Enhanced anticancer activity of multiwalled carbon nanotube-methotrexate conjugates using cleavable linkers

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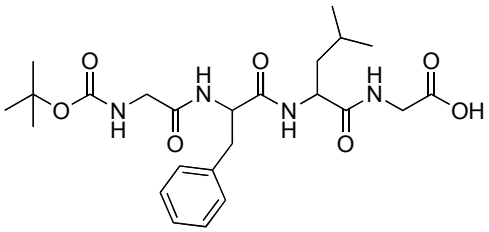
Supporting Information

Materials and Instrumentation. All reagents and solvents were obtained from commercial suppliers and used without further purification. 2-Chlorotrityl resin (1.55 mmol/g) was obtained from CBL (Patras, Greece). Amino acids were purchased from NeoMPS (Strasbourg, France). All reactions were performed under a dry atmosphere of argon using oven-dried glassware. Tetrahydrofuran, toluene and ethyl ether were distilled from sodium benzophenone ketal. Dichloromethane and DMF were distilled from calcium hydride. All other solvents were HPLC grade. Reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with E. Merck silica gel 60-F254 plates. The peptide was synthesized manually in a 30 ml fritted glass tube. RP-HPLC analyses were carried out on a Macherey-Nagel C_4 column (5 μm, 150 × 4.6 mm) using a linear gradient of A: 0.1% TFA in water and B: 0.08% TFA in acetonitrile, 0-100% B in 20 min at 1.2 mL/min flow rate. Chromatograms were recorded on a Varian ProStar 330 photodiode array detector. LC-MS was performed on a Finnigan LCQ Advantage MAX. Infrared spectra (IR) were measured on a Perkin Elmer Spectrum One ATR-FT-IR Spectrometer. UV-Vis-NIR spectra were recorded on a Varian Cary 5000. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX 300 spectrometer, the peak values were given as ppm (δ), using the tetramethylsilane or the residual deuterated solvent protons as reference. Chromatographic purifications were done with silica gel Merck (Kiesegel 60, 40-60 μm, 230-400 mesh ASTM) in standard column. Transmission electron microscopy (TEM) analyses were performed on a TEM Hitachi 600 HS. TGA was performed under N_2 using a TGA Q500 analyzer (TA Instruments) by equilibrating at 100 °C and then following a ramp of 10 °C/min up to 900 °C.

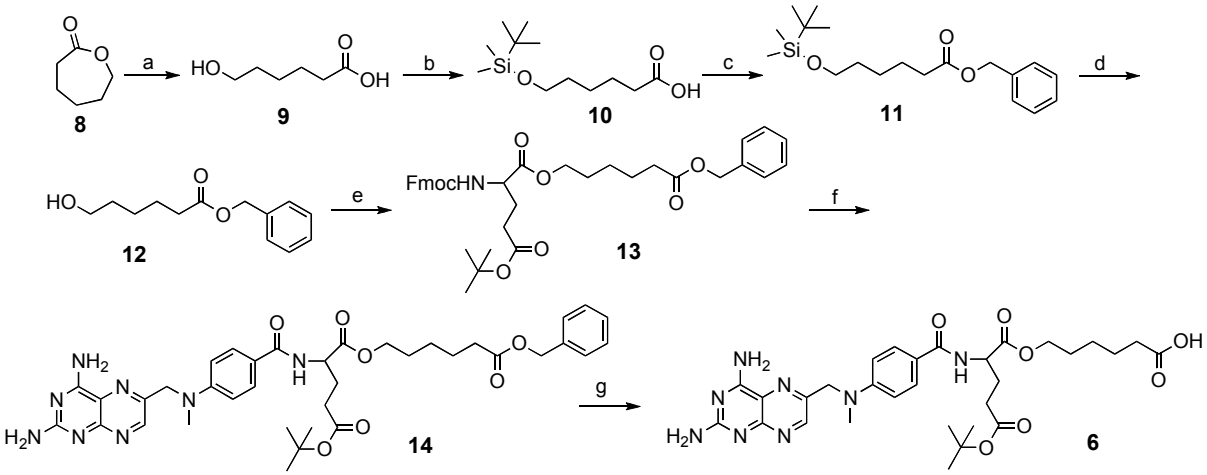
Abbreviations. Symbols and abbreviations for amino acids and peptides are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977). Other abbreviations used are: Boc, *tert*-butyloxycarbonyl; BOP, benzotriazole-1-yl-oxy-tris-(dimethylmino)-phosphonium hexafluorophosphate; DIEA, diisopropylethylamine; Fmoc: 9-fluorenyl-methyloxycarbonyl; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol.

Synthesis of Boc-Gly-Phe-Leu-Gly-OH (5).



The peptide was prepared on 2 mmol of 2-chlorotrityl resin (1.3 gr). Two-fold excess of Fmoc-Gly-OH in 12 ml of DCM/DMF 5:1 was added to the resin, followed by 6 eq. of DIEA. The resin was shaken 5 hours at room temperature. After elimination of the solution, the resin was re-suspended in 10 ml DCM/MeOH 1:1 for 30 min. The resin was washed and the Fmoc group was removed using 20% piperidine in DMF (10 ml) for 15 min. The treatment was repeated twice. Five-fold excess of Fmoc-Leu-OH in DMF (10 ml), activated with BOP/DIEA at a 1:1:3 ratio, was added and the resin shaken for 5 h. The coupling was repeated using three-fold excess overnight. The resin was washed and the Fmoc group was removed using 20% piperidine in DMF (10 ml) for 15 min. This treatment was repeated twice. Three-fold excess of Fmoc-Phe-OH in DMF (10 ml), activated with BOP/DIEA at a 1:1:3 ratio, was added and the resin shaken for 3 h. The coupling was repeated using two-fold excess for 2 h. The resin was washed and the Fmoc group was removed using 20% piperidine in DMF (10 ml) for 15 min. This treatment was repeated twice. Five-fold excess of Boc-Gly-OH in DMF (10 ml), activated with BOP/DIEA at a 1:1:3 ratio, was added and the resin shaken overnight. Completeness of each coupling was confirmed by negative Kaiser test. Cleavage of the final peptide from the resin was done using 10 ml of distilled DCM:HFIP (3:2) at room temperature for 2 h. The cleavage mixture was evaporated and the peptide was re-precipitated from diethyl ether and recovered by filtration as pale yellow solid (763 mg, 95% yield). The crude peptide was characterized by RP-HPLC, LC-MS and ^{1}H-NMR. The crude peptide was >91% pure. The impurity corresponded to the pentapeptide with an additional Leu residue. The peptide was used without further purifications. RP-HPLC: R_t 12.6 min. LC-MS (MW 492.6): *m/z* 515.2 $[M+Na]^+$, 493.1 $[M+H]^+$, 437.1 $[M-t\text{Bu}+H]^+$, 393.2 $[M\text{-Boc}+H]^+$. ^{1}H-NMR (CD_3OD): δ (ppm) 7.83 (d, 1H, NH, Leu/Phe), 7.58 (d, 1H, NH, Phe/Leu), 7.36 (t, 1H, NH, Gly4), 7.25-7.14 (m, 5H, CH-Ar, Phe), 6.30 (t, 1H, NH, Gly1), 4.45-4.35 (m, 2H, αCH, Leu/Phe) 3.91-3.59 (m, 4H, αCH_2, Gly), 3.09-2.93 (m, 2H, βCH_2, Phe), 1.63-1.45 (m, 3H, βCH_2, γCH, Leu), 1.38 (s, 9H, CH_3, Boc) 0.85 (m, 6H, δCH_3, Leu).

