SUPPORTING INFORMATION

for

Hybrids of iron-filled multi-wall carbon nanotubes and anticancer agents as potential magnetic Drug Delivery Systems – *in vitro* studies against human melanoma, colon carcinoma and colon adenocarcinoma

Sławomir Boncel[1,*], Anna Pluta[1], Magdalena Skonieczna[2], Andrzej Gondela[1], Barbara Maciejewska[3], Artur P. Herman[1], Rafał G. Jędrysiak[1], Sebastian Budniok[1], Kamila Komędera[4], Artur Błachowski[4], Krzysztof Z. Walczak[1]

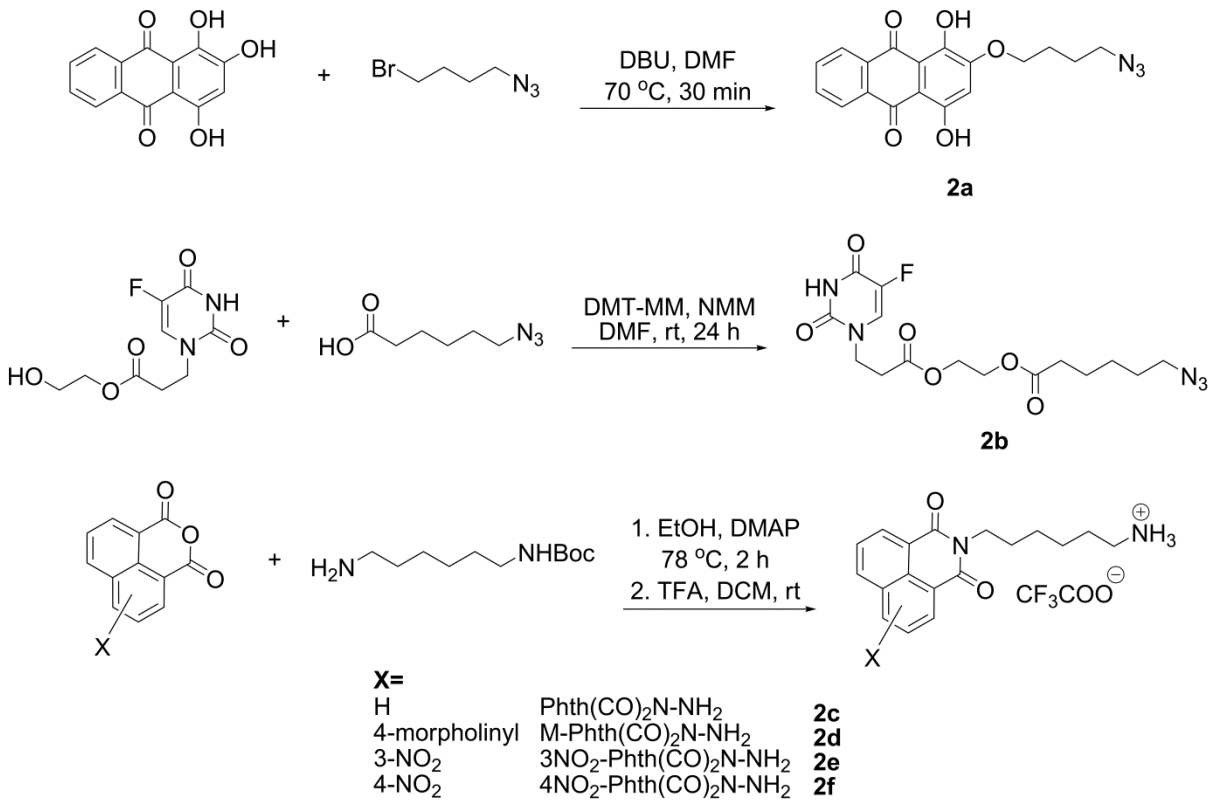
[1] Silesian University of Technology, Department of Organic Chemistry, Bioorganic Chemistry and Biotechnology, Krzywoustego 4, 44-100 Gliwice, Poland

