Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

The "click-on-tube" approach for the production of efficient drug carriers based on oxidized multi-walled carbon nanotubes

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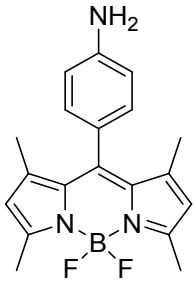
Unless otherwise stated, common reagents or materials were obtained from commercial sources and used without further purification. Compounds 10-(4-aminophenyl)-BODIPY**, 1, 2, 4**, and **10** were synthesized according to literature procedures.[1]



10-(4-aminophenyl)-BODIPY

Multi walled carbon nanotubes were purchased from Sigma-Aldrich (Lot. n. MKBH7743V). R_f values, unless otherwise stated, refer to TLC on 0.25-mm silica gel plates (Merck F254) obtained using the same eluent as in the separation of the compound by flash column chromatography. ^{1}H and ^{13}C NMR spectra were recorded with Varian Gemini instrument at 400 MHz and 100 MHz, respectively. The chemical shift for ^{1}H and ^{13}C NMR spectra are given in ppm from TMS. The resonance multiplicity is indicated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or combinations of these. Broad resonances are designated with b. Thermogravimetric analyses (TGA) were performed under N_2 atmosphere (50 mL min^{-1}) on an EXSTAR thermogravimetric analyzer (TG/DTA) Seiko 6200 coupled with a ThermoStar GSD 301T (TGA-MS) for MS analysis of volatiles. Multielemental analysis were recorded using an Optima 2000 Perkin Elmer Inductively Coupled Plasma (ICP) Optical Emission Spectrophotometry Dual Vision. ESI spectra were recorded with instrumentation LCQ-Fleet Thermo Scientific Electron Spray Ionization; spectra were recorded on 10^{-4}-10^{-5} M samples in HPLC grade MeOH. Mass spectra (EI, 70 eV) were recorded with instrumentation Shimadzu GM-MS-QP505A by direct inlet. IR spectra were recorded using a Perkin-Elmer FR-IR 881 spectrometer: absorption bands are expressed by wavenumbers (cm^{-1}). UV-visible spectra were recorded using a Varian Cary 4000 uv-vis spectrophotometer, absorption peaks are listed. Fluorescence spectra were recorded using a Jasco FP-750 spectrofluorometer. Elemental analysis were performed with Perkin-Elmer 240 instrumentation. Centrifugation were performed on a Hettich Universal 320 centrifuge at 1400 *g* for the time stated.

Cell Culture

Breast cancer cells (MCF-7), were routinely grown in 60 mm plates and maintained in monolayers to 80% confluence using DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% glutamine solutions, in a 5% CO_2 controlled humidified atmosphere at 37°C.

Flow Cytometric Analysis

MCF-7 cancer cells were grown in 35 mm dishes until 60% of confluence and treated with 10 μg/ml of fluorescent carbon nanotubes for 3 h at 37°C. Then, cells were detached by trypsinization and resuspended in DMEM medium. Cells were collected by centrifugation and washed with PBS before flow cytometric analysis using a FACSCanto flow cytometer (Becton-Dickinson).

Analysis of carbon nanotubes cells internalization with confocal microscopes

To evaluated the carbon nanotubes incorporation in MCF-7 cells, $0.5x10^6$ cells were plated on 35-mm dishes containing glass cover slips on the bottom and growth at 37 °C under controlled atmosphere for 24 hours. Then, BODIPY decorated carbon nanotubes (10 μg/ml, final concentration) were added to cell cultures, and stored at 37°C for three hours. After this time, cells were extensively washed with PBS to remove the non-internalized nanotubes, and then cells were fixed in 3% paraformaldehyde for 30 min at room temperature before the analysis with the confocal fluorescence microscope. The analysis was made using a Leica TCS SP5 confocal scanning microscope (Leica Microsystems, Mannheim, Germany), equipped with an argon laser source and a Leica Plan Apo 639 oil immersion objective.

Staining of cell nuclei with Hoechst dye

MCF7 cells nuclei were stained using Hoechst dye. Fixed cells were incubated with a staining solution containing modified Dulbecco's PBS, 0.1 % Triton X-100 and 1 μg/ml of Hoechst dye. After 20 minutes, staining solution was removed, and cells were washed with PBD to remove excess Hoechst. The analysis of sample was made using a confocal scanning microscope.

Toxicity tests

MCF-7 cells were grown in the presence of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin-streptomycin, at 37 °C in controlled atmosphere with 5% CO_2. For the experiments, cells were seeded at a density of 80000 cells per well in P35 mm well plates; the day after, compounds were added to growth medium and cells stored at 37 °C in 5% $C0_2$ for 24 h. After this time, the growth medium was removed and plates extensively washed with PBS. Then the media were replaced with medium containing 0.5 mg/mL of 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the cells were incubated for 1 h at 37 °C in 5% CO_2. Finally, the number of viable cells was quantified by the estimation of their dehydrogenase activity, which reduces MTT to water-insoluble formazan. Growth medium was removed and substituted with 1 ml of DMSO to dissolve the formazan produced. The quantification was carried out measuring the absorbance of samples at 570 nm.

The test to evaluate the toxic effects induced by high drug concentration was carried out incubating MCF-7 cells for 1 hour with 50 μg/ml of DOX or Ox-MWCNT bearing equivalent dose of DOX. After 1 hour at 37°C, medium containing drug or nanotubes was withdrawn; samples were washed with PBS to completely remove drugs and non internalized nanotubes. Fresh growth medium was added to culture dishes and then cells were incubated at 37°. After 48 or 72 hours incubation, cells viability was evaluated by MTT test, as previously described.

***In vivo* tests**

In vivo experiments were carried out in agreement with national guidelines and approved by the Ethics Committee of the Animal Welfare Office of the Italian Health Ministry (Authorization 181/2015 to Paola Chiarugi) and conformed to the legal mandates and Italian guidelines for the care and maintenance of laboratory animals. Experiments were performed using 6-week-old nude mice (Charles River Laboratories International). Briefly, 10^7 MCF-7 cells were diluted in a solution containing 100 μl PBS + 100 μl Matrigel and then subcutaneously injected in animals. After 30 days, all animals have developed subcutaneous tumors of about 5 mm^3 volume. Then, mice were divided in three groups (5 mice per group) and treated with DOX, biotinylated Ox-MWCNT loaded with DOX or biotinylated CNT alone, respectively. Each mouse received a dose of 5 μg/ml of DOX every week through tail injection (volume injection 200 μl/mouse). Tumor size was measured every 7 days by a caliper. Tumor volumes were determined by the length (L) and the width (W): $[W^2*L/2]$. After 21 days, mice were sacrificed and the tumor weight was evaluate. Statistical analysis of the data was performed by Student *t*-test. P values of ≤ 0.05 were considered statistically significant. To evaluate the effectiveness of drug treatment, paraffin-embedded samples were prepared to immunohistochemistry analysis. For each sample, H&E staining and Ki67 expression level were assayed.

Synthetic procedures:

Compound 9: Modified carbon nanotubes **3** (5 mg) together with Bodipy **7** (5 mg, 0.010 mmol) and $CuI{\cdot}P(OEt)_3$ (1 mg, 0.003 mmol) as catalyst were dispersed in anhydrous DMF (3.5 mL), the suspension was sonicated for 10 min and *N*,*N*-diisopropylethylamine (5.8 μL, 0.03 mmol) was added.