Synthesis of methotrexate-α-hexanoic acid linker (6).



a) NaOH (0.5 M) b) TBDMSCl, Im, DMF c) BnBr, K_2CO_3, Acetone d) $Et_3N{\cdot}3HF$, THF e) Fmoc-NH-Glu(OtBu)-OH, DCC, HOBt, DMAP f) APA, HATU, DMSO g) Pd/C 10%, MeOH

Synthesis of 6-hydroxyhexanoic acid (9).

A solution of ε-caprolactone **8** (10.0 g, 87.6 mmol) in 0.5 M NaOH (200 mL) was stirred at room temperature for 12 h. The mixture was neutralized with Amberlite IR-120 (H^+), filtered and concentrated. The residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH/AcOH (20:1:0.5) as an eluent to provide 6-hydroxyhexanoic acid (**9**) (11.3 g, 98%) as a colorless oil: R_f 0.45 (CH_2Cl_2/MeOH/AcOH 10:1:0.5); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.68 (bs, 1 H), 3.59 (t, 2 H, J = 6.4 Hz), 2.30 (t, 2 H, J = 7.5 Hz), 1.65-1.48 (m, 4 H), 1.40-1.26 (m, 2 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.9, 62.3, 34.0, 31.9, 25.1, 24.4.

Synthesis of 6-(*tert*-butyldimethylsilyloxy)hexanoic acid (10).

To a solution of **9** (2.0 g, 15.2 mmol) in dry DMF (20 mL), imidazole (2.48 g, 36.4 mmol) was added. After 10 min *tert*-butyldimethylsilyl chloride (2.97 g, 19.7 mmol) was introduced. The reaction was stirred at room temperature until TLC analysis CH_2Cl_2-MeOH (30:1) showed the complete conversion (30 h). Excess of solvents was removed under diminished pressure and water was added to the residue. The aqueous layer was extracted with AcOEt (70 mL x 3). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting oil residue (3.4 g, 90%) was sufficiently pure for the following step. FT-IR (neat) (cm^{-1}): 3340, 2943, 2864, 1721. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.88 (bs, 1 H), 3.57 (t, 2 H, J = 6.4 Hz), 2.30 (t, 2 H, J = 7.5 Hz), 1.71-1.58 (m, 2 H), 1.57-1.45 (m, 2 H), 1.38-1.24 (m, 2 H), 0.89 (s, 9 H), 0.04 (s, 6 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.1, 63.0, 34.2, 32.5, 25.6, 25.4, 24.7, 18.3, -5.3.

Synthesis of benzyl 6-(tert-butyldimethylsilyloxy)hexanoate (11).

To a solution of **10** (3.4 g, 13.6 mmol), in Acetone (60 mL), potassium carbonate (9.4 g, 68.2 mmol) was added and after 10 min benzyl bromide (2.1 mL, 17.7mmol) was added dropwise. The reaction was brought to reflux and stirred until TLC analysis *c*-Hexane-AcOEt (5:1) showed the complete conversion (6 h). The reaction was cooled at room temperature and filtrated, the excess of solvents was removed under vacuum and the crude oil product (4.4 g, 96%) was sufficiently pure for following step. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40-7.20 (m, 5 H), 5.11 (s, 2 H), 3.59 (t, 2 H, J = 6.4 Hz), 2.36 (t, 2 H, J = 7.5 Hz), 1.70-1.58 (m, 2 H), 1.56-1.44 (m, 2 H), 1.38-1.26 (m, 2 H), 0.89 (s, 9 H), 0.04 (s, 6 H) ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 136.0, 128.8, 128.6, 128.2, 66.1, 63.0, 34.3, 32.5, 25.6, 24.8, 24.7, 18.3, -5.3.

Synthesis of benzyl 6-hydroxyhexanoate (12).

To a solution of **11** (4.4 g, 13.1 mmol) in dry THF 50 (mL), $Et_3N{\cdot}HF$ (6.40 mL, 39.3 mmol) was added drop wise. The reaction was stirred at room temperature till disappearance of the starting material (36 h). TLC analysis *c*-Hexane-AcOEt (2:1). The solvent was evaporated under vacuum and the crude material was purified by silica gel flash chromatography *c*-Hexane-AcOEt (2:1) to afford (**12**) (2.90 g, ~100%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40-7.28 (m, 5 H), 5.11 (s, 2 H), 3.61 (t, 2 H, J = 6.6 Hz), 2.37 (t, 2 H, J = 7.5 Hz), 1.91 (bs 1 H), 1.71-1.60 (m, 2 H), 1.56-1.48 (m, 2 H), 1.42-1.32 (m, 2 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 136.0, 128.6, 128.2 (2C), 66.2, 62.5, 34.2, 32.3, 25.3, 24.7.

Synthesis of 1-(6-(benzyloxy)-6-oxohexyl) 5-*tert*-butyl 2-FmocNH-glutamate (13).

DCC (459 mg, 2.23 mmol), and N-hydroxybenzotriazole (301 mg 2.23 mmol) were added to a mixture of Fmoc-NH-Glu(O^{t}Bu)-OH (450 mg, 1.06 mmol) in dry CH_2Cl_2 (10 mL) at 0 °C. After 1 h at 0 °C Benzyl 6-hydroxyhexanoate **12** (400 mg 1.80 mmol) was solubilized in dry CH_2Cl_2 (2 mL) and added drop wise, followed the addition of catalytic DMAP (10 mg, 0.08 mmol). The reaction mixture was stirred for additional hour at 0 °C and then warmed at room temperature for further 5h. Et_2O was added and the precipitate was filtered, the solution was concentrated under vacuum. The crude material was purified by silica gel flash chromatography (step gradient elution *c*-Hexane-AcOEt = 5:1, 3:1), to afford **13** (600 mg, 90%). LC-MS (MW 629.3): *m/z* 652.4 $[M+Na]^+$, 574.4 $[M-t\text{Bu}+H]^+$, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (d, 2 H, J = 7.2 Hz), 7.60 (d, 2 H, J = 7.2 Hz), 7.44-7.26 (m, 9 H), 5.50 (d, 1 H, J = 8.1 Hz), 5.21 (bs, 1 H), 5.11 (s, 2 H), 4.44-4.34 (m, 3 H), 4.22 (t, 1 H, J = 6.9 Hz), 4.14 (t, 2 H, J = 6.3 Hz), 2.40 (m, 4 H) 2.20-2.06 (m, 1 H), 2.00-1.92 (m, 1 H), 1.74-1.58 (m, 4 H), 1.47-1.33 (m 11 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 172.1 (2C), 156.0, 143.9, 143.7, 141.3, 136.0, 128.6, 128.2, 127.7, 127.1, 125.1, 120.0, 80.9, 67.1, 66.2, 65.4, 53.6, 47.2, 34.1, 31.5, 28.2, 28.1, 27.6, 25.4, 24.6.

Synthesis of methotrexate γ-*tert*-butyl *α*-*n*-hexylbenzyl ester (14).

