Supporting Information

Functionalized Single-walled Carbon Nanotubes as Rationally Designed Vehicles for Tumor-Targeted Drug Delivery

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Experimental Section

Chemicals and reagents, if not specified, were used as received from the relevant commercial sources. *N*-(*N*-Boc-aminoethyl)glycine (**5**)[1] 4-phthalimidobutylamine (**7**)[2], and 7-(3-fluorescein-*O*-ylpropanoyl)-10-cyclopropanecarbonyl-2'-{2-(2-succinimidyloxycarbonyl)-ethyldisulfanyl}phenylacetyl]-3'-dephenyl-3'-(2-methylbut-2-enyl)docetaxel (**13**)[3] were prepared according to the literature methods. Solvents were purified either by distillation after drying with respective dessication agents according to either standard protocols or by using PureSolv^{TM} solvent purification system (Innovative Technology, Inc) under N_2. Degassed solvents were used whenever necessary.

Thin layer chromatography (TLC) analysis was performed on Merck DC-aluminfolien with Kieselgel 60F-254 and visualized with UV light (for fluorescein derivatives), 4-*N*,*N*-dimethylamino-cinnamaldehyde solution (for biotin derivatives) and 10 % sulfuric acid in ethanol (for taxoid derivatives), which was used to confirm the removal of organic molecules from the SWNT conjugates. The TLC plate was developed with CH_2Cl_2-MeOH (9:1) as mobile phase.

The quantitative Kaiser test was performed using the modified procedure as described in the literature.[4] The reagents **a** and **b** for the Kaiser test were prepared as follows: For reagent **a**, 40 g of phenol dissolved in 10 mL of ethanol were mixed with 2 mL of 10 mM KCN aqueous solution in 100 mL pyridine. For reagent **b**, 2.5 g of ninhydrin were dissolved in 50 mL of ethanol and stored in the dark under nitrogen. Functionalized SWNT (~1 mg) were added to a 10 × 75-mm test tube, followed by adding 100 μL of reagent a and 25 μL of reagent **b**. The same amount of reagent **a** and **b** were added to another test tube in the absence of the SWNT sample as a control. After the solution mixed well, both test tubes were heated in 100 ^{o}C oil bath for 10 min, and then placed in cold water. 1 mL of 60% ethanol in water was added to each test tubes and mix thoroughly. The solution in each test tube was filtered through a Pasteur pipette containing a tight plug of glass wool. The glass wool was rinse twice with 0.5 mL of 60% ethanol. The filtrate solution was diluted with 60% ethanol and measured the absorbance (< 1 a.u.) at 570 nm against the reagent blank. The concentration of free amine was calculated by using $c = a/(\varepsilon \times b)$, where c is the concentration (M), a is the absorbance, ε is the effective extinction coefficient (1.5×10^4 $M^{-1}cm^{-1}$)[5], and b is the optical path length of the cuvette (1 cm). The primary amine content (mol) per sample was calculated using concentration multiplied by

the volume of solution. Finally, number of amine in the functionalized SWNT sample was indicated as mmol/g. Three parallel tests were done for each sample and the standard deviation value was obtained using Microsoft Excel.

^{1}H NMR spectra were measured on a Varian 300 or Varian 400 NMR spectrometer. Infrared spectra were obtained on a Nexus 670 (Thermo Nicolet) equipped with a single reflectance ZnSe ATR accessory, a KBr beam splitter, and a DTGS KBr detector. UV-visible spectra were recorded on a UV1 (Thermo Spectronic) spectrometer. For transmission electron microscopy (TEM), samples were prepared by drying droplets of a sample in ethanol dispersed onto 300 mesh carbon-coated copper grids (Ted Pella). Low magnification TEM images were taken at an accelerating voltage of 80 kV on a FEI Tecnai 12 BioTwinG^2 instrument, equipped with an AMT XR-60 CCD Digital Camera System.