The suspension was stirred at 60 °C under N_2 atmosphere for 48 h. Every 16 h the mixture was sonicated for 5 min. The reaction was then cooled to room temperature, diluted with (i-Pr)$_2$O (50 mL), and centrifuged for 5 min at 1400 *g*. The centrifugation and dispersion steps were repeated four times, with fresh (i-Pr)$_2$O and sonication each time, to completely remove unreacted materials. The last supernatant appear clear and colorless. Finally the precipitate was dried under vacuum to obtain carbon nanotubes **9** as a blue-black powder (5.8 mg). UV-Vis (nm, DMF): 350, 573, 627. Fluorescence (nm, DMF): 630. ICP analysis: B, 3.1 mg/g.

Compound 12: Modified carbon nanotubes **3** (15 mg) together with alkyno-biotin **10** (3.4 mg, 0.012 mmol), BODIPY **6** (5 mg, 0.012 mmol) and CuI·P(OEt)$_3$ (2 mg, 0.006 mmol) as catalyst were dispersed in anhydrous DMF (5 mL), the suspension was sonicated for 10 min and *N*,*N*-diisopropylethylamine (8.6 μL, 0.05 mmol) was added. The suspension was stirred at 60°C under N_2 atmosphere for 48 h. Every 16 h the mixture was sonicated for 5 min. The reaction was then cooled to room temperature, diluted with (i-Pr)$_2$O (50 mL), and centrifuged for 5 min at 1400 *g*. The centrifugation and dispersion steps were repeated for three times, with fresh (i-Pr)$_2$O and sonication each time. The last supernatant appear clear and colorless. The residue was then redispersed in water and washed by centrifugation for 20 min at 15.000 *g* four times to completely remove the biotin unbound to carbon nanotubes. The precipitate was dried under vacuum to obtain carbon nanotubes **12** as a dark powder (16.2 mg). UV-Vis (nm, DMF): 500. Fluorescence (nm, DMF): 509. IR (KBr): 3422, 2918, 1580, 1099, 1255, 1630, 2846, 1382, 1537, 1510, 806. ICP analysis: B, 6.3 mg/g; S, 6.8 mg/g.

DOX-loaded CNT, Compound 15: Nanotubes **11** (6 mg) were dissolved in a phosphate buffer solution 0.2 M at pH 7.4 (6 mL), the dispersion was sonicated for 5 min and doxorubicin hydrochloride (4 mg, 0.007 mmol) was added. The mixture was stirred vigorously overnight at room temperature and in the dark. The reaction mixture was filtered on a Teflon filter (Whatmann, pore size 0.2 μm) washing six times with phosphate buffer to completely remove the unbound doxorubicin. The solid residue was dried under vacuum to obtain DOX-loaded CNT **15** as a black-brown powder (7.9 mg). DOX loading (evaluated by difference from filtration waters): 36.3% w/w (0.62 mmol/g). UV-Vis (nm, phosphate buffer pH 7.4): 233, 253, 481, 493.

DOX-loaded CNT, compound 16: Nanotubes **3** (6 mg) were dissolved in a phosphate buffer solution 0.2 M at pH 7.4 (6 mL), the dispersion was sonicated for 5' and doxorubicin hydrochloride (4.2 mg, 0.007 mmol) was added. The mixture was stirred vigorously overnight at room temperature and in

the dark. The reaction mixture was filtered on a Teflon filter (Whatmann, pore size 0.2 μm) washing six times with phosphate buffer to completely remove the unbound doxorubicin. The solid residue was dried under vacuum to obtain DOX-loaded CNT **16** as a black-brown powder (9.6 mg). DOX loading (evaluated by difference from filtration waters): 35.1% w/w (0.61 mmol/g). UV-Vis (nm, phosphate buffer pH 7.4): 233, 253, 481, 493.

TGA analysises of Ox-MWCNT derivatives

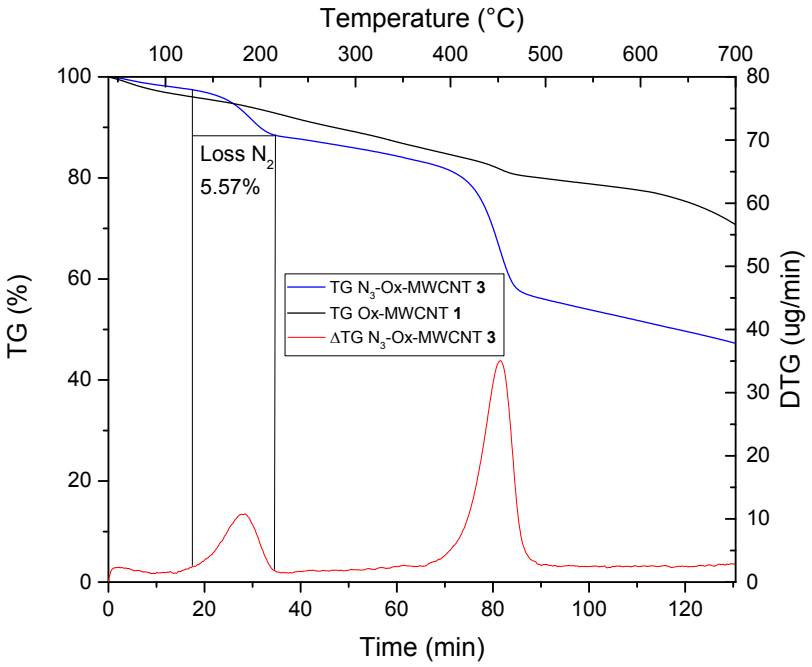


Figure 1S: TGA analysis of compounds **1** and **3**

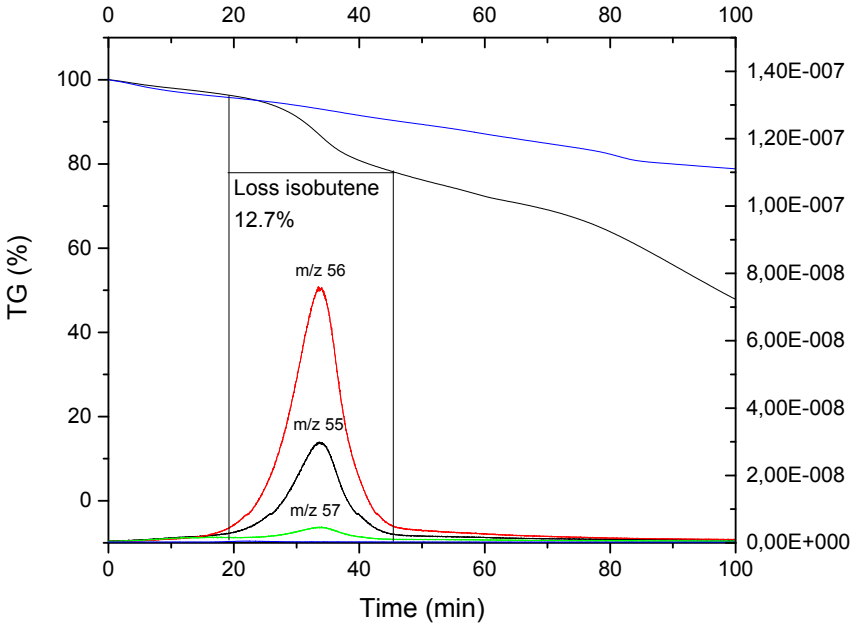


Figure 2S: TGA analysis of compounds **1** and **5**

UV and fluorescence spectra

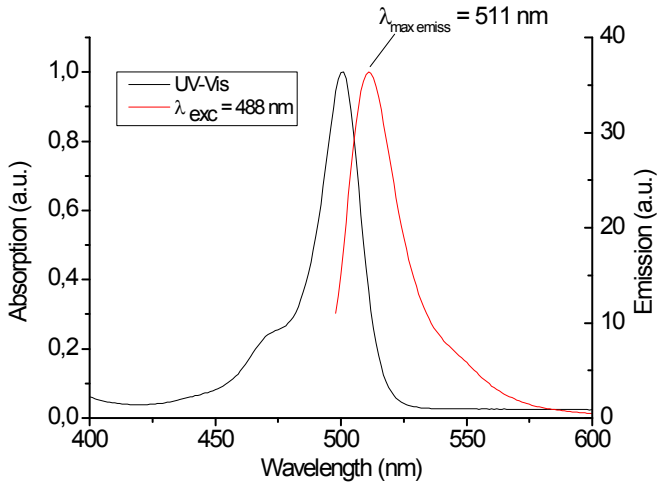


Figure 3S. UV-Vis (black) and Fluorescence (red) spectrum of compound **6** (10^{-5}, CH_2Cl_2)