[2] Silesian University of Technology, Institute of Automatic Control, Biosystems Group Department of Automatics, Electronics and Informatics, Akademicka 16, Gliwice, Poland

[3] NanoBioMedical Centre, Adam Mickiewicz University, Umultowska 85, 61-614 Poznań, Poland

[4] Pedagogical University, Institute of Physics, Mössbauer Spectroscopy Laboratory, Podchorążych 2, 30-084 Kraków, Poland

Synthesis of anticancer agents (and their pro-drugs)



Scheme S1 Synthetic pathways towards anticancer agents and its precursors

Synthesis of pure anticancer agents, their pro-drugs, MWCNT vehicles and 'drug-nanotube' hybrids

2-(4-azidobutoxy)-1,4-dihydroxyanthracene-9,10-dione = Purp-N_3 (2a)

To a solution of 1,2,4-trihydroxyanthraquinone (purpurin) (256 mg, 1 mmol) in DMF (5.0 mL), a deprotonating agent 1,8-diazabicycloundec-7-ene (DBU) (145 mg, 0.95 mmol) was added and the resulting mixture was stirred at 70 °C for 30 min. Next, 1-azido-4-bromobutane (1.5 mmol) was added and the stirring at 70 °C was continued. The progress of the reaction was monitored by TLC ($CHCl_3$). The reaction mixture was evaporated to dryness and the residual oil was purified on a silica gel packed column using $CHCl_3$ as the eluent. The fractions containing product were collected, combined and evaporated giving crystalline

orange solids. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.81-1.90 (m, 2H, H-3'), 1.98 (m, 2H, H-2'), 3.44 (t, J=6.6 Hz, 2H, H-4'), 4.14 (t, J=6.6 Hz, 2H, H-1'), 6.62 (s, 1H, H-3), 7.75-7.84 (m, 2H, H-5, H-6), 8.26-8.31 (m, 2H, H-7, H-8), 13.40 (s, 1H, 4-OH), 13.47 (s, 1H, 1-OH). ^{13}C NMR ($CDCl_3$, 75 MHz), δ: 25.7, 26.0, 51.1, 68.9, 106.1, 107.4, 112.4, 126.8, 126.9, 133.2, 133.7, 134.0, 134.5, 150.4, 156.9, 160.7, 184.3, 187.1. ESI-MS m/z=352.6 $[M\text{-}H^+]^-$.

2-(3-(5-fluorouracil-1yl)propanoyloxy)ethyl 6-azidohexanoate = 5-FU-N_3 (2b)

To a solution of 2-hydroxyethyl 3-(5-fluorouracil-1-yl)propanoate [1] (0.492 g, 2 mmol) in anhydrous DMF (10 mL) placed in a round-bottom flask (25 mL) on magnetic stirrer, 6-azidohexanoic acid (0.314 g, 2 mmol), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM, 1.106 g, 4 mmol), *N*-methylmorpholine (NMM) (0.202 g, 2 mmol, d=0.91 g mL^{-1}). The reaction mixture was stirred for 24 h. Next, DMF was removed from the post-reaction mixture using rotary evaporator and the solid residue was purified by column chromatography. A white semi-solid was obtained in 28% yield (0.216 g).^{1}H NMR (400 MHz, $CDCl_3$), δ ppm): 9.98 (br s, 1H, N-H), 7.43 (d, 1H, J_{HF}=5.6 Hz, H-6), 4.31 (m, 4H, -OCH_2CH_2O-), 3.99 (t, 2H, J=6.0 Hz, >NCH_2), 3.28 (t, 2H, J=7.2 Hz, -CH_2N_3), 2.83 (t, 2H, J=6.0 Hz, >NCH_2CH_2-), 2.35 (t, 2H, J=7.2 Hz, CH_2CO), 1.64 (m, 4H, 4-CH_2, 2-CH_2), 1.41 (m, 2H, 3-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$), δ ppm): 173.1, 171.0, 157.5, 149.6, 141.1, 130.1 (d, J_{CF}=32.7 Hz), 63.0, 61.6, 51.1, 45.3, 33.7, 32.6, 28.4, 26.0, 24.2.