Synthesis of functionalized SWNTs

(a) Preparation of oxidized SWNT 4. Pristine SWNTs (Carbon Nanotechnologies Inc.) used in this experiment were CNI grade (Lot No. P0279). Per product specifications, mean diameters of the SWNTs were about 1 nm and corresponding lengths ranged between 300 and 1,000 nm. Accurate SWNT length and diameter determination after functionalization was hampered by the presence of aggregation of the dispersed tubes. Pristine SWNTs (10 mg) were oxidized to yield a functionalized SWNT **4** using 5 mL of a 3:1 (v/v) concentrated H_2SO_4 and HNO_3 solution by sonicating at 40 ºC for 2 h, followed by heating at 70 ºC for 30 min. The reaction mixture was diluted to 200 mL with water and filtered through a 0.2 μm polycarbonate membrane. The product was then washed extensively by water until the pH reached neutral conditions and further oven dried at 120 ºC under vacuum for 2 h (9 mg, 90 % yield).

(b) Synthesis of SWNT-FITC conjugate 1 (Scheme 2)**.** To a suspension of oxidized SWNT **4** (15 mg) in DMF (5 mL) was added *N*-(2-*N*-Boc-aminoethyl)glycine (**5**)[1] (70 mg, 0.32 mmol) and paraformaldehyde (47 mg, 1.57 mmol). The reaction mixture was subsequently heated overnight at 125°C under a N_2 atmosphere. Excess amino acid **5** and paraformaldehyde were removed by filtration. The resulting residue was purified by centrifugation-precipitation, using methanol/ether 5 times (12 mL x 4 and 3 mL x 1) to thoroughly remove organic reactants,[6,7] and dried under vacuum to afford product **3** (11 mg, 73 % yield). The resulting functionalized SWNT **6** was treated with 2 mL of TFA/CH_2Cl_2 (1:1) and then stirred at room

temperature for 2 h. The solvent was evaporated *in vacuo*. The crude product was purified was purified by centrifugation-precipitation, using methanol/ether 5 times (12 mL x 4 and 3 mL x 1) to thoroughly remove organic reactants,[6,7] and subsequently dried under vacuum. The amount of loading of functional groups per gram was estimated *via* quantitative Kaiser test. To a solution of functionalized SWNTs obtained above (10 mg with 1.8 μmol of primary amine loading, based on the loading calculated using the quantitative Kaiser test) in 1 mL of DMF, was added a solution of FITC (50 mg, 0.13 mmol) and DIPEA (0.1 mL) in 1 mL of DMF. The mixture was then stirred overnight at room temperature. The resulting SWNT-FITC (**1**) was washed 5 times through centrifugation-precipitation process, using methanol/ether (12 mL x 4 and 3 mL x 1) to thoroughly remove free FITC.[6,7] The absence of free FITC was confirmed by TLC analysis and finally dried under vacuum for 5 h to give the pure conjugate **1** (7.0 mg, 70 % yield). The loading of FITC in **1** was estimated to be 0.2 ± 0.02 mmol/g based on the quantitative Kaiser test.

(c) Synthesis of biotin-SWNT-FITC conjugate 2 (Scheme 3)**.** A solution of functionalized SWNT **6 (**20 mg)**,** *N*-(4-aminobutyl)phthalimide (**7**)[2] (100 mg, 0.46 mmol), DIPEA (0.3 mL) and HATU (175 mg, 0.46 mmol) in 2 mL of anhydrous DMF was stirred at room temperature overnight. An excess of amine **7** was removed by washing 5 times with methanol/ether (4/1, 12 mL/3 mL) to afford modified SWNT **8**. A mixture of 10 mg of modified SWNT **8** and of hydrazine hydrate (25%, 0.2 mL) in 5 mL of ethanol was heated overnight under reflux in a nitrogen atmosphere. The resulting phthalhydrazide was removed by dialysis (Spectro/Por dialysis tubing with molecular weight cut-off of 3,500) to yield amine-functionalized SWNT **9**. [Dialysis procedure: Product in ethanol (20 mL) was applied to the dialysis tubing and the dialysis was run against 500 mL water for 5 h for one operation. Then, we ran 5 operations, i.e., total 2.5 L water, 25 h. The resulting product was lyophilized overnight to give pure **9**.] The loading of amine groups per gram was estimated using the quantitative Kaiser test.