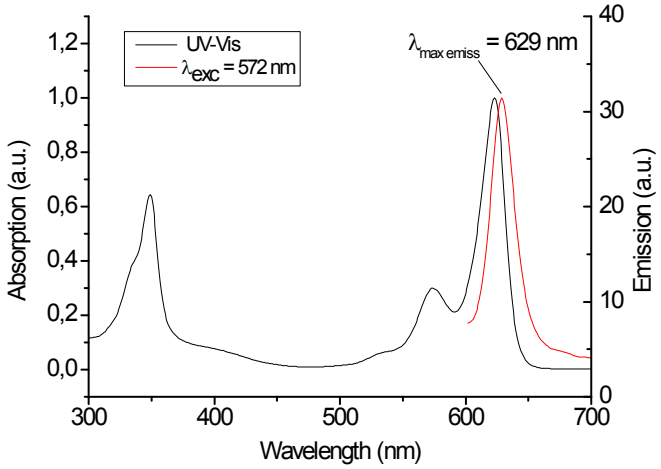


Figure 4S. UV-Vis (black) and Fluorescence (red) spectrum of compound **7** (10^{-5}, DMF)

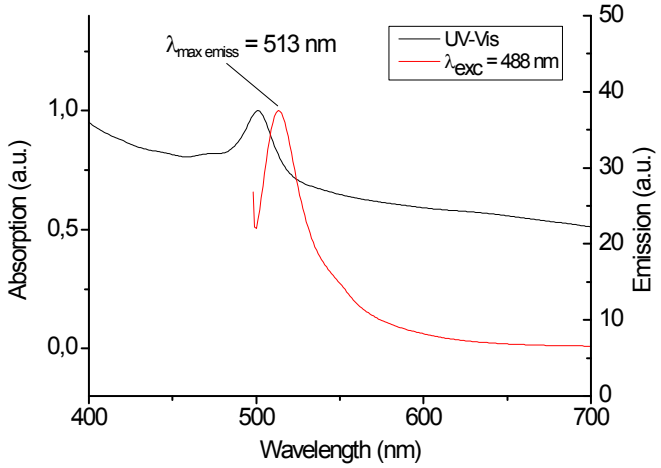


Figure 5S. UV-Vis (black) and Fluorescence (red) spectrum of compound **8** (0.5 mg/mL, DMF)

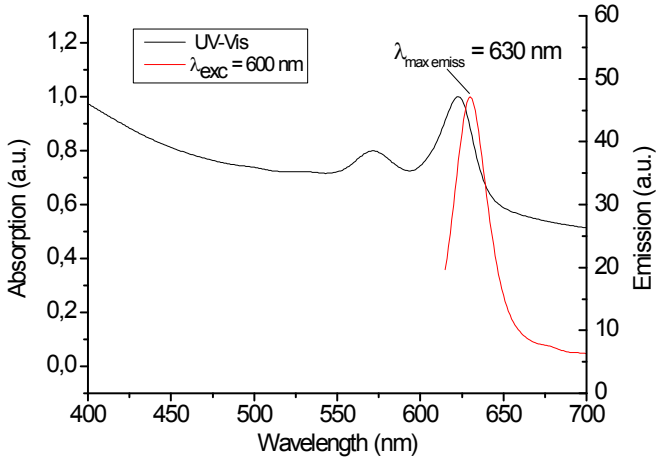


Figure 6S. UV-Vis (black) and Fluorescence (red) spectrum of compound **9** (0.5 mg/mL, DMF)

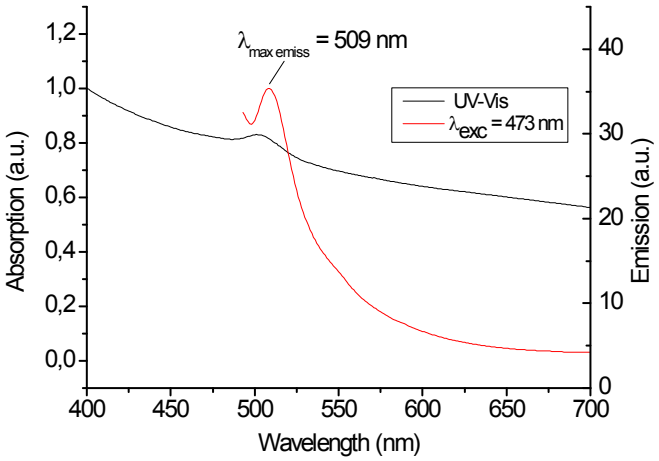


Figure 7S. UV-Vis (black) and Fluorescence (red) spectrum of compound **12** (0.5 mg/mL, DMF)

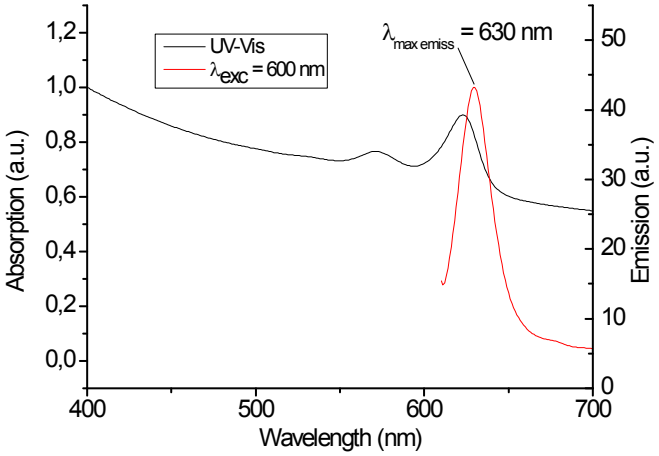


Figure 8S. UV-Vis (black) and Fluorescence (red) spectrum of compound **13** (0.5 mg/mL, DMF)

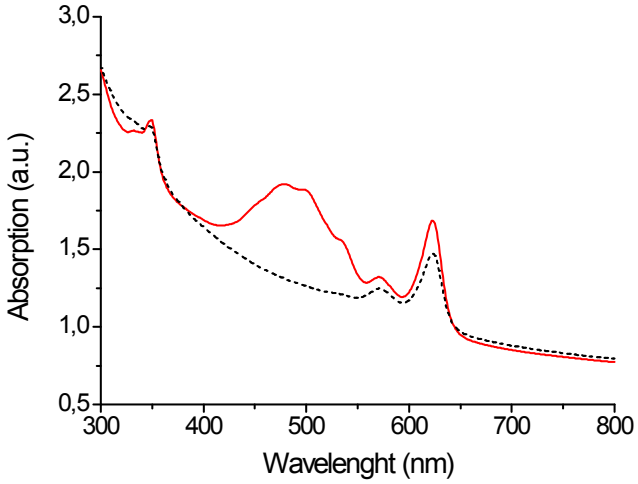


Figure 9S: UV-visible spectra of compound **14** (solid line), and **13** (dashed line) (0.5 mg/mL, DMF solution).