2-(6-Aminohexyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (2c)

4-(*N,N*-Dimethylamino)pyridine (DMAP) (0.001 g) was introduced into a solution of hexylene-1,6-diamine (2.320 g, 20.0 mmol) in anhydrous EtOH (50 mL) in a round-bottomed flask (100 mL). The mixture was refluxed under inert atmosphere (nitrogen) and then, within the next 1 h, 1,8-naphthalenedicarboxylic acid anhydride (0.198 g, 1.0 mmol) was added in small portions while a thorough stirring. The reaction mixture was refluxed for 4 h at monitoring the progress using TLC. The volatiles were removed using rotary evaporator and

the product was isolated using column chromatography. The reaction was performed using 1,6-hexylenediamine (2.320 g, 20.0 mmol), anhydrous EtOH (50 mL) and 1,8-naphthalenedicarboxylic acid anhydride (2.000 g, 10.0 mmol). Yield 1.642 g (55%) (also 0.930 g of bisnaphthalimide derivative was obtained); white crystals, mp 70-71 °C; ^{1}H NMR ($CDCl_3$, 600 MHz), δ ppm): 1.41-1.48 (m, 8H, H-3", H-4", H-5", NH_2), 1.75 (dd, 2H, *J*=7.4; 15.0 Hz, H-2"), 2.68 (dd, 2H, *J*=7.2; 13.8 Hz, H-6"), 4.18 (dd, 2H, *J*=7.4; 15.0 Hz, H-1"), 7.75 (dd, 2H, *J*=7.2; 13.2 Hz, H-3, H-6), 8.21 (dd, 2H, *J*=1.2; 3.6 Hz, H-4, H-5), 8.60 (dd, 2H, *J*=4.2; 7.2 Hz, H-2, H-7). ^{13}C NMR ($CDCl_3$, 600 MHz), δ ppm): 26.71 (C-4"), 27.01 (C-3"), 28.21 (C-2"), 33.86 (C-5"), 40.49 (C-1"), 42.28 (C-6"), 122.89 (C-9), 127.06 (C-1, C-8), 128.30 (C-10), 131.32 (C-3, C-6), 131.74 (C-2, C-7), 133.98 (C-4, C-5), 164.35 (C-1', C-3').

_t_-Butyl 6-(6-morpholino-1,3-dioxo-1_H_-benzo[_de_]isoquinolin-2(3_H_)-yl)hexylcarbamate (2d)

To a solution of *N*-Boc-1,6-diaminohexane (0.65 g, mmol) in EtOH (15 mL) 4-morpholino-1,8-naphthalenedicarboxylic acid anhydride [2] (0.71 g, mmol) and DMAP (50 mg) were added. The reaction mixture was refluxed for 2 h. The solvent was removed using rotary evaporator and the residue was recrystallized from MeOH yielding yellow fluorescent precipitate. Yield 88% (1.059 g). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.59 (dd, J = 7.3, 1.2 Hz, 1H, H-7), 8.53 (d, J = 8.1 Hz, 1H, H-2), 8.42 (dd, J = 8.4, 1.2 Hz, 1H, H-5), 7.70 (dd, J = 8.4, 7.3 Hz, 1H, H-6), 7.23 (d, J = 8.1 Hz, 1H, H-3), 4.56 (s, 1H, NH), 4.18 – 4.14 (m, 2H, H-1'), 4.04 – 4.00 (m, 4H, H-3"), 3.29 – 3.25 (m, 4H, H-2"), 3.14 – 3.06 (m, 2H, H-6'), 1.77 – 1.70 (m, 2H, H-2'), 1.55 – 1.47 (m, 2H, H-5'), 1.45 – 1.38 (m, 13H, H-3', H-4', Me). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.41, 163.96, 155.98, 155.59, 132.49, 131.16, 129.99, 129.90, 126.19, 125.85, 123.41, 117.29, 114.98, 67.00, 53.48, 40.52, 40.12, 29.94, 29.93, 28.46, 28.04, 26.72, 26.47.