Amine-functionalized SWNT **9** (10 mg with 5.0 μmol of primary amine loading, based on the loading calculated with the quantitative Kaiser test) and biotin-OSu **(10**, 110 mg, 0.32 mmol) were suspended in 3 mL of anhydrous DMSO. The resulting suspension was stirred overnight at room temperature. Excess biotin-OSu (**10**) was removed by dialysis, in the same manner as that described above, to yield biotin-*N*-Boc-SWNT conjugate **11** (10 mg, quantitative). The absence of free biotin-OSu presence was confirmed by TLC analysis.

SWNT conjugate **11** was treated with 2 mL of TFA/CH_2Cl_2 (1:1) and the reaction mixture was stirred at room temperature for 2 h. Upon evaporation of the solvent *in vacuo*, the crude product was purified by centrifugation-precipitation, using methanol/ether 5 times (12 mL x 4 and 3 mL x 1),[6,7] and dried under vacuum. To a suspension of the resulting biotin-amine-SWNT conjugate **12** (10 mg with 1.8 μmol primary amine groups, based on the loading calculated with the quantitative Kaiser test) in 2 ml of anhydrous DMF was added FITC (50 mg, 0.13 mmol) and DIPEA (0.1 mL). The resulting mixture was stirred overnight at room temperature. The excess of FITC was removed by washing 5 times with methanol/ether (12 mL x 4 and 3 mL x 1) through centrifugation and precipitation.[6,7] The absence of FITC was confirmed by TLC analysis, and the resulting product was dried under vacuum at room temperature for a few hours to afford biotin-SWNT-FITC conjugate **2** (7.5 mg, 75 % yield). The loadings of biotin and FITC in **2** were estimated to be 0.5 ± 0.03 mmol/g and 0.2 ± 0.02 mmol/g, respectively, based on the quantitative Kaiser test.

(d) Synthesis of biotin-SWNT-linker-(taxoid–fluorescein) 3 (Scheme 4)**.** To a suspension of biotin-amine-SWNT **12** (10 mg with 1.8 μmol of primary amine loading), based on the loading calculated with the quantitative Kaiser test) in 4 mL of anhydrous DMF was added SuO-linker-(taxoid-fluorescein) **13**[3] (taxoid = SB-T-1214[3]) (160 mg, 0.099 mmol) and DIPEA (0.1 mL). The resulting mixture was stirred overnight at room temperature. Excess **13** was removed by washing 5 times with methanol/ether (12 mL x 4 and 3 mL x 1) through centrifugation and precipitation.[6,7] The absence of FITC was confirmed by TLC analysis and the product was dried under vacuum at room temperature for 5 h to afford biotin-SWNT-linker-(taxoid-fluorescein) conjugate **3** (6.0 mg, 60 % yield). The loadings of biotin and taxoid-fluorescein in **3** were estimated to be 0.5 ± 0.03 mmol/g and 0.2 ± 0.02 mmol/g, respectively, based on the quantitative Kaiser test.

Biological experiments

(a) Cell culture. L1210 (ATCC) and L1210FR (a gift from Dr. Gregory Russell-Jones, Access Pharmaceuticals Australia Pty Ltd., Australia) cell lines were grown as a suspension in a RPMI-1640 cell culture medium (Gibco) in the absence of folic acid (FA) but supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) as well as 1 % (v/v) penicillin and streptomycin (P/S) at 37°C in a humidified atmosphere with 5% CO_2. WI-38 human lung

fibroblast cells (ATCC) were cultured as monolayers on a 100 mm tissue culture dishes in a DMEM cell culture medium (Gibco) that was supplemented with 10 % (v/v) heat-inactivated FBS and 1 % (v/v) P/S at 37 °C in a humidified atmosphere with 5% CO_2. The cells were harvested, collected by centrifugation at 1,000 rpm for 6 min, and finally resuspended in fresh PBS medium containing different cell densities for subsequent biological experiments and analysis.

(b) Incubation of cells with SWNT conjugate 1 and 2. The cell suspension (1 mL) at 5×10^5 cells/mL was added to a 1.5 mL microcentrifuge tube first. The SWNT conjugates **1** or **2** at 1.0 mg/mL in DMSO (10 μL) were then injected to the microcentrifuge tube to provide the final concentration of 10 μg/mL and the resultant suspension was incubated at 37 ^oC for 3 h. For low temperature experiments, the incubation of cells (L1210FR) with conjugates **1** or **2** was carried out in the cold room at 4 ^oC for 3 h. For the assessment of the effect of NaN_3 addition, the cells were initially pre-incubated at 37°C for 0.5 h with 0.05 % (w/v) of NaN_3, prior to the addition of a SWNT conjugate **1** or **2**. For the assessment of the effect of excess biotin addition, cells were treated with 2 mM of biotin (at final concentration) at 37°C for 0.5 h prior to incubation with the conjugates.

(c) Incubation of cells with conjugate 3. The cell suspension (1 mL) at 5×10^5 cells/mL was added to a 1.5 mL microcentrifuge tube. The conjugate **3** at 5.0 mg/mL in DMSO (10 μL) was subsequently added to the microtube at a final concentration of 50 μg/mL. After incubation at 37 °C for 3 h, cells were washed twice with PBS to remove excess conjugates and subsequently resuspended in the medium. For observation of the release of taxoid in a short period of time, glutathione ethyl ester (10 μL) was added to the L1210 FR cell suspension at a final concentration of 2 mM and incubated for another 2 h. Excess glutathione ethyl ester was removed by washing twice with PBS and the cells were then resuspended in 100 μL PBS prior to imaging. In the control experiment, DMSO (10 μL) was added to the suspension and incubated for another 2 h. After incubation, cells were washed with PBS, twice collected by centrifugation, and finally resuspended in 100 μL PBS prior to imaging.

Immunofluorescence study. The cell suspension (1 mL) at a concentration of $5x10^5$ cells/mL was added to a 1.5 mL microcentrifuge tube. The conjugate **3** (10 μL) in DMSO was subsequently added to the microcentrifuge tube at the final concentration of 50 μg/mL and

incubated at 37ºC for 12 h. Cells were then extracted using a PEM microtubule stabilizing buffer [100 mM PIPES, 2 mM EGTA, and 2 mM $MgCl_2$ (pH 6.8)] for 4 min, fixed in 3% formaldehyde in PEM for 40 min, blocked with 20% normal goat serum for 30 min, and then incubated with 1:100 α-tubulin monoclonal antibody (Aldrich) for 1 h. After removal of the excess primary antibody with PBS, Texas Red® goat anti-mouse IgG (Invitrogen, 1:100) was used as the secondary antibody and also incubated for 1 h. Excess IgG was removed by washing the cells with PBS. The cells were then resuspended in 100 μL PBS prior to imaging. (see Figure S4)

Confocal microscopy imaging. Cells treated as described above were resuspended in 100 μ L of PBS after each experiment, and dropped onto an uncoated bottom glass dish (MatTek Corp.). CFM experiments were subsequently performed using a Zeiss LSM 510 META NLO two-photon laser scanning confocal microscope system: for green fluorescence, the system was operated at 488 nm excitation wavelength and at 527±23 nm detecting emission wavelength using a 500-550 nm bandpass filter; for red fluorescence, the system was operated at 543 nm excitation wavelength and at 615±20 nm detecting emission wavelength using a 560 nm longpass filter.