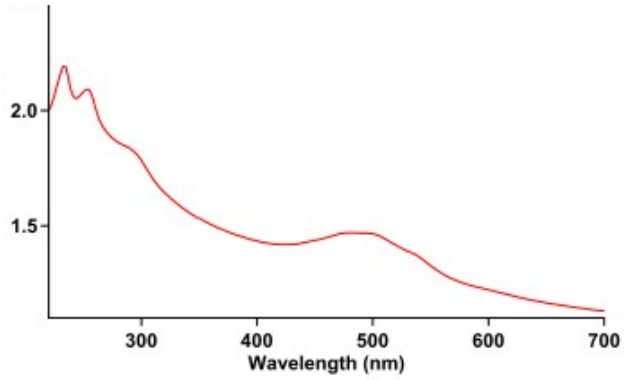


Figure 10S: UV spectrum of compound **15** (0.5 mg/mL, phosphate buffer solution)

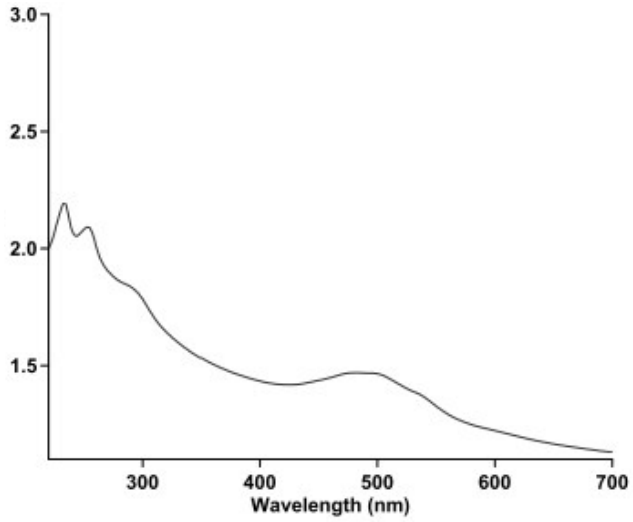


Figure 11S: UV spectrum of compound **16** (0.5 mg/mL, phosphate buffer solution)

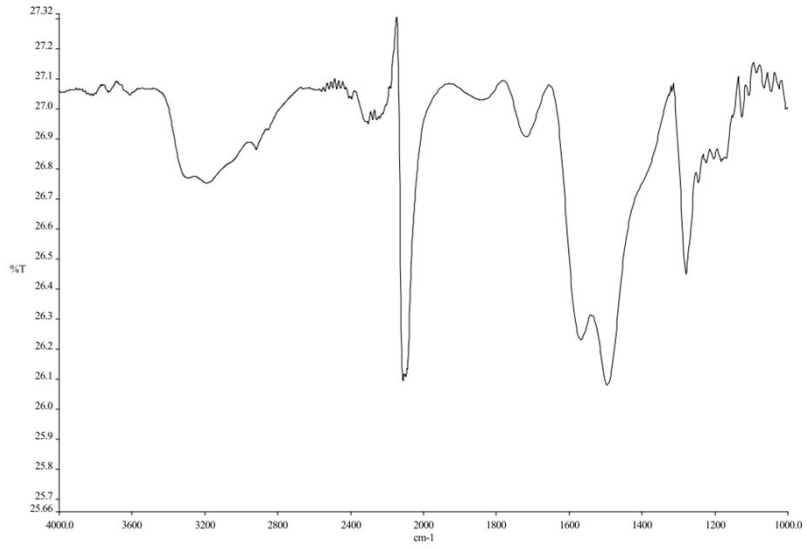


Figure 12s: IR spectrum of compound **3**

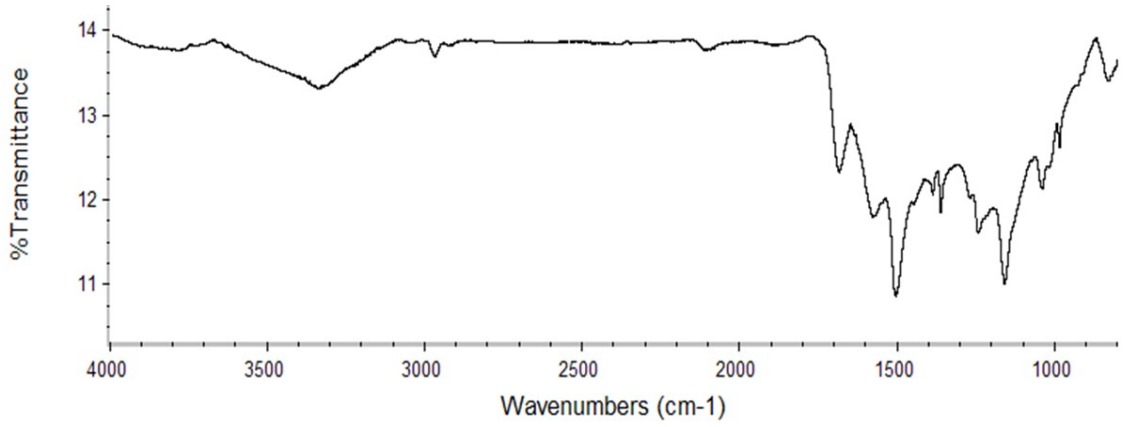


Figure 13S. IR spectrum of compound **5**

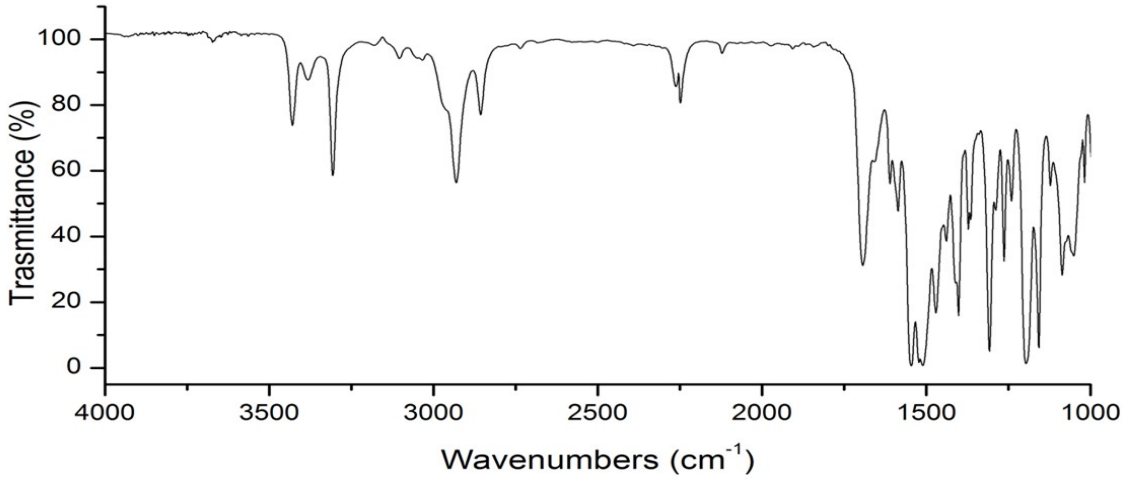


Figure 14S. IR spectrum of compound **6**

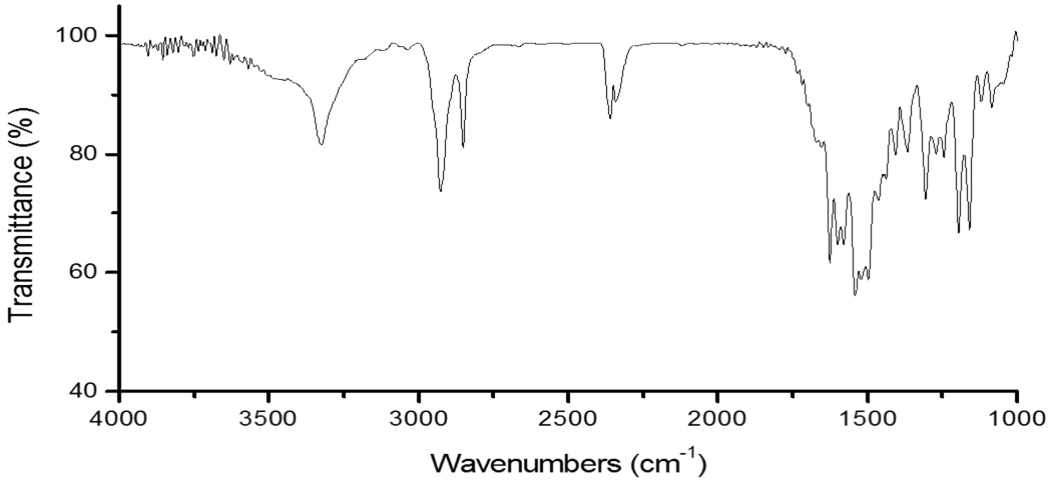


Figure 15S. IR spectrum of compound **7**

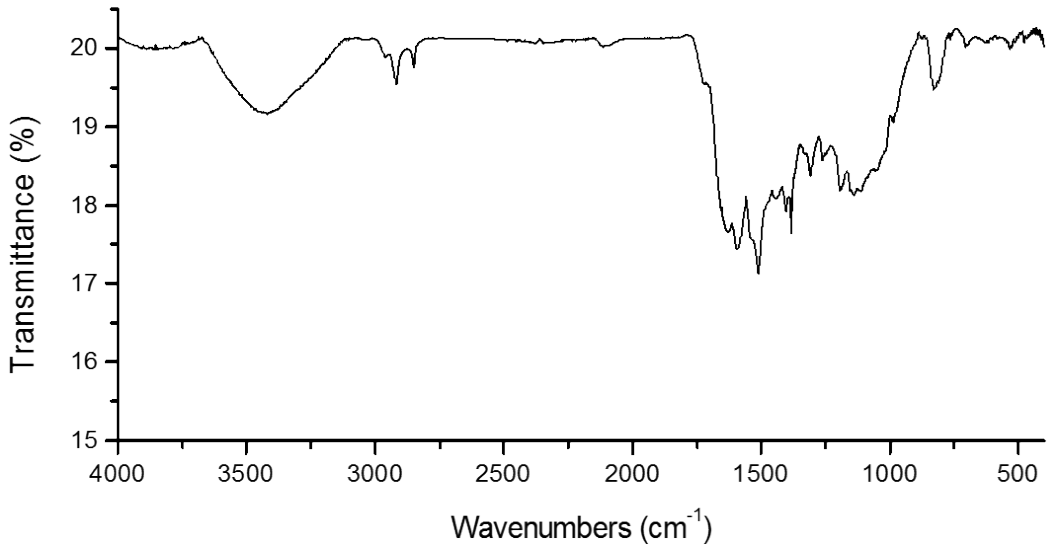


Figure 16S. IR spectrum of compound **11**

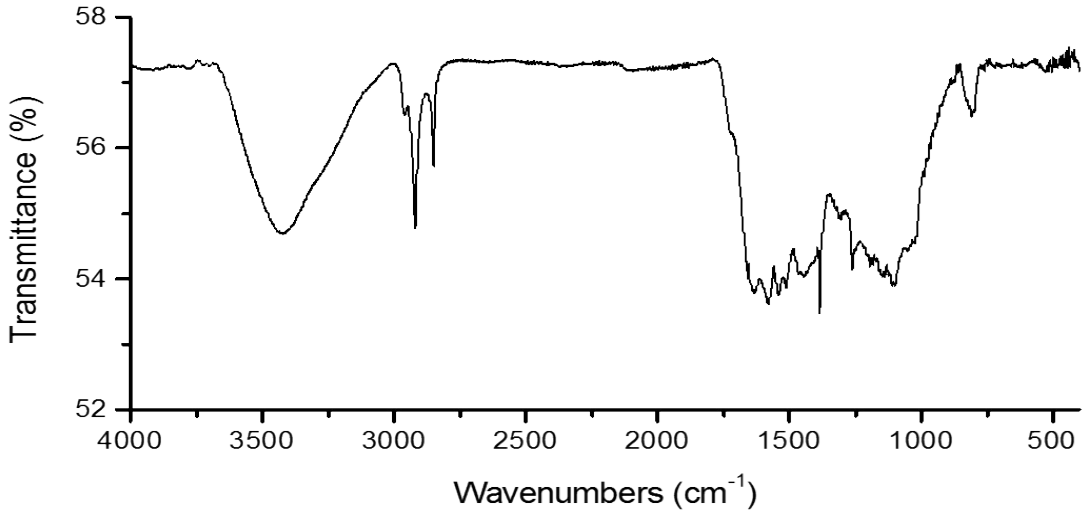


Figure 17S. IR spectrum of compound **13**

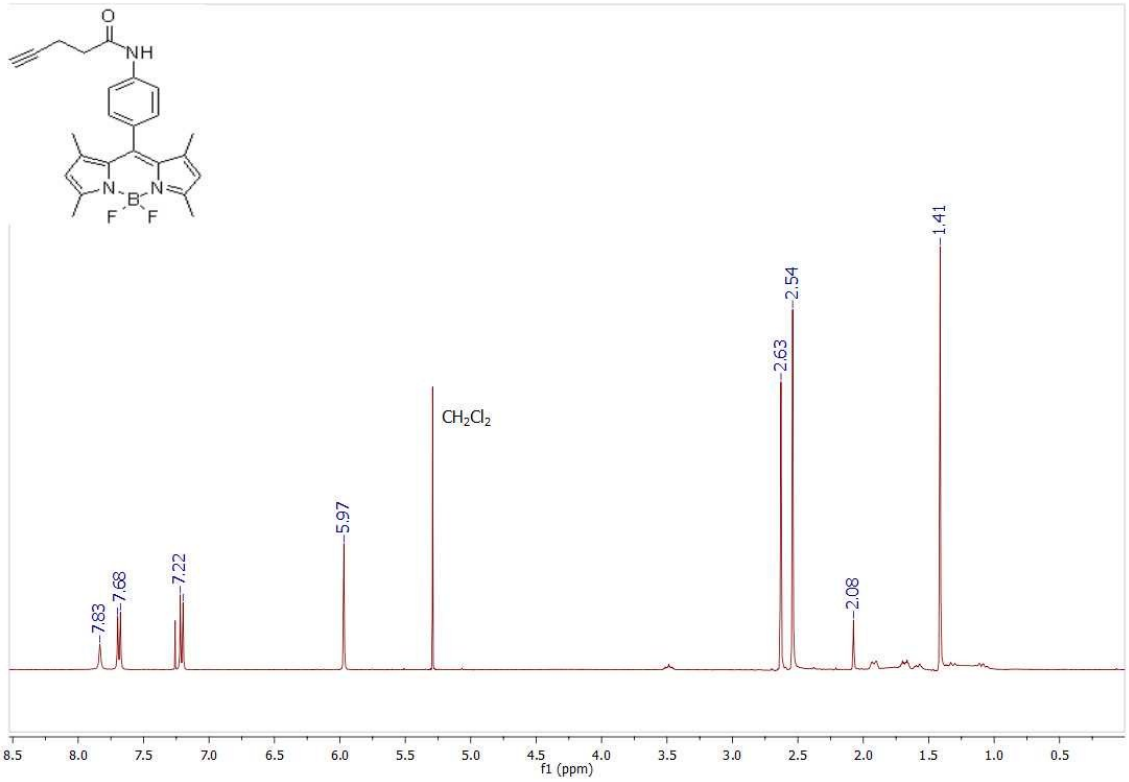


Figure 1s. ^{1}H NMR spectrum of compound **6**.

