Supplementary Information

for

Ratiometric Delivery of Cisplatin and Doxorubicin using Tumour-Targeting Carbon-Nanotubes Entrapping Platinum(IV) Prodrugs

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Experimental Section

Materials. Unless otherwise noted, all procedures were carried out without taking precautions to exclude air and moisture. All solvents and chemicals were used as received without further treatment. K_2PtCl_4 was obtained from Strem Chemicals. Cisplatin, oxoplatin, and **1** were synthesized and purified accordingly to literature procedures.[1-3] Doxorubicin was purchased from Merlin Chemicals Ltd. c(RGDfK) peptide was purchased from ChinaPeptides Co. Ltd. RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA, USA). 2,2'-(Ethylenedioxy)diethylamine and dextran-coated charcoal were obtained from Sigma Aldrich. Penicillin, streptomycin and thiazolyl blue tetrