were performed as described by Hwang JH et al.[1] The cells were treated with 5-FU-TPGS (15 μM) and 5 μM PTX in different formulation (PTX alone, PTX/TPGS NPs and the MFNPs) for 24 h and then lysed with hypertonic solution buffer (1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, 20 mM Tris HCl pH6.8) containing protease inhibitors. The samples were centrifuged at 13,000 xg for 10 min at 37°C and supernatant containing depolymerised μs was transferred to a new tube. The pellet (polymerized tubulin) was re-suspended in RIPA buffer and centrifuged at 13,000 xg for 10 min at 4°C. The amounts of tubulin were detected using a Western blot analysis.

Western blot analysis

The total protein in the lysate was quantified by Pierce BCA protein assay kit. Approximately 40 μg of protein from each sample were separated by NuPAGE 12% gel and then transferred onto an Immobilon-P Transfer Membrane (Millipore, Billerica, MA). The mouse monoclonal antibody to p53 with HRP was used at a 1:200 dilution, β-tubulin was used at a 1:500 dilution and the rabbit monoclonal anti-GAPDH clone was used at a 1:200 dilution. The secondary antibody, anti-mouse conjugated with HRP was at a dilution of 1:10000 or anti-rabbit IgG at a dilution of 1:2000. The specific protein bands were visualized using a Pierce ECL

Western Blotting Substrate kit, and the chemiluminiscent signals were detected with the use of high-performance chemiluminescence film (GE Healthcare).

Measurement of 5-FU by HPLC

The HPLC system was equipped with a Waters 2478 Dual λ Absorbance detector and a Waters 600 pump (Waters Corporation, MA, US). The separation was carried out on a ZORBAX column (5µm, 150 mm × 4.6 mm, Agilent Technologies, US) with a mobile phase of methanol/H_2O (5:95, v/v, 50 mM ammonium acetate containing 0.1% formic acid) and the flow rate was set to 0.2 mL/min. Compound concentration was determined at an ultraviolet wavelength of 266 nm and the injection volume was 20 µL. The retention time of 5-FU was 3.5 min (20.7 min for 5-FU-TPGS). The linear regression of the peak area ratios versus concentrations was fitted over the concentration range 28.3–9040 ng/mL, and the typical equation for the calibration curve was as follows: $Y = 576.89 + 42.624\ X$ ($R^2 = 0.9999$), where Y is the peak area of 5-FU, and X is the concentration of 5-FU (ng/mL).

Statistical analysis

All data were represented as mean value ± standard deviation (SD). Statistical comparisons were made using a one-way analysis variance (ANOVA) and Tukey's test. Results were considered significant when a confidence interval of 95% ($p < 0.05$) was achieved.

REFERENCE

(1) Hwang, J. H.; Takagi, M.; Murakami, H.; Sekido, Y.; Shin-ya, K. Induction of tubulin polymerization and apoptosis in malignant mesothelioma cells by a new compound JBIR-23. *Cancer Lett.* **2011,** *300*, (2), 189-96.