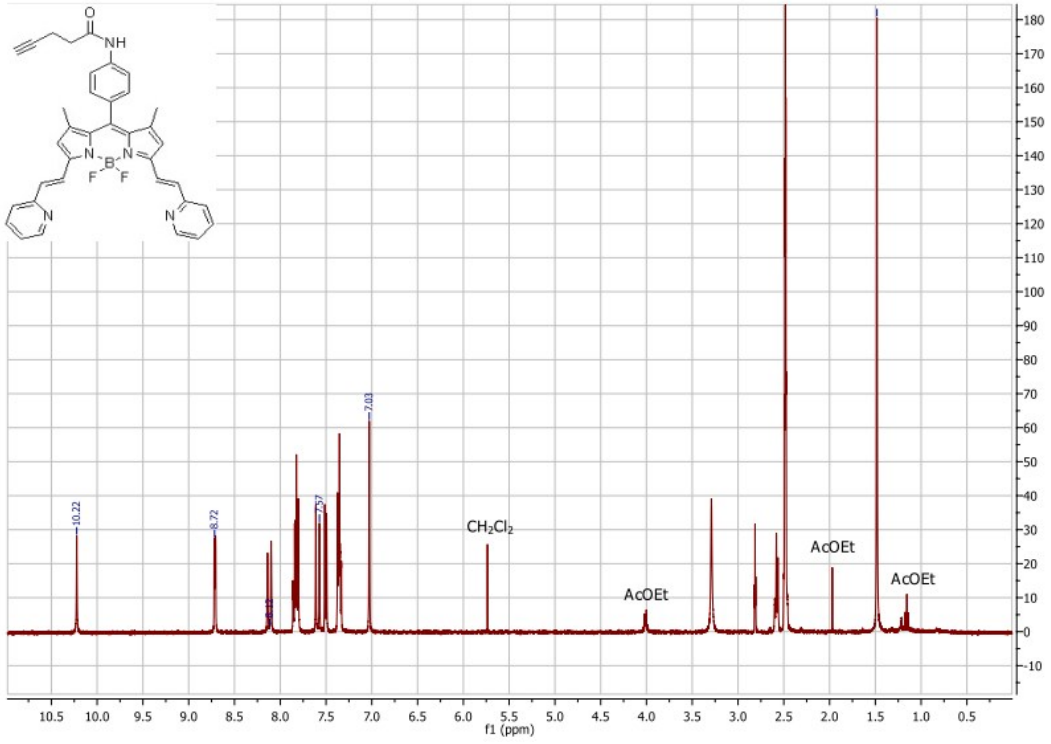


Figure 2. ^{1}H NMR spectrum of compound **7**.

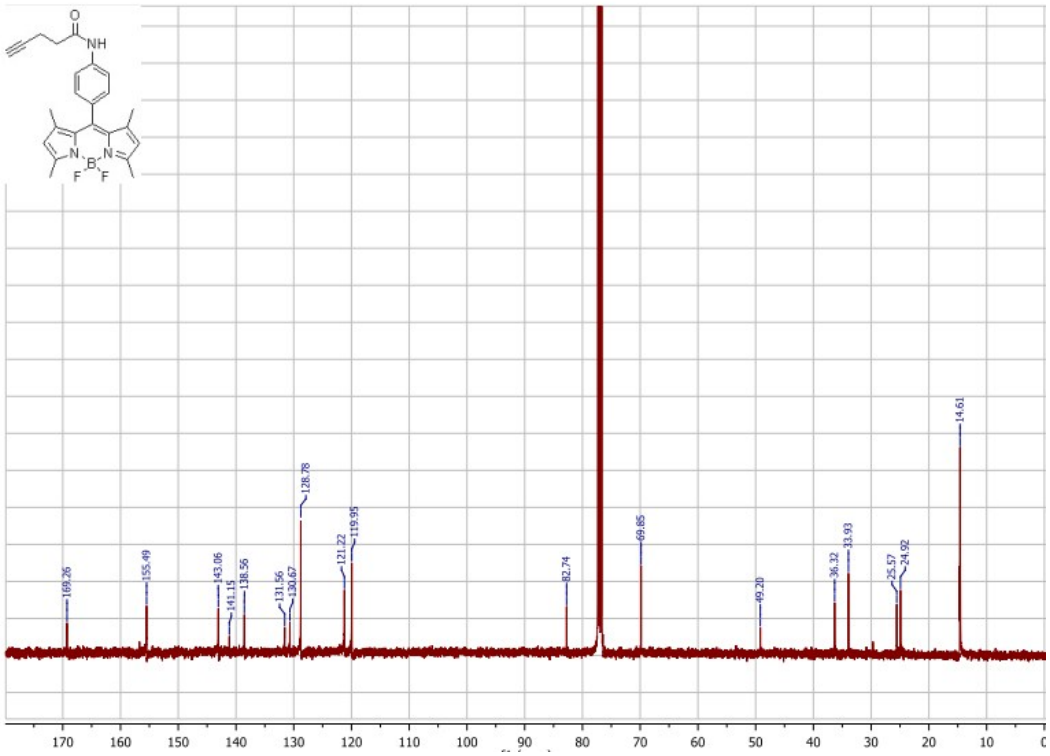


Figure 20S. ^{13}C NMR spectrum of compound **6**.