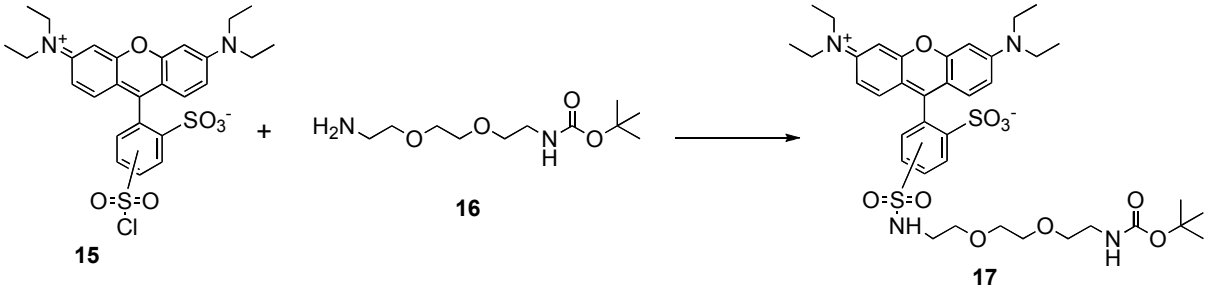
To a solution of 1-(6-(Benzyloxy)-6-oxohexyl) 5-^{t}Butyl 2-FmocNH-Glutamate **13**, (600 mg, 0.96 mmol), in dry CH_2Cl_2 (15 mL), Et_2NH (3.6 mL) was added. The reaction was stirred at room temperature until TLC analysis *c*-Hexane-AcOEt (3:1) showed the complete conversion (8 h). Excess of solvents was evaporated under diminished pressure. The crude oil material was sufficiently pure for direct use in the next step (390 mg ~100%). FT-IR (neat) (cm^{-1}): 3340, 2953, 1728, 1681. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35–7.26 (m, 5 H), 5.12 (s, 2 H), 4.17-4.02 (m, 3 H), 3.51-3.38 (m, 1 H), 3.36-3.22 (m, 1 H), 2.58-2.52 (m, 2 H), 2.48-2.22 (m, 4 H), 1.70-1.58 (m, 4 H), 1.44 (s, 9 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.6, 173.1, 172.2, 136.0, 128.5, 128.1, 127.0, 80.2, 66.1, 64.5, 53.7, 34.0, 31.8, 28.4, 28.2, 27.6, 25.4 24.6.

DIPEA (76 μL, 0.437 mmol), and O-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU, 89 mg, 0.233 mmol), were added to a solution of 4-Amino-4-deoxy-N^{10}-methylpteroic acid (APA, 50 mg, 0.146 mmol) in 1.5 mL of dry DMSO. After 25 min of stirring the above diester (83 mg, 0.204 mmol) was solubilized in dry DMSO (0.5 mL) and added drop wise to the reaction. The solution was stirred at room temperature for 12 h. The reaction mixture was diluted with water (50 mL) and then extracted with CH_2Cl_2 (3 x 20 mL). The combined organic extracts were washed with brine, and dried over Na_2SO_4, and concentrated under reduce pressure. The crude material was purified by silica gel chromatography (CH_2Cl_2-MeOH = 20:1) to afford **14**, as a yellow solid (73 mg, 70%). LC-MS (MW 714.3): *m/z* 737.5 $[M+Na]^+$, 715.4 $[M+H]^+$, 659.5 [M-*t*Bu+H]$^+$, ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.64 (s, 1 H), 7.70 (d, 2 H, J = 8.7 Hz), 7.37-7.28 (m, 5 H), 6.89 (d, 1 H, J = 7.5 Hz), 6.74 (d, 2 H, J = 9.0 Hz), 5.38 (bs, 2 H), 5.10 (s, 2 H), 4.74 (bt, 2 H), 4.21 (dd, 1 H, J_1 = 6.0 Hz, J_2 = 3.0 Hz), 4.13 (t, 2 H, J = 6.6 Hz), 3.17 (s, 3 H), 2.48-2.13 (m, 5 H), 2.12-1.96 (m, 1 H), 1.74-1.54 (m, 6 H), 1.40-1.30 (m, 11 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 172.6, 172.5, 166.9, 162.9, 162.3, 151.4, 149.7, 147.2, 137.5, 136.0, 128.9, 128.8, 128.5, 128.2, 121.8, 111.5, 80.9, 66.2, 65.3, 55.9, 52.4, 39.2, 34.1, 31.7, 29.7, 28.2, 27.3, 25.4, 24.5.

Synthesis of methotrexate γ-*tert*-butyl *α*-*n*-hexanoic acid (6).

To a solution of **14**, (87 mg, 0.122 mmol), in a THF-MeOH (2:1), dry 10% Pd/C (48 mg) was carefully added to the reaction mixture, and the dissolved oxygen was removed under vacuum. Then, a balloon of hydrogen was mounted and the reaction was stirred for 14 h at room temperature. The catalyst was removed by filtration through a Celite pad, and the filtrate was concentrated under reduced pressure, to afford **6** (73 mg, 96%). RP-HPLC: R_t 6.90 min. LC-MS (MW 624.3): *m/z* 647.5 $[M+Na]^+$, 625.4 $[M+H]^+$; FT-IR (neat) (cm^{-1}): 3359, 2955, 1736, 1641; ^1H NMR (300 MHz, CD_3OD) δ 8.55 (s, 1 H), 7.73 (d, 2 H, J = 9.0 Hz), 6.84 (d, 1 H, J = 9.0 Hz), 4.83 (s, 2 H), 5.58 (dd, 1 H, J_1 = 9.3 Hz, J_2 = 5.1 Hz), 4.19-4.09 (m, 2 H), 3.23 (s, 3 H), 2.37 (bt, 2 H, J = 7.2 Hz), 2.28-2.17 (m, 1 H), 2.14 (t, 2 H, J = 7.5 Hz), 2.09-1.96 (m, 1 H), 1.74-1.50 (m, 4 H), 1.48-1.34 (m, 2 H), 1.41 (s, 9 H). ^{13}C NMR (75 MHz, CD_3OD) δ 172.5, 172.3, 172.1, 168.9, 162.9, 162.3, 151.8, 148.8, 147.7, 135.1, 128.8, 120.9, 111.3, 80.5, 65.5, 55.2, 52.4, 38.2, 37.1, 31.4, 28.1, 26.9, 26.1, 25.7, 25.6.

Synthesis of t-Boc-NH-triethylenglicole-sulfonamide-rhodamine B (17).



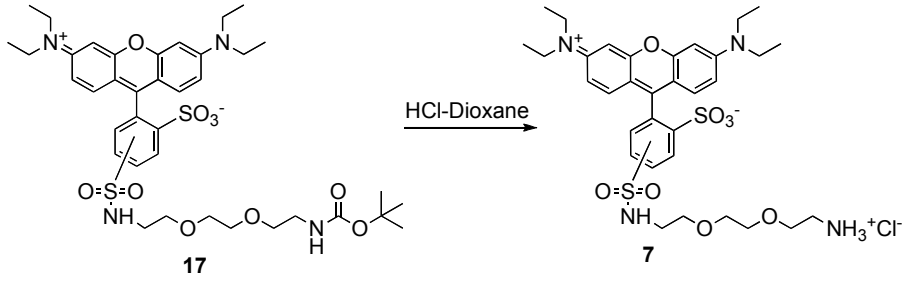
To a solution of {2-[2-(2-Amino-ethoxy)-ethoxy]-ethyl}-carbamic acid tert-butyl ester[1] (**16**), (129 mg, 0.520 mmol) in a CH_2Cl_2-DMF (5:1), Et_3N (315 μL, 2.26 mmol) was added and the reaction mixture was cooled at 0 °C. Lissamine Rhodamine B sulfonyl chloride (**15**), (200 mg, 0.347 mmol) was added in portions over 20 min. The solution was allowed to warm up to room temperature and stirred overnight. The crude mixture was concentrated under reduced pressure. Compounds **17a** and **17b** were isolated by silica gel flash chromatography (step gradient elution CH_2Cl_2-MeOH = 25:1, 20:1, 15:1).