Images were captured using a C-Apochromat 63×/1.2 Water (corr.) objective or a Plan-Apochromat 100×/1.45 oil objective. Acquired data were analyzed using LSM 510 META software.

Flow cytometry fluorescent measurements. Cells treated as previously described were resuspended in 0.5 mL of PBS. Cells were analyzed using a flow cytometer, FACSCalibur, operating at 488 nm excitation wavelength and at 530±30 nm detecting emission wavelength using a 500-560 nm bandpass filter. At least 10,000 cells were counted for each experiment using CellQuest 3.3 software (Becton Dickinson) and the distribution of the FITC fluorescence was analyzed using the WinMDI 2.8 freeware (Joseph Trotter, Scripps Research Institute). Propidium iodide staining was used in all experiments to rule out dead cell count in the flow cytometry analysis.

Cytotoxicity assay of conjugate 3. Cells were harvested, collected, and resuspended in 100 μL medium at a concentration of $2x10^4$ cells per well over a 96-well plate. For the adhesive cell type, cells were allowed to descend to the bottom of the plates overnight and fresh medium was added to each well upon removal of the old medium. SWNT conjugates were diluted to a series of concentrations in medium without FBS to prepare test solutions. Test solutions of SWNT

conjugates (10 μL) were added to the wells in the 96-well plate and cells were subsequently cultured for 72 h. At the end of the cell culture, the number of viable cells in each of the wells was determined by a quantitative colorimetric staining assay using a tetrazolium salt – based analysis ("MTT assay"; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.). The inhibitory concentration (IC_{50} nM) of each compound was defined as the concentration required for inhibiting 50% of the growth of the L1210FR, L1210 and WI38 cells. The final concentration of DMSO per well was ≤1% in all cases. The control experiment, adding 1% DMSO to the cells, indicated that 1% DMSO was not cytotoxic and thus all the cells treated remained virtually 100% positive. Each data point on the curve in Figure S5 is indicated as a median value and its error bar based on three parallel experiments. IC_{50} values and its standard errors were determined by the Four Parameter Logistic Model using software Sigma plot.