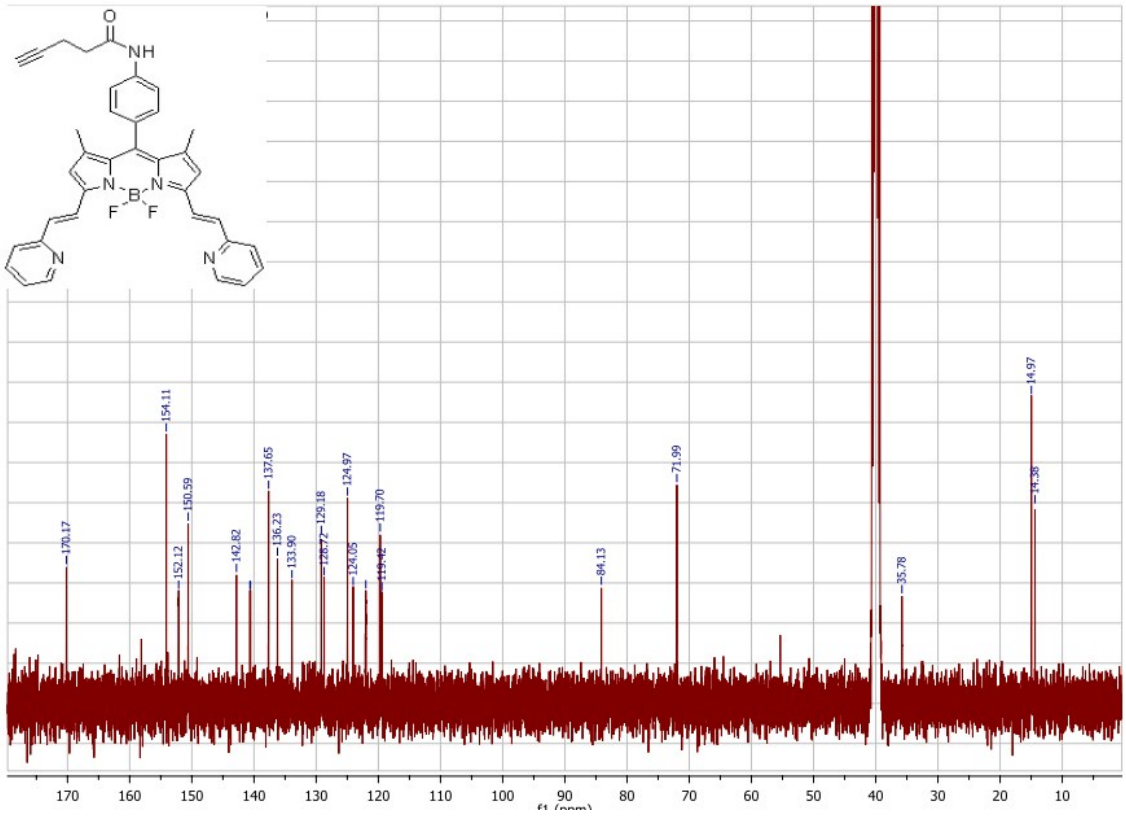


Figure 21S. ^{13}C NMR spectrum of compound **7**.

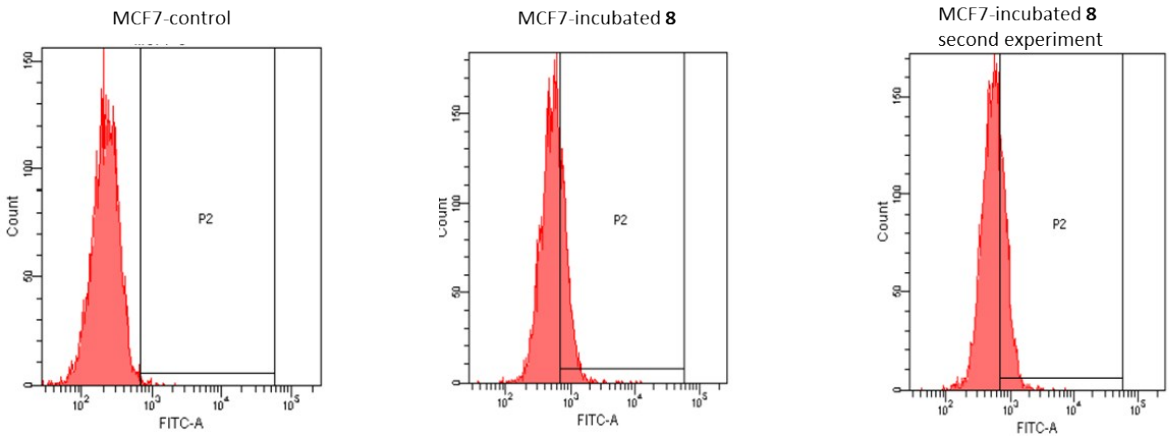


Figure 22S. Citofluorimetric analysis of MCF-7 cells incubated with compound **8**.

CNT-11	DOX	CNT-15
0.63g	0.53g	0.48g
0.68g	0.61g	0.52g
0.57g	0.54g	0.42g
0.61g	0.49g	0.44g
0.73g	0.55g	0.49g