17a red dark solid (70 mg, 26%); LC-MS (MW 788.3): *m/z* 811.3 $[M+Na]^+$, 789.2 $[M+H]^+$, 689.6 $[M-{}^t\text{BOC}]^+$; ^1H NMR (300 MHz, $CDCl_3/CD_3OD$ 9:1) δ 8.57 (s, 1 H), 7.94 (d, 1 H, J = 7.2 Hz), 7.18 (d, 1 H, J = 7.2 Hz), 7.04 (d, 2 H, J = 9.3 Hz), 6.75 (d, 2 H, J = 9.3 Hz), 6.63 (s, 2 H), 3.54-3.38 (m, 16 H), 3.18-3.04 (m, 4 H), 1.41 (s, 9 H), 1.29 (bt, 12 H, J = 7.5 Hz).^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ 9:1) δ 158.0, 155.7, 147.7, 142.6, 132.4, 131.3, 130.9, 127.8, 126.4, 113.9, 113.6, 95.6, 80.2, 70.0, 69.9, 69.8, 69.5, 46.9, 45.5, 42.7, 39.9, 27.6, 11.7.

17b red dark solid (101 mg, 37%); LC-MS (MW 788.3): *m/z* 811.3 $[M+Na]^+$, 789.2 $[M+H]^+$, 689.6 $[M-{}^t\text{BOC}]^+$; ^1H NMR (300 MHz, CD_3OD) δ 8.60 (s, 1 H), 8.19 (dd, 1 H, J_1 = 7.8 Hz, J_2 = 0.6 Hz), 7.51 (dd, 1 H, J_1 = 7.8 Hz, J_2 = 0.6 Hz), 7.17 (dd, 2 H, J_1 = 9.3 Hz, J_2 = 2.1 Hz), 7.05 (dd, 2 H, J_1 = 9.3 Hz, J_2 = 2.1

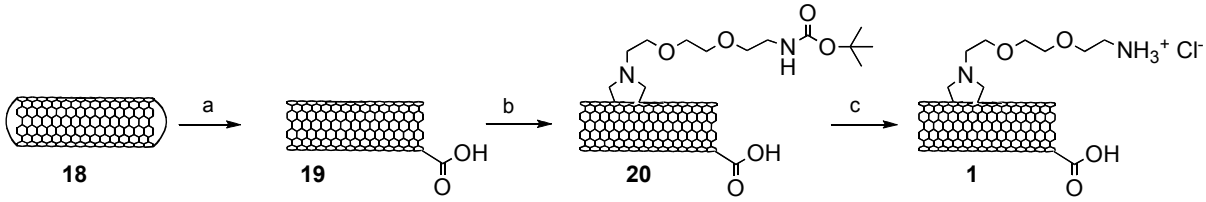
Hz), 6.97 (d, 2 H J = 1.5 Hz), 3.68 (q, 8 H, J = 7.5 Hz), 3.51-3.34 (m, 8 H), 3.15 (t, 2 H, J = 7.5 Hz), 3.00 (t, 2 H, J = 7.5 Hz), 1.40 (s, 9 H), 1.31 (bt, 12 H, J = 7.5 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 157.9, 155.8, 147.7, 141.2, 132.3, 131.8, 131.4, 129.3, 126.3, 114.0, 113.9, 95.8, 78.6, 70.0, 69.8 (2C), 69.5, 69.4, 46.5, 45.5, 42.4, 39.7, 27.4, 11.6.

Synthesis of rhodamine B sulfonamide-N-triethylenglicole hydrochloride (7).



Compound **17a** (18 mg, 0.023 mmol), dissolved in dry CH_2Cl_2 (5 mL), was treated with HCl (25 μL, 0.1 mmol, (4.0 M in 1,4-Dioxane)). The reaction was stirred for 5 h at room temperature, and then the solvent was removed under reduced pressure to obtain Rhodammine B Sulfonamide-N-triethylenglicole hydrochloride **7**, (17 mg, ~100%), as red dark solid. RP-HPLC: R_t 13.1 min. purity > 95%, LC-MS (MW 740.3): *m/z* 689.5 $[M+H]^+$; ^{1}H NMR (300 MHz, CD_3OD) δ 8.64 (d, 1 H, J = 1.2 Hz), 8.11 (dd, 1 H, J_1 = 8.1 Hz, J_2 = 1.2 Hz), 7.52 (d, 1 H, J = 8.1 Hz), 7.11 (d, 2 H, J = 9.6 Hz), 6.99 (d, 2 H, J = 9.6 Hz), 6.94 (s, 2 H), 3.81-3.61 (m, 14 H), 3.58 (t, 2 H, J = 5.4 Hz) 3.24 (t, 2 H, J = 5.4 Hz), 3.14 (t, 2 H, J = 4.5 Hz), 1.29 (bt, 12 H, J = 7.5 Hz).

Synthesis of MWNT 1.



a) H_2SO_4-HNO_3 (3:1);))))), rt 24 h b) ^{t}BOCNH-TEG-$NHCH_2CO_2H$ (**21**), $(CH_2O)_n$, DMF, 130 °C d) HCl (4.0 M in 1,4-Dioxane)

Synthesis of *ox*-MWNT (19).

Pristine MWNTs **18** (1 g), (p-MWNTs, Nanocyl 3100, Batch n° 071005) were sonicated in a water bath (characteristics 20W, 40 kHz) for 24 h in 150 mL of sulphuric acid/nitric acid (3:1 v/v, 98% and 65%, respectively) at room temperature.[2] Deionized water was then added and the MWNTs were filtered (Omnipore® membrane filtration 0.45 μm), re-suspended in water and filtered again until pH became neutral. The resulting oxidized MWNTs **19** (*ox*-MWNTs) were dried in vacuum (0.911 g).