***t*-Butyl 6-(5-nitro-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)hexylcarbamate (2e)**

To a solution of *N-Boc*-1,6-diaminohexane (1.28 g, 5.26 mmol) in anhydrous EtOH (10 mL) 3-nitro-1,8-naphthalenedicarboxylic acid anhydride (1.23 g, 5.53 mmol) and DMAP (50 mg) were added. The reaction mixture was refluxed for 3 h. The reaction mixture was cooled down and the precipitate was washed with EtOH. The raw product was recrystallized from MeOH (18 mL) to give product as white needles. Yield 69% (1.61 g). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 9.31 (d, J = 2.2 Hz, 1H, H-2), 9.13 (d, J = 2.2 Hz, 1H, H-4), 8.78 (dd, J = 7.3, 1.2 Hz, 1H, H-7), 8.42 (ddd, J = 8.2, 1.2, 0.4 Hz, 1H, H-5), 7.95 (dd, J = 8.2, 7.3 Hz, 1H, H-6), 4.54 (s, 1H, NH), 4.24 – 4.17 (m, 2H, H-1'), 3.15 – 3.07 (m, 2H, H-6'), 1.79–1.71 (m, 2H, H-2'), 1.56 – 1.48 (m, 2H, H-5'), 1.47 – 1.36 (m, 13H, H-3', H-4', Me). ^{13}C NMR (151 MHz, $CDCl_3$) δ 163.10, 162.48, 155.98, 146.45, 135.47, 134.40, 131.04, 130.22, 129.08, 128.86, 124.78, 124.24, 123.29, 40.70, 40.49, 29.95, 28.45, 27.93, 26.68, 26.43.

***tert*-Butyl 6-(6-nitro-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)hexylcarbamate (2f)**

To a solution of *N*-Boc-1,6-diaminohexane (0.65 g, mmol) in anhydrous EtOH (10 mL) 4-nitro-1,8-naphthalenedicarboxylic acid anhydride (0.75 g, mmol) and DMAP (50 mg) were added. The reaction mixture was refluxed for 1.5 h. The formed precipitate after cooling down the reaction mixture was filtered off and recrystallized from EtOH yielding product (0.966 g). Additionally, solvent was removed from the filtrate using rotary evaporator and the precipitate was recrystallized from EtOH yielding additional fraction of product (0.090 g). Yield 78% (1.06 g). ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.84 (dd, J=8.7, 1.1 Hz, 1H, H-5), 8.73 (dd, J=7.3, 1.1 Hz, 1H, H-7), 8.69 (d, J = 8.0 Hz, 1H, H-3), 8.40 (d, J=8.0 Hz, 1H, H-2), 7.99 (dd, J=8.7, 7.3 Hz, 1H, H-6), 4.53 (s, 1H, NH), 4.23-4.12 (m, 2H, H-1'), 3.17-3.04 (m, 2H, H-6'), 1.79-1.70 (m, 2H, H-2'), 1.55-1.47 (m, 2H, H-5'), 1.48-1.35 (m, 13H, H-3', H-4', Me). ^{13}C NMR ($CDCl_3$) δ: 163.30, 162.48, 155.95, 149.60, 132.44, 129.93, 129.80, 129.30, 129.13, 127.03, 123.88, 123.71, 123.07, 40.69, 40.48, 29.94, 28.45, 27.91, 26.68, 26.42.