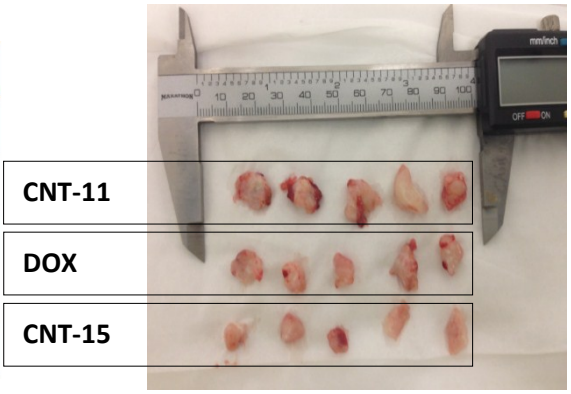


Figure 23S. Weight of the tumor masses for the three series of experiments

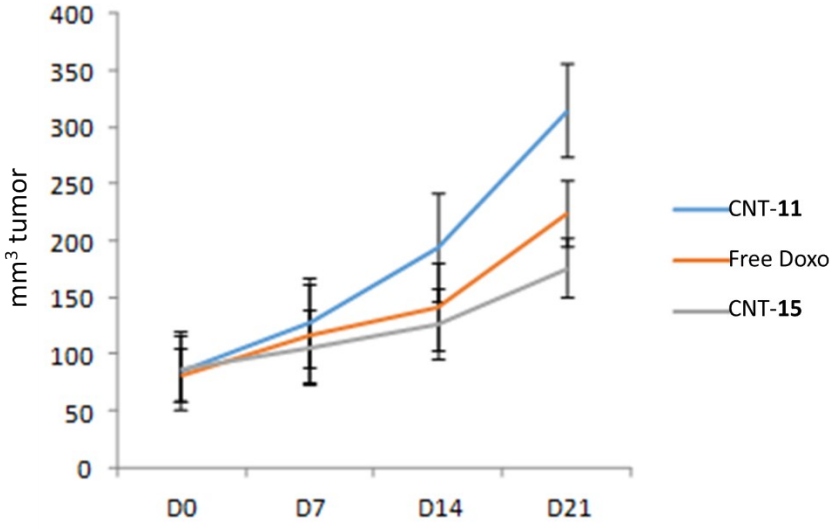


Figure 24S. Growth of the tumor volumes in the three groups of mice

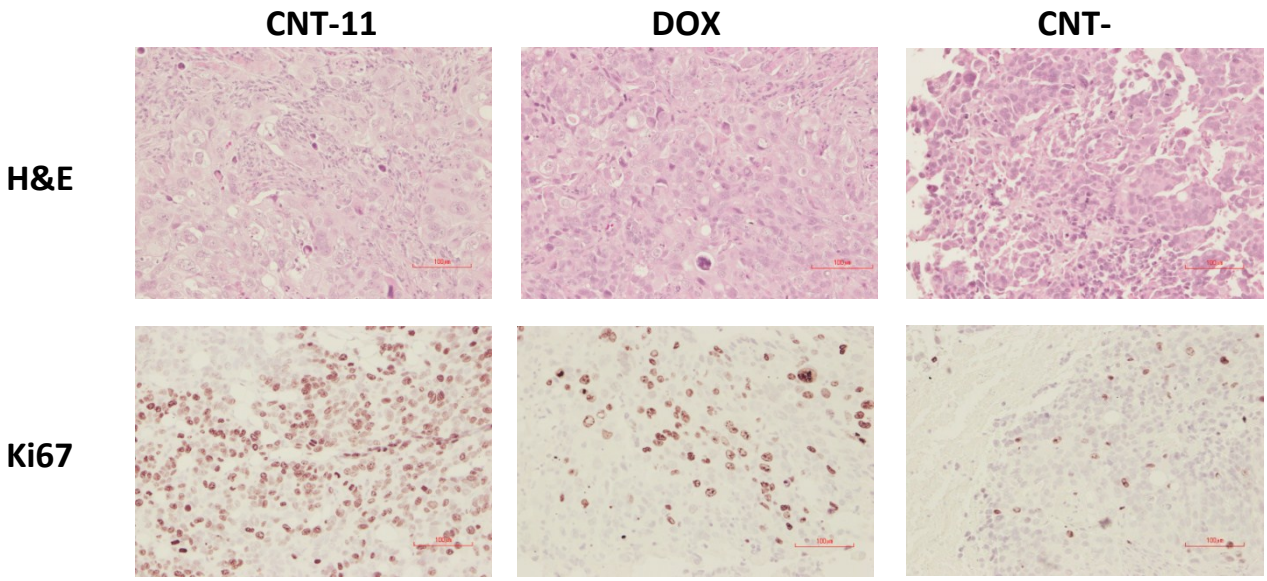


Figure 25S. Histological analysis of tissues from tumors of the three series

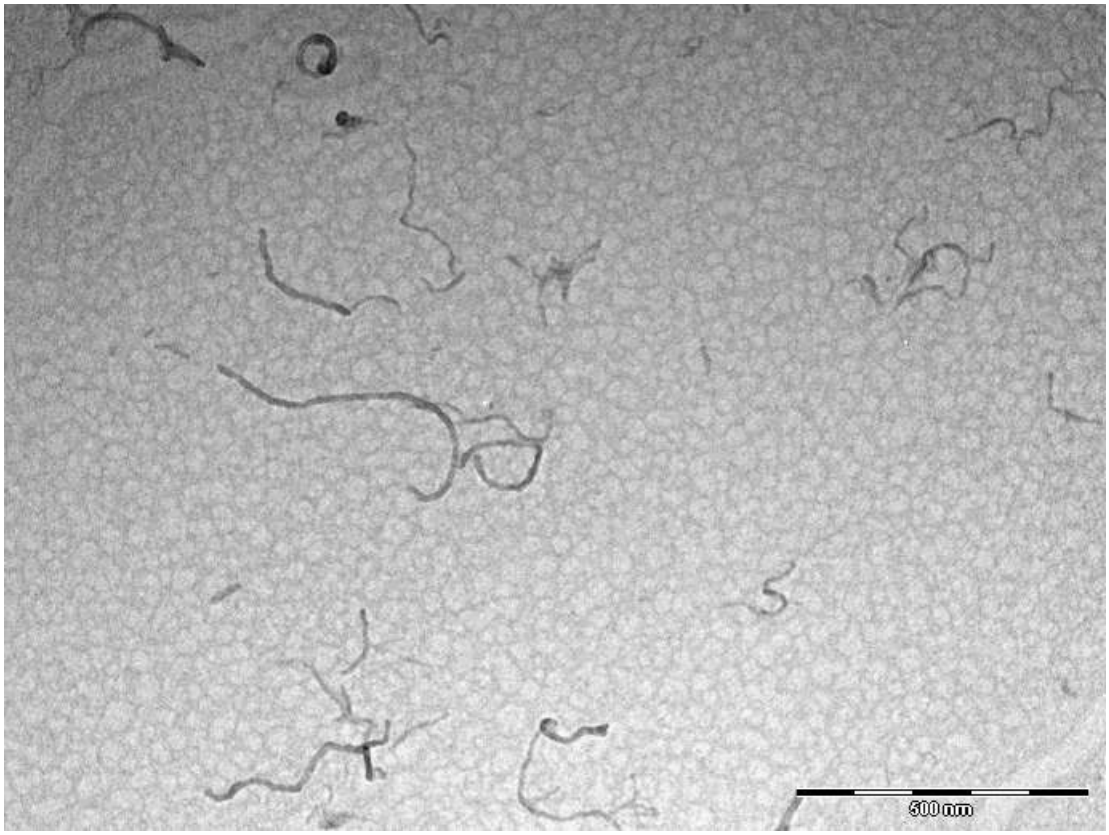


Figure 26S. TEM image of a dispersion of OX-MWCNTs **1**.

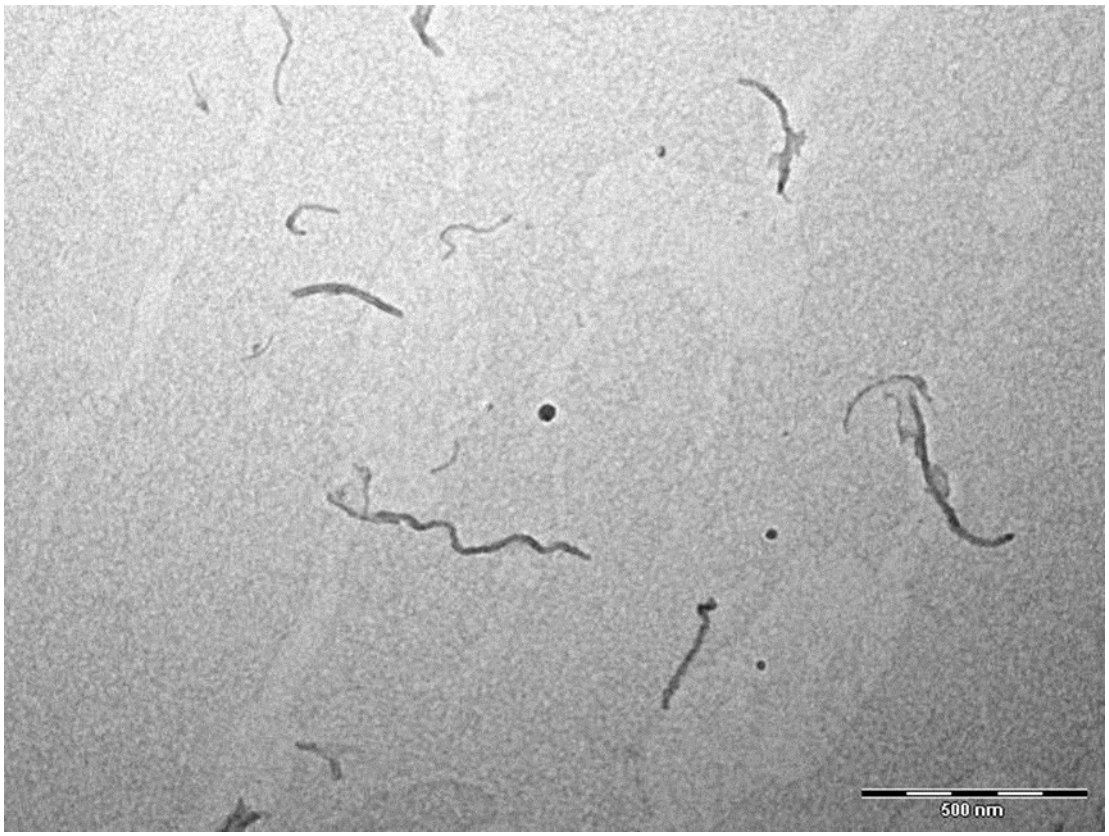


Figure 27S. TEM image of a dispersion of functionalized OX-MWCNTs **13**.

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[1]Compound 10-(4-aminophenyl)-BODIPY : , E. Maligaspe; N. V. Tkachenko, N. K. Subbaiyan,; R. Chitta, M. E. Zandler, H. Lemmetyinen, F. D'Souza, *J. Phys. Chem. A* **2009,** *113,* 8478–8489; Compound **1**: S. Fedeli, P. Paoli, A. Brandi, L. Venturini, G. Giambastiani, G. Tuci, S. Cicchi, *Chem. Eur. J.* **2015**, *21*, 15349–15353; Compound **2**: M. A. Fazio, O. P. Lu, D. I. Schuster, *Org. Lett.* **2008**, *10*, 4979-4982; Compound **4**: T. T. Denton, X. Zhang, J. R. Cashman, *J. Med. Chem.* **2005**, *48*, 224. Compound **10**: F. M. Cordero, P. Bonanno, M. Chioccioli, P. Gratteri, I. Robina, A. J. Moreno Vargas, A. Brandi, *Tetrahedron* **2011**, *67*, 9555.