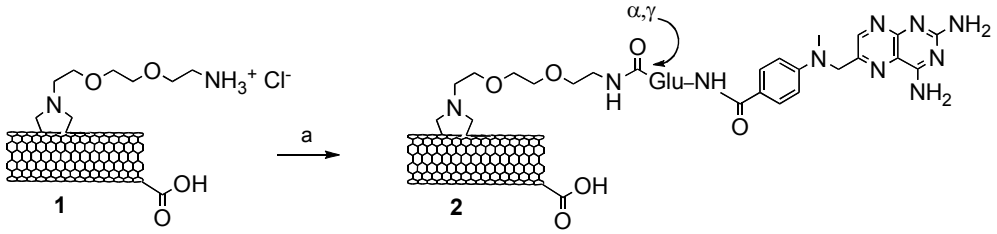
Synthesis of Boc-protected amino-functionalized MWNT (20).

ox-MWNT **19** (100 mg), and paraformaldehyde (5 × 150 mg every 24 hours) were suspended in DMF (130 mL). [2-[2-(2-tert-butoxycarbonylamino-ethoxy)-ethoxy]-ethylamino]-acetic acid **21** (synthesized as previously reported)[1,3] (300 mg, 0.98 mmol), was dissolved in DMF (5 mL) and subsequently added to the reaction. The reaction mixture was heated at 125 °C under Ar and magnetically stirred for 120 h. Excess of solvent was evaporated under reduced pressure. The resulting residue was purified by centrifugation-precipitation using MeOH-Et_2O V_{tot}= 600 mL (step gradient centrifugation starting from pure MeOH adding increasing volumes of Et_2O) until TLC analysis showed complete disappearance of amino acid (**21**). The resulting functionalized MWNTs **20** were dried in vacuum (130 mg).

Synthesis of MWNT (1).

A solution of HCl 4.0 M in 1,4-dioxane (10 mL) was added to MWNT **20** (35 mg) and the mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the resulting product was reprecipitated several times from MeOH-Et_2O. MWNT **1** was then diluted with about 35-40 mL of MilliQ water, and added to a dialysis membrane (12-14,000 MWCO, Spectra/Por®). The solution was then dialyzed for 72 h with several water changes and lyophilized to dryness (31 mg). Quantitative Kaiser test: 0.22 mmol/g of free NH_2. MWNT **1** were characterized by TEM and TGA.

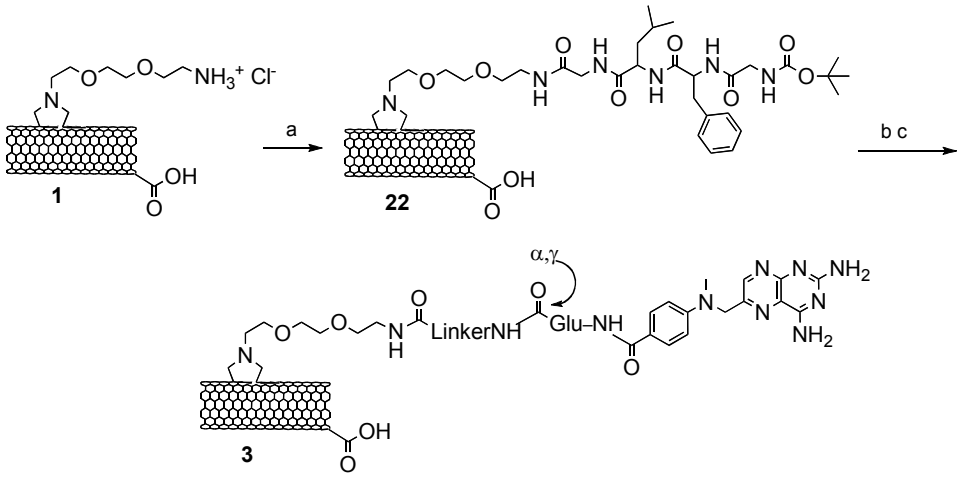
Synthesis of MWNT 2.



a) MTX, HATU, DIPEA, DMF, 60 °C

To a suspension of MWNT **1** (8 mg, 1.8 μmol), in dry DMF (2.5 mL), DIPEA (21 μL, 70 eq.) and HATU (10 mg, 15 eq.) were added. Subsequently, MTX (10 mg, 12 eq.) was added and the reaction was stirred at 60 °C under Ar atmosphere for 50 h. The crude product was purified by centrifugation-precipitation, using step gradient centrifugation DMF-MeOH, MeOH, MeOH-Et_2O, to thoroughly remove excess of organic reactants. The absence of free MTX was confirmed by TLC analysis and finally dried under vacuum (7.1 mg). The amount of MTX was estimated *via* quantitative Kaiser test (see Table S1). MWNT **2**, were also characterized by TEM and TGA (see Supplementary Figures).

Synthesis of MWNT 3.



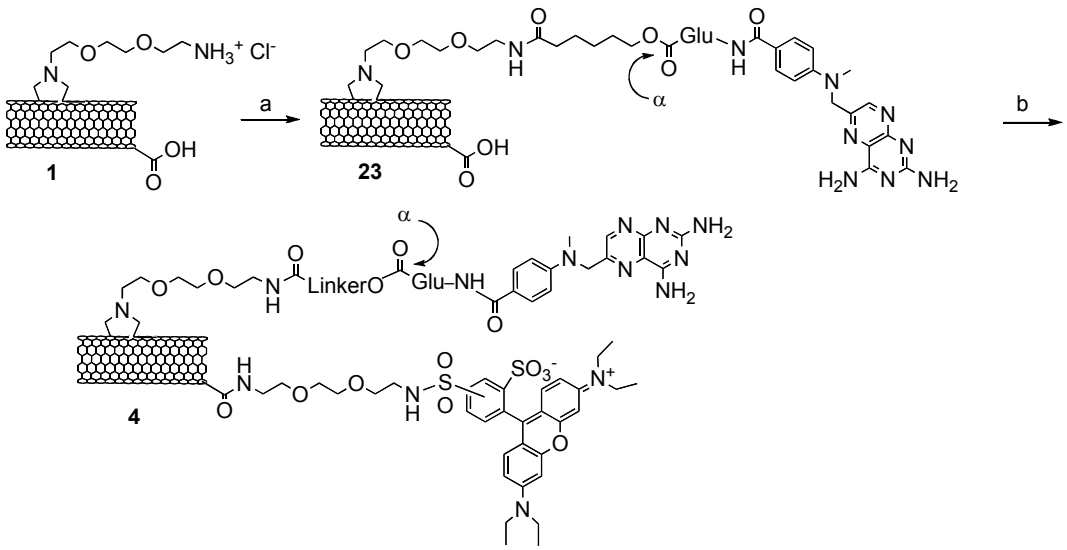
a) **5**, DIPEA, HATU, DMF, 60 °C b) HCl (4.0 M in 1,4-Dioxane) c) MTX, HATU, DIPEA, DMF, 60 °C

To a suspension of MWNT **1** (10 mg, 2.2 μmol), in dry DMF (3 mL), DIPEA (30 μL, 70 eq.) and HATU (25 mg, 30 eq.) were added. Subsequently, BOC-NH-Gly-Phe-Leu-Gly-OH (**5**) (21 mg, 20 eq.) was added and the reaction was stirred at 45 °C under Ar atmosphere for 60 h. To thoroughly remove excess of organic reactants, the crude product was purified by centrifugation-precipitation, using step gradient centrifugation DMF-MeOH, MeOH, MeOH-Et_2O, to afford the modified MWNT **22** (9.3 mg). The absence of free tetrapeptide **5** was confirmed by TLC analysis and finally dried under vacuum. MWNT **22** was characterized by TGA.

MWNT **22**, (9.3 mg), was suspended in 1 mL of 1,4-dioxane and then a solution of HCl 4.0 M in 1,4-dioxane (6 mL) was added. The mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the resulting product was reprecipitated several times from MeOH-Et_2O. The amount of free amine was estimated *via* Kaiser test (see Table S1).