Synthesis of Fe@O-MWCNTs (1b) from Fe@MWCNTs (1a)

Fe@MWCNTs (0.50 g) were placed in round-bottom flask and 300 mL of a mixture containing concentrated sulphuric (VI) (98%) and nitric (V) acid (68%) prepared in a ratio 3:1 (v/v) was added. The flask was equipped with a reflux condenser and the reaction mixture was heated to boiling and a vigorous evolution of brown nitrogen (IV) oxide started. The boiling was continued for 20 minutes. Subsequently, the mixture was allowed to cool to room temperature and was quenched in a beaker with 500 mL of distilled water. Then, the post-reaction mixture was filtrated using a Teflon® membrane (0.45 μm pore size, EMD Milipore) under reduced pressure and the precipitate was rinsed with deionised water until neutral pH. The product – Fe@O-MWCNTs – was dried in an electric dryer at 80 °C under atmospheric pressure for 120 h.

Anchoring anticancer agents and their pro-drugs onto the nanotube vehicles

MWCNT>N-Purp (3aa)

MWCNTs (50 mg) were added to a solution of Purp-N_3 (**2a**) (100 mg, 0.3 mmol) in 1,1,2,2-tetrachloroethylene (TCE, 15 mL), and the reaction mixture was ultrasonicated for 1 h. The resulting dispersion was then refluxed for 4 h and allowed to cool to room temperature. Solid product was filtered off over a PTFE membrane (0.45 μm pore size), washed thoroughly with methanol (120 mL) and chloroform (120 mL) and dried in a laboratory oven for 24 h at 100 °C.

MWCNT>N-5FU (3ab)

MWCNTs (27 mg) and tetrachloroethylene (TCE) (14 mL) were introduced to a round-bottom flask (50 mL) and the flask was placed in an ultrasonic bath for 15 min. The appropriate azide (108 mg, 0.28 mmol) was dissolved in TCE and the solution was added to the reaction mixture. The mixture was refluxed for 8 h. The drug-nanotube hybrid was filtered

off from the post-reaction mixture (Teflon® filter 0.45 μm) and washed with copious amounts of chloroform (250 mL) and MeOH (250 mL) until colorless filtrate. The product was dried in the electric oven at 75 °C overnight; yield 66 mg.

MWCNT-COO$^-$ Phth(CO)$_2$N-NH$_3^+$ (3bc_i)

To 2-(6-aminohexyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (**2bc**) (0.071 g, 0.23 mmol) dissolved in DMF (2.9 mL), O-MWCNTs (0.040 g) was added followed by the addition of DMF (38 mL). The reaction mixture was ultrasonicated for 20 h. Afterwards, the precipitate was filtered off, washed with MeOH (160 mL) and $CHCl_3$. (100 mL). The product was dried in an electric oven at 70 °C. The 'drug-nanotube' hybrid was received in yield of 0.041 g.

MWCNT-CONH-N-(OC)$_2$Phth (3bc_c)

To O-MWCNTs (0.100 g) $SOCl_2$ (1.30 g, 109 mmol, 8.00 mL) was added. The dispersion was ultrasonicated for 30 min with a drying tube placed at the outlet of flask, and then for 21 h cork-stopped. The volatiles were removed by rotary evaporator (1 h, 40 °C) and subsequently using a diaphragm vacuum pump (30 min, 6 mmHg). The solid residue was combined with a mixture of 2-(6-aminohexyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (0.100 g, 0.34 mmol), triethylamine (0.728 g, 7.19 mmol, 1.00 mL) and DMF (8 mL), and it was ultrasonicated for 7 h. Afterwards, the solid was filtered off and washed with EtOH (120 mL) and $CHCl_3$ (mL). The product was dried at 90 °C. The 'drug-nanotube' hybrid was received in yield of 0.104 g.

MWCNT-CONH-N-(OC)$_2$Phth-M (3bd)

To *t*-Butyl 6-(6-morpholino-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)hexylcarbamate (0.240 g, 0.50 mmol), trifluoroacetic acid (0.199 g, 1.75 mmol, 0.13 mL) and dichloromethane (5 mL) were added. The reaction mixture was stirred for 6 days. After the reaction was completed, the volatiles were removed using rotary evaporator (40 min, 50 °C).