References

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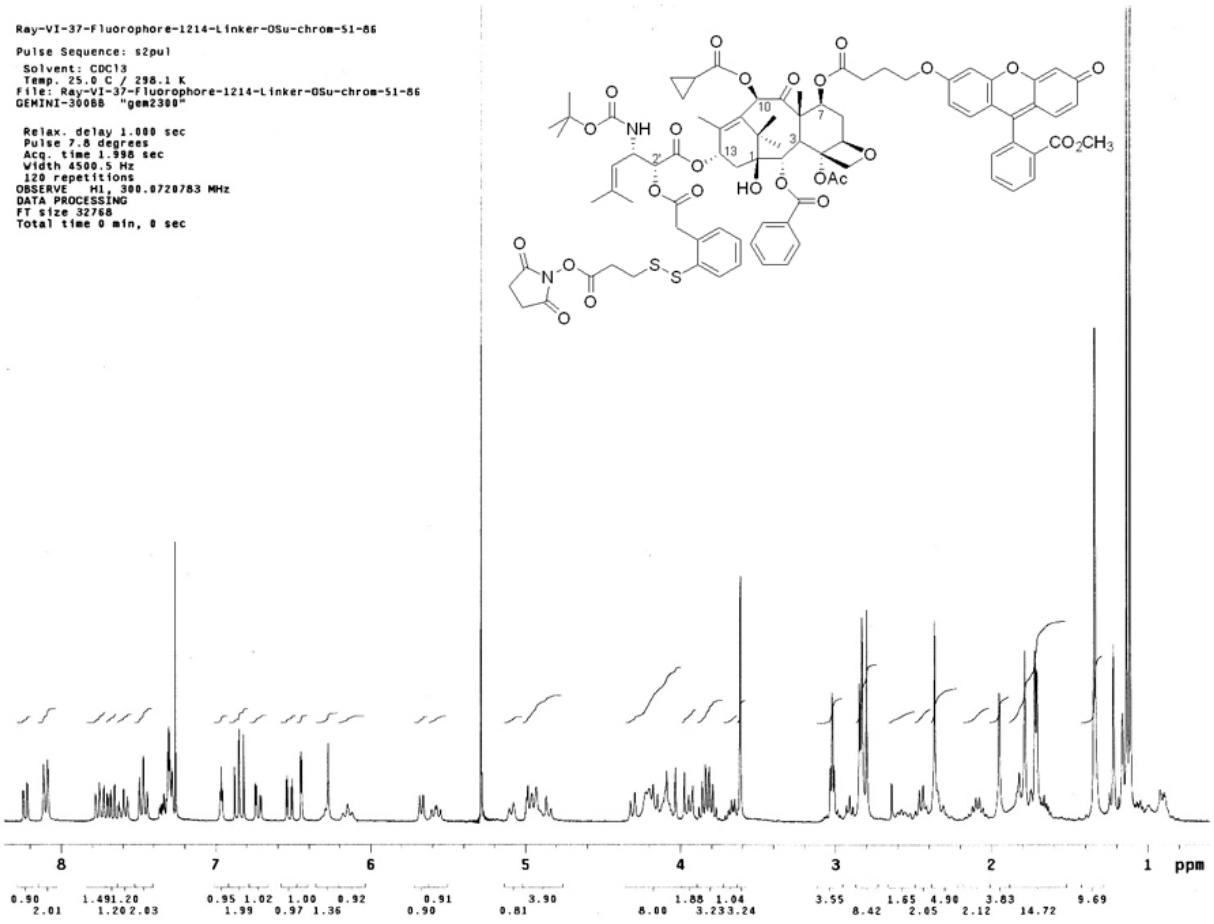

Figure S1. ^{1}H NMR spectrum of SuO-linker-taxoid (SB-T-1214)-fluorescein (**13**).

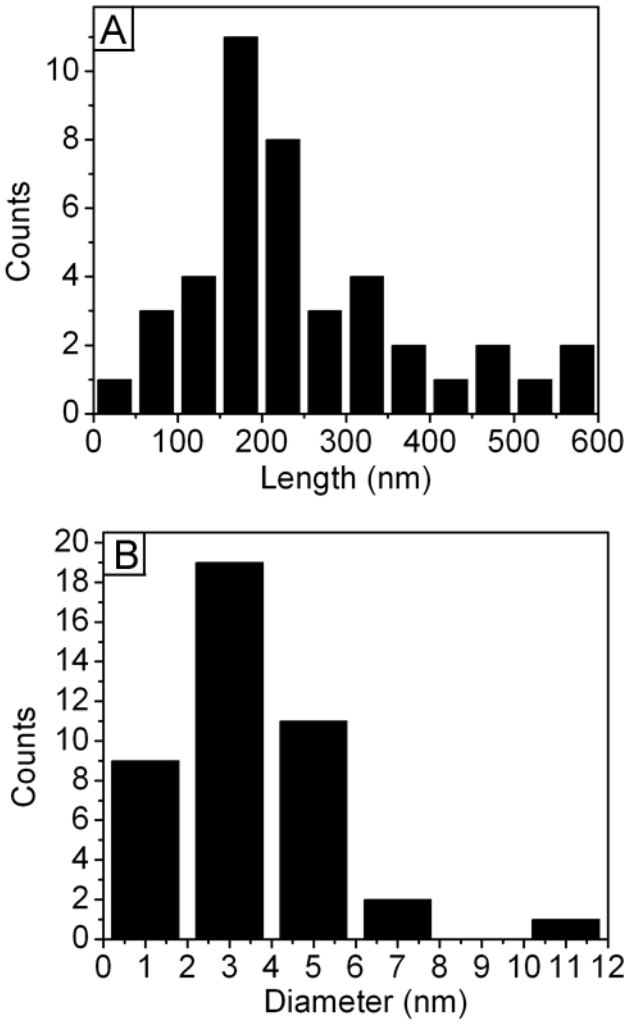

Figure S2. Histograms of length (A) and height (B) measurements on 50 oxidized SWNTs **4** recorded by AFM.