To a suspension of amine-functionalized MWNT **22**, (9.3 mg, 1.0 μmol) in dry DMF (2.5 mL), DIPEA (10 μL, 70 eq.) and HATU (8 mg, 25 eq.) were added. Subsequently, MTX (7 mg, 18 eq.) was added and the reaction was stirred at 60 °C under Ar atmosphere for 50 h. The crude product was purified by centrifugation-precipitation, using step gradient centrifugation DMF-MeOH, MeOH, MeOH-Et_2O, to thoroughly remove excess of organic reactants. The absence of free MTX was confirmed by TLC analysis and HPLC (supernatant) (see Figure S8), finally dried under vacuum, to afford MWNT **3** (8.5 mg). The amount of MTX was estimated *via* quantitative Kaiser test (see Table S1). MWNT **3**, were also characterized by TEM and TGA (see Supplementary Figures).

Synthesis of MWNT 4.



a) **6**, DIC, DMF, 60 °C b) **7**, DIPEA, HATU, DMF, 60 °C, 50 h

DIPEA (20 μL, 100 eq.), and Diisoprorylcarbodiimide (DIC, 12 μL, 70 eq.), were added to a suspension of MWNT **1** (6 mg, 1.1 μmol) in dry DMF (2 mL), then Linker-O-MTX (**6**) (23 mg, 35 mmol) was added and the reaction was brought at 60 °C under Ar atmosphere and magnetically stirred for 60 h. The crude was purified by centrifugation-precipitation, to remove the excess of reactants using a step gradient centrifugation DMF-MeOH, MeOH, MeOH-Et_2O, to afford modified MWNT **23** (5.6 mg). The absence of free Linker-O-MTX **6** was confirmed by TLC analysis and then dried under vacuum. MWNT **23** was characterized by TGA.

To a suspension of MWNT **23**, (5.6 mg) in dry DMF (2 mL), DIPEA (24 μL, 50 eq.) and HATU (10 mg, 10 eq.) were added. Subsequently, Rhodamine B Sulfonamide-N-triethylenglicole hydrochloride **7** (8 mg, 4 eq.) was added and the reaction was stirred at 60 °C under Ar atmosphere for 50 h. The crude product was purified by centrifugation-precipitation, using DMF-MeOH, MeOH, MeOH-Et_2O, to thoroughly remove excess of organic reactants. The absence both of free MTX and Rhodamine was confirmed by TLC analysis and HPLC analysis of the supernatant (see Figure S8) and finally dried under vacuum (5.1 mg). The amount of MTX was estimated *via* quantitative Kaiser test (see Table S1). MWNT **4**, were also characterized by TEM and TGA (see Supplementary Figures).

Table S1.

	Loading NH_2 (μmol/g) from Kaiser test	Efficiency of MTX coupling from Kaiser test (%)	Amount of bound MTX (μg/mg of conjugate)	Amount of bound MTX (μmol/g of conjugate)
MWNT **1**	180 or 220[a]	—	—	—
MWNT **2**	220	90	89.9	198
MWNT **3**	100[b]	80	36.4	80
MWNT **4**	180	100	81.8	180

a) Reaction was performed twice giving slightly different loading as indicated; b) Loading calculated after Boc deprotection of peptide-MWNT **22**.

Supplementary Figures

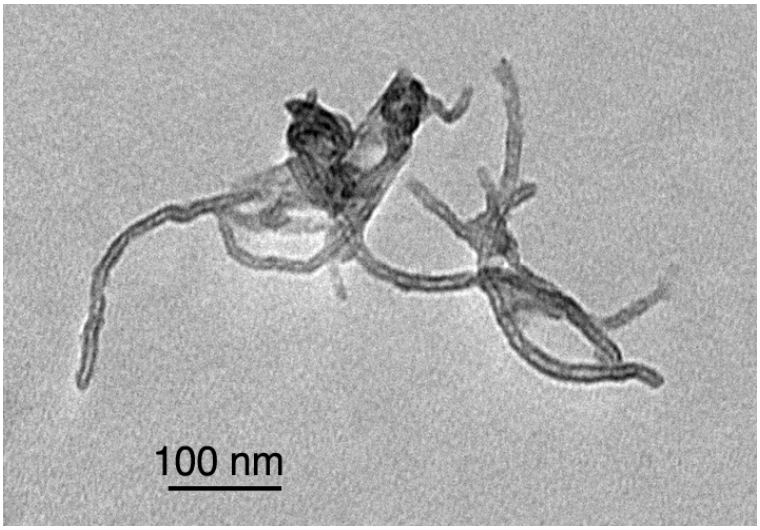


Figure S1. TEM image of MWNT **1**.

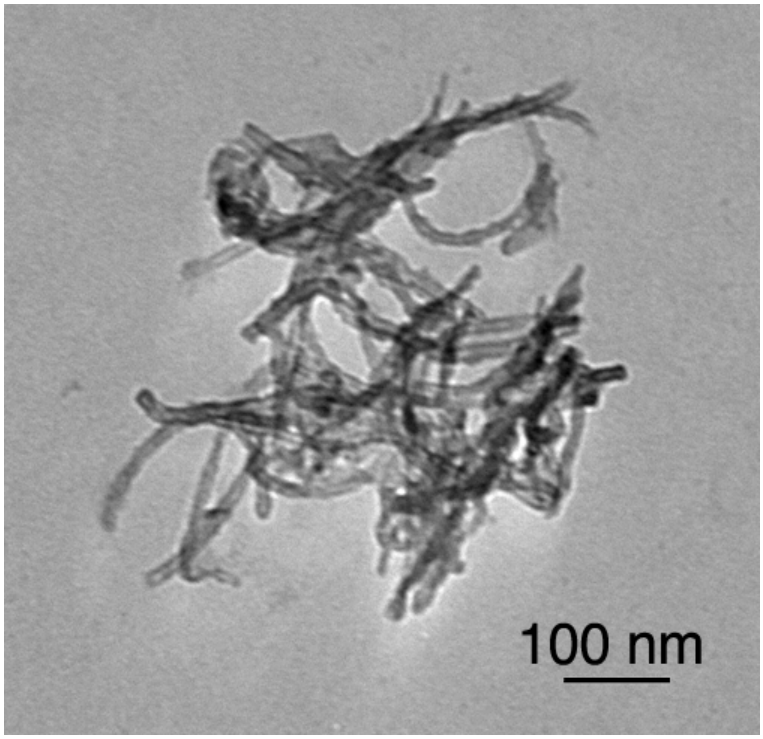


Figure S2. TEM image of MWNT **2**.

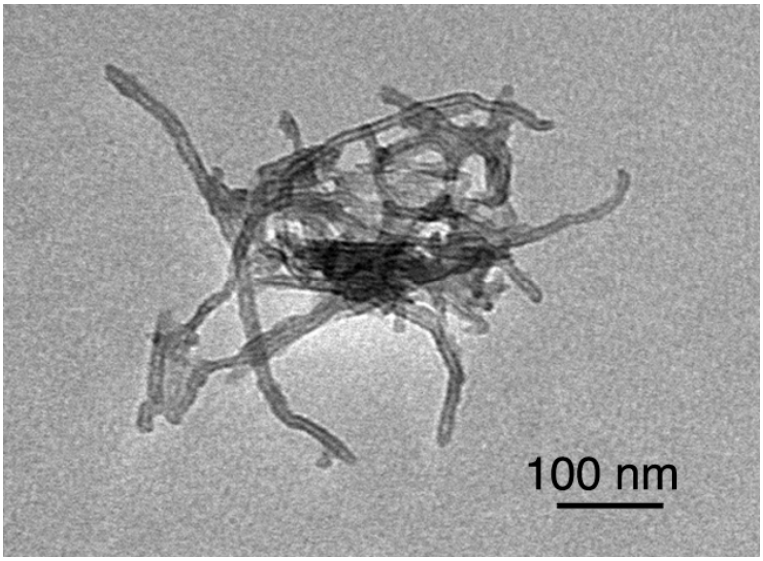


Figure S3. TEM image of MWNT **3**.