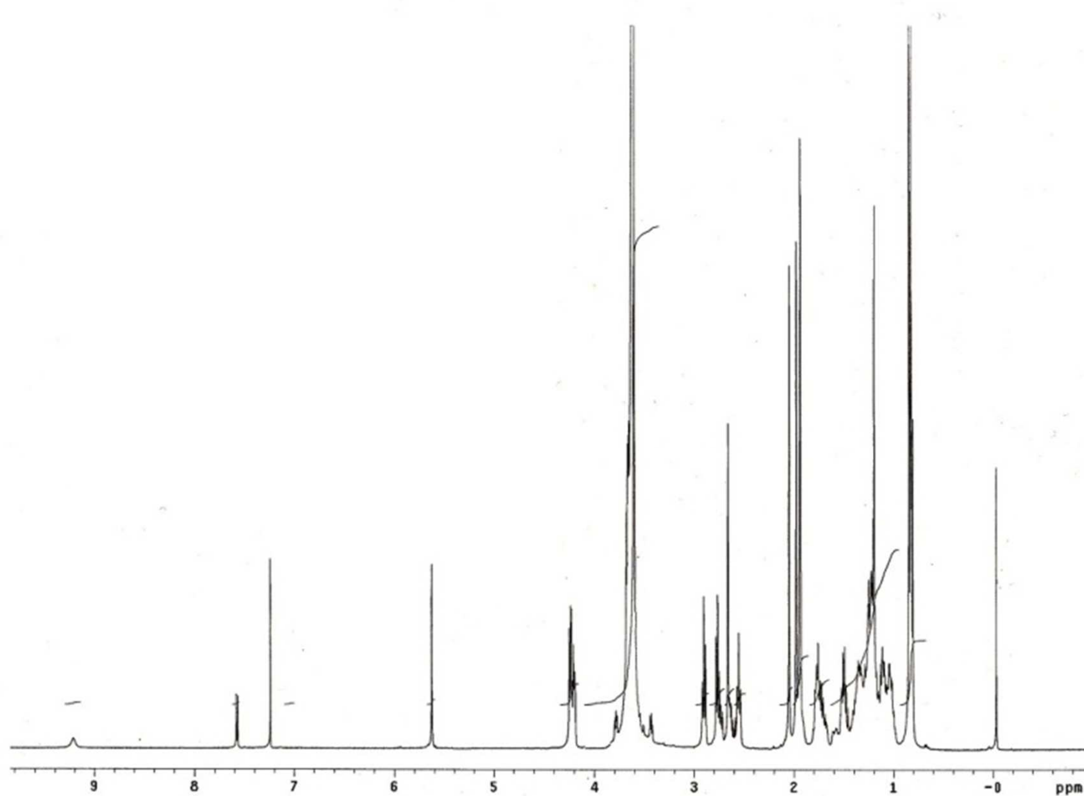
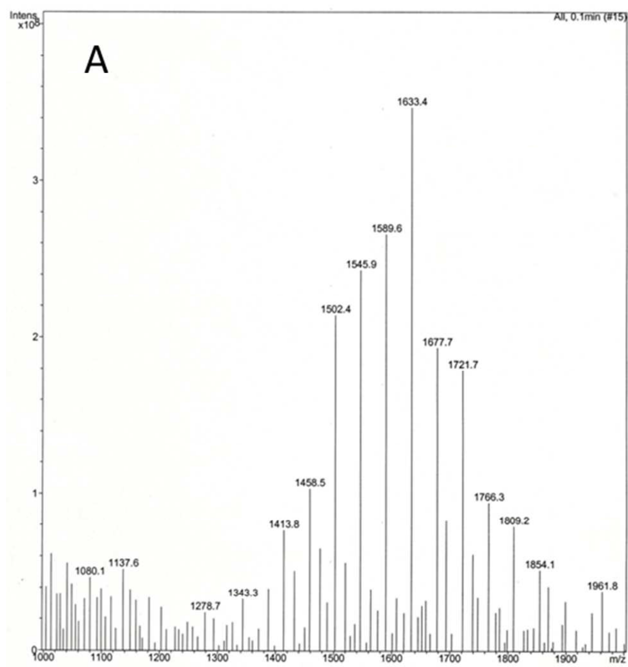


Figure S1 ^{1}H-NMR spectrum of the 5-FU-TPGS



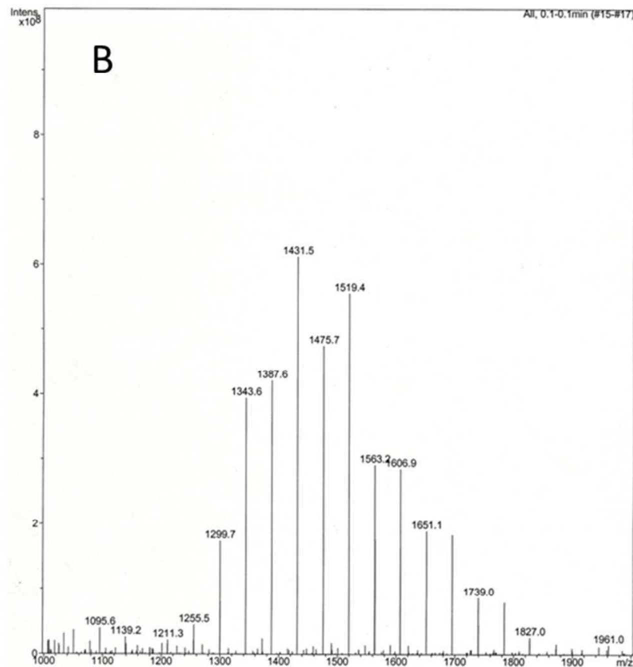


Figure S2. ESI mass spectra of 5FU-TPGS (A) and TPGS (B)