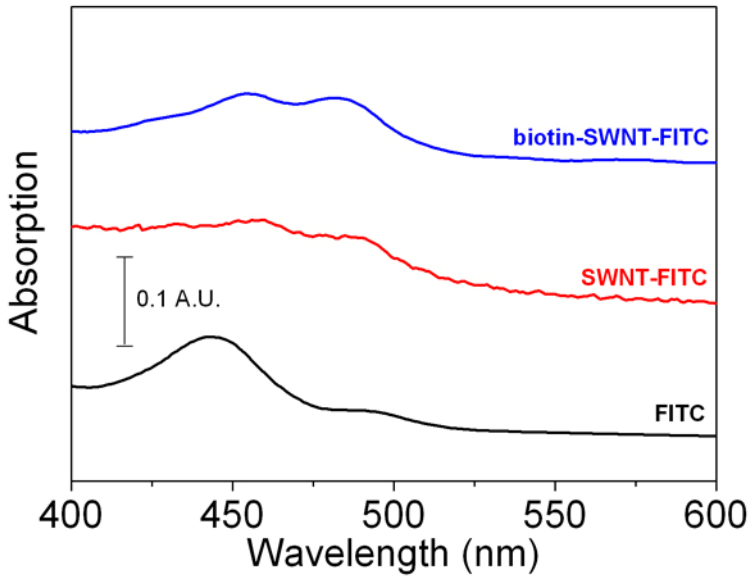

Figure S3. UV-visible spectra of SWNT-FITC **1** (in red) and biotin-SWNT-FITC **2** (in blue). UV-visible spectrum of FITC (in black) shows two peaks at absorption peaks at ~450 and ~490 nm.