Afterwards, dispersion of O-MWCNTs (0.234 g) in DMF (30 mL) was added to the reaction mixture. The mixture was ultrasonicated for 10 min with a subsequent addition of NMM (0.051 g, 0.50 mmol, 0.07 mL). Then DMT-MM (0.152 g, 0.55 mmol) was added and the reaction mixture was ultrasonicated for 24 h. The product was filtered off and washed with MeOH (190 mL) and $CHCl_3$ (95 mL). The product was dried at 70 °C. The 'drug-nanotube' hybrid was received in yield of 0.214 g.

MWCNT-CONH-N-$(OC)_2$Phth-3NO_2 (3be)

To *Boc*-protected 2-(6-aminohexyl)-5-nitro-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (0.800 g, 1.81 mmol), trifluoroacetic acid (1.787 g, 15.67 mmol, 1.20 mL) and dichloromethane (15 mL) were added. The reaction mixture was stirred for 5 days. Then, the volatiles were removed using a rotary evaporator (45 min, 50 °C). Afterwards, to the reaction mixture, dispersion of O-MWCNTs (0.702 g) in DMF was added (40 mL). The reaction mixture was ultrasonicated for 10 min with a subsequent addition of NMM (0.182 g,1.81 mmol, 0.22 mL). Afterwards, 0.548 g (1.98 mmol) of DMT-MM was added and the reaction mixture was ultrasonicated for 46 h. The solid product was filtered off and washed with $CHCl_3$ (210 mL) and CH_2Cl_2 (50 mL). Product was dried in 70 °C. The 'drug-nanotube' hybrid was received in yield of 0.834 g.

MWCNT-CONH-N-$(OC)_2$Phth-3NO_2 (3bf)

To 2-(6-aminohexyl)-6-nitro-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (0.800 g, 1.81 mmol), trifluoroacetic acid (1.787 g, 15.67 mmol, 1.20 mL) and dichloromethane (15 mL) were added. The reaction mixture was stirred for 5 days. The volatiles were removed using a rotary evaporator (45 min, 50 °C). Afterwards, to the reaction mixture, dispersion of O-MWCNTs (0.708 g) in DMF (40 mL) was added. The reaction mixture was ultrasonicated for 10 min with a subsequent addition of NMM (0.182 g, 1.81 mmol, 0.22 mL). Afterwards, DMT-MM (0.548 g, 1.98 mmol) was added and the reaction mixture was ultrasonicated for 46 h. Then

the solid product was filtered off through a PTFE membrane filter and washed with $CHCl_3$ (210 mL) and CH_2Cl_2 (50 mL). The product was dried at 70 °C. The 'drug-nanotube' hybrid was received in yield of 0.780 g.