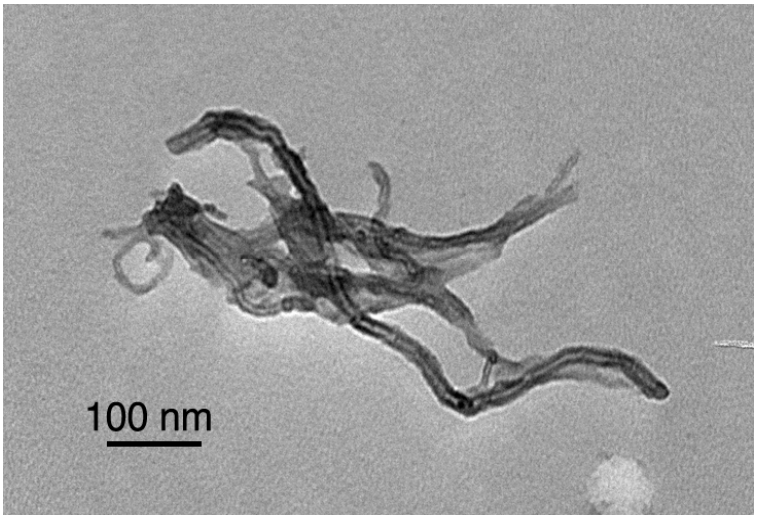


Figure S4. TEM image of MWNT **4**.

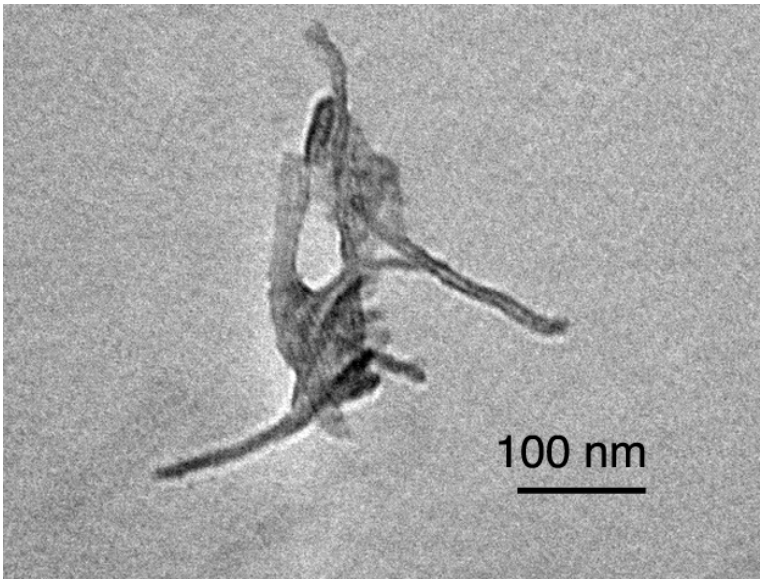


Figure S5. TEM image of MWNT **22**.

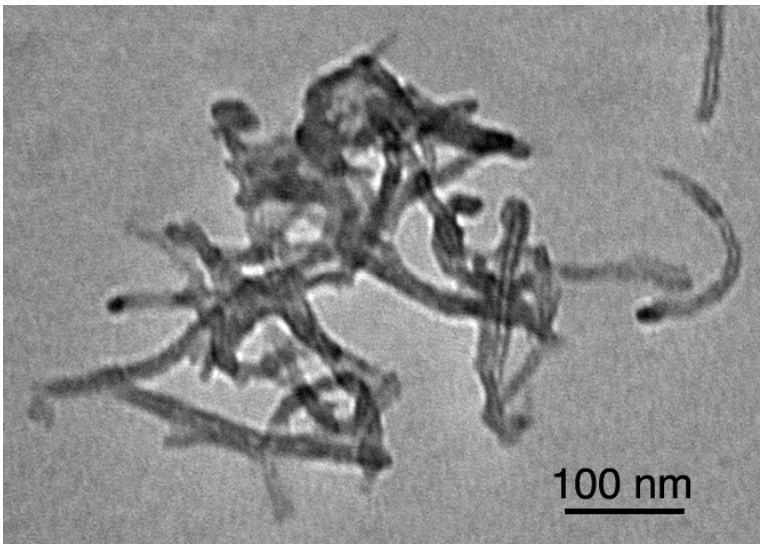


Figure S6. TEM image of MWNT **23**.

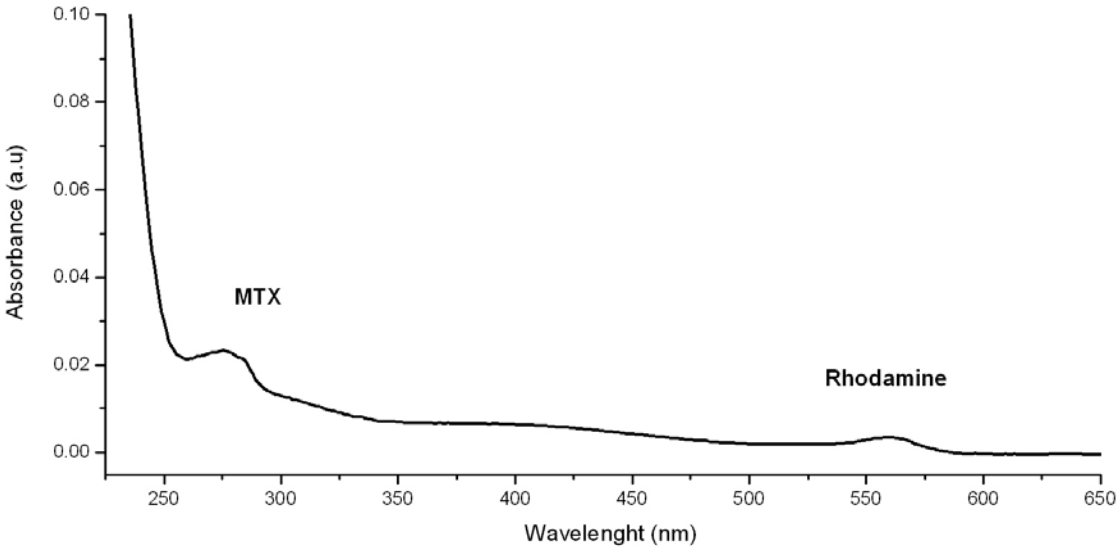


Figure S7. UV-Vis spectrum of MWNT **4** in methanol.