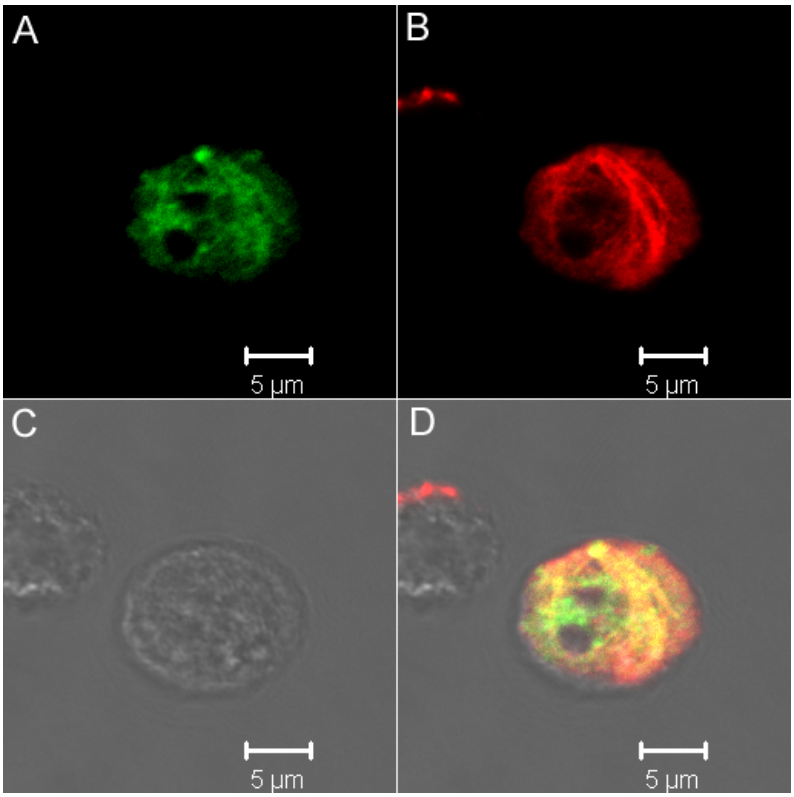

Figure S4. CFM images taken at the same thin focal plane of an individual L1210FR cell stained with conjugate **3** in green fluorescence (A) and with α-tubilin antibody in red fluorescence (B). (C) Phase contrast image of the cell. (D) Overlay of the images in A, B and C (double-staining image). Microtubule networks in the cell are clearly observable in the images. The result clearly indicates that the taxoid molecules were released from the conjugate **3** and bound to the microtubules.

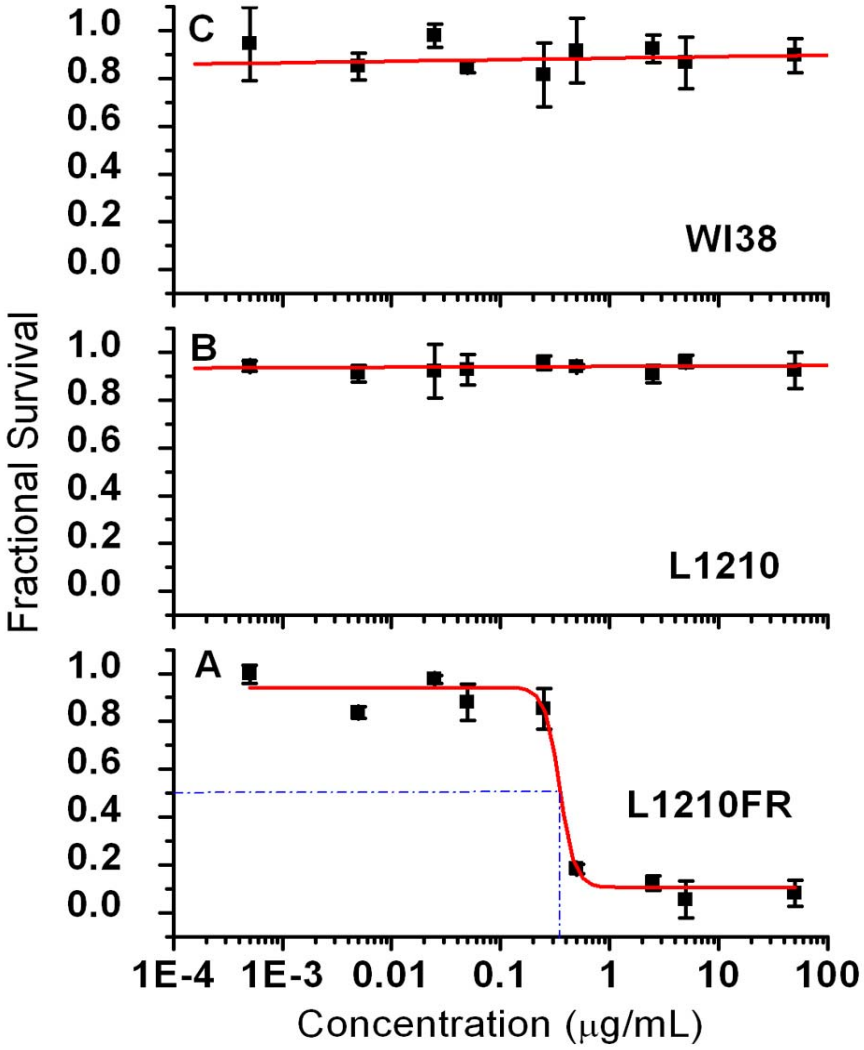

Figure S5. Results of the MTT cytotoxicity assay of a biotin-SWNT-taxoid conjugate **3** in the presence of different cell lines: (A) L1210FR; (B) L1210; and (C) WI38 (human normal) cell lines.