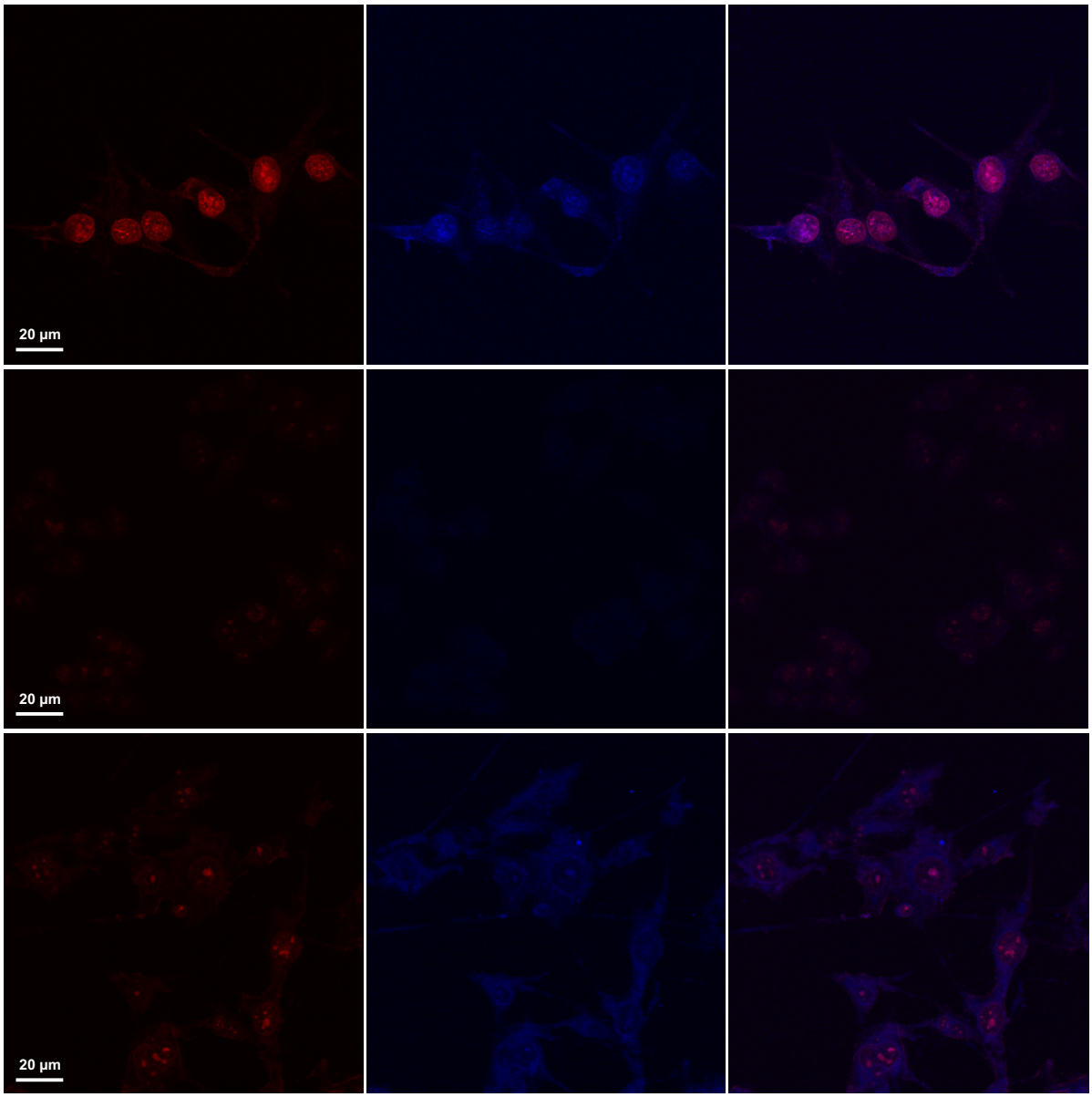


Fig. S1 Confocal microscopy images of 'drug-nanotube' hybrids in the presence of cancer cells; the samples were incubated for 24 h under standard conditions at 5 µg/mL. *Upper panel*: Me45 *vs* MWCNT>N-5FU (**3ab**); *Middle panel*: HCT116+ *vs* MWCNT>N-5FU (**3ab**); *Below panel*: Caco-2 *vs* MWCNT-CONH-N-$(OC)_2$Phth-$4NO_2$ (**3bf**); From left to right: cell nuclei dyed by ethidium bromide (*red*); areas dyed by 'drug-nanotube' hybrids and anticancer agents released therefrom (*blue*); merged images.

References

[1] Boncel, S.; Osyda, D.; Walczak, K. Z. *Beilstein J. Org. Chem.* **2007**, *3*, 40.

[2] Synthesized *via* a modified method from: Wu, J.; Zawistowski, A.; Ehrmabb, M.; Yi, T.; Schmuck, C. *J. Am. Chem. Soc.* **2011**, *133*, 9720–9723.