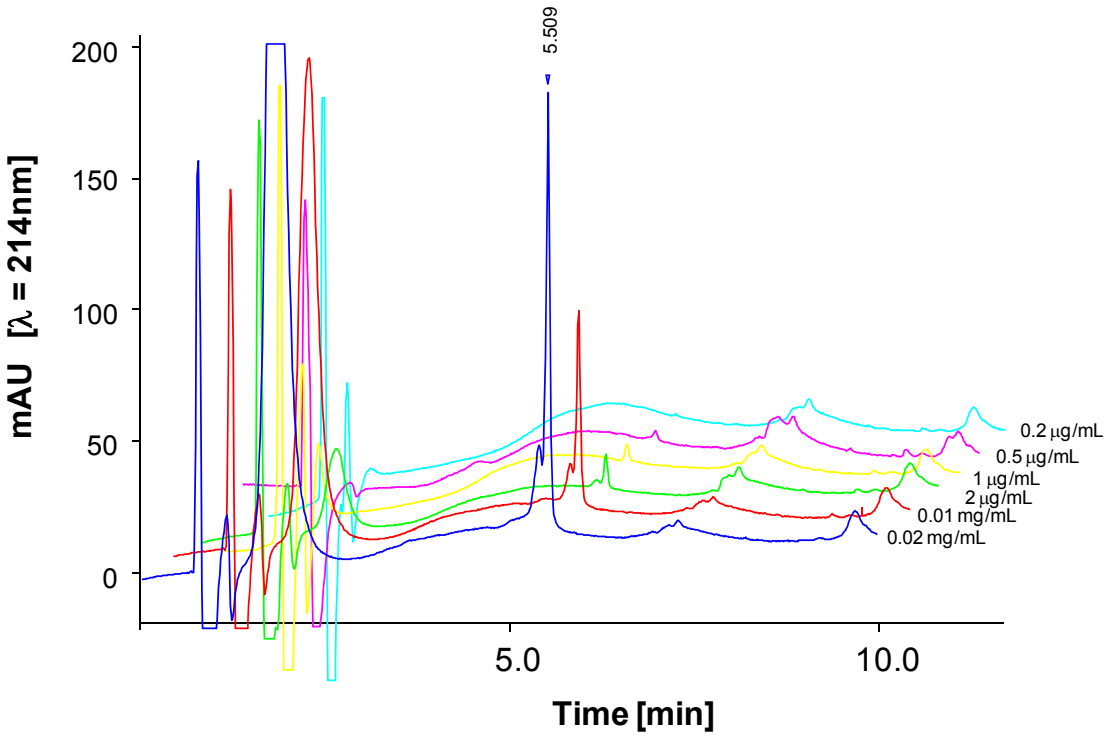


Figure S8. To establish the limit detection of MTX in the washing solutions of the functionalized MWNT **3** and **4** we have performed a series of HPLC analysis of MTX in MeOH at different concentrations. For this experiment, a stock solution of 1 mg/ml of MTX in MeOH/DMF (100:1 v/v) was prepared and further diluted in MeOH. Injections of 20 µl were performed using a linear gradient of A: 0.1% TFA in

water and B: 0.08% TFA in acetonitrile, 0-100% B in 10 min at 1.0 mL/min flow rate, detection at λ = 214 or 300 nm. Column: Macherey-Nagel C_{18} (3 μm, 70 × 4 mm).
The peak at 5.509 min corresponds to the commercially available MTX (Apollo Scientific LTD; Lot n. 40138). Subsequent dilutions (starting from 0.02 mg/ml to 0.2 μg/ml) display the decrease of MTX peak. The HPLC detection limit of the MTX is lower than 0.5 μg/ml. This concentration is 10-fold lower than the concentration of the free MTX tested for its cytotoxic activity on MCF-7 cells (4.54 μg/ml – 10 μM, see Figure 4 of the main text).

Cell Culture and Confocal Microscopy studies

Human breast cancer cells (MCF-7; ATCC®.HTB-22™) were grown in RPMI 1640 folate free medium (Invitrogen, UK) and supplemented with 10% fetal bovine serum (FBS, Invitrogen UK) and 50 U/ml penicillin (Invitrogen, UK) and 50 μg/ml streptomycin (Invitrogen, UK) at 37 °C in 5% CO_2. Cells were passaged to reach 80 % confluency.

For confocal laser scanning microscopy studies, MCF7 cells were plated onto microscope coverslips at a density of 30000 cells and left to attach overnight. The cells were then incubated with the different MWNT conjugates (MWNT **1** and MWNT **4**) in folate-free medium at a final MWNT concentration of 10μg/ml for 24 h. The cells were then washed with phosphate buffer saline (1×PBS, Invitrogen, UK) and fixed using 4 % paraformaldehyde for 20 min, followed by 3 cycles of washes with PBS after which the coverslips were mounted onto slides. The cellular uptake of MWNT-MTX-rhodamine B (MWNT **4**) was imaged using a Zeiss Axiovert LSM510 confocal with a 63× oil immersion objective (Carl Zeiss Inc., Thornwood, NY). Rhodamine B was excited at 543 nm laser power with pinhole of 500 nm. All images in Figure 2 were collected using identical acquisition parameters.

For the cytotoxicity assessment of the MWNT-MTX conjugates, a modified lactate dehydrogenase (LDH) assay was used. This assay was performed using the Promega Cytotox 96 ® Non-radioactive cytotoxicity assay (Promega UK Ltd) according to the manufacturer instructions. The assay was modified to avoid interference between CNT and the LDH in the survived cells, and was assessed after artificially lysing all cells instead of detecting the LDH released from the treated cells alone as this would contain CNT.

MCF 7 cells were seeded at 7000 cells in a 96 well plate and left to attach overnight. Cells were then treated with the MTX alone and the MWNT-MTX conjugates (MWNT **2-4**) at which the concentration of MTX was kept constant at 10μM with and without the MWNTs. MWNTs without MTX were used as control to exclude any intrinsic toxicity from the MWNTs alone. In addition, 10 % DMSO was used as a

positive control for cytotoxicity. After 24 h treatment, cells were lysed with 10 µl of lysis buffer per 100 µl serum free media and left for 45-60 min at 37 ºC. 50 µL of cell lysate after centrifuging (13000 rpm, 5 min) were mixed with 50 µl substrate mixture in a new microtiter plate and incubated for 15 minutes at room temperature. Absorbance at 490 nm was read in ELISA plate reader. The amount of LDH released was an indication of the number of cells, which survived treatment. Hence the percentage cell survival is expressed as [LDH released from tested cells- Blank (media alone) / control cells] ×100.

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2. (a) Liu, J.; Rinzler A. G.; Dai H.; Hafner J. H.; Bradley R. K.; Boul P. J.; Lu A.; Iverson T.; Shelimov K.; Huffman C. B.; Rodriguez-Macias F.; Shon Y.-S.; Randall L. T.; Colbert D. T.; Smalley R. E.; *Science* **1998**; *280,* 1253–1256. (b) Li S.; Wu W.; Campidelli S.; Sarnatskaïa V.; Prato M.; Tridon A.; Nikolaev A.; Nikolaev V.; Bianco A.; Snezhkova E.; *Carbon,* **2008**, *46*, 1091–1095.
3. Pastorin, G.; Wu, W.; Wieckowski, S.; Briand, J.-P.; Kostarelos, K.; Prato, M.; Bianco, A.; *Chem. Commun.,* **2006**, 1182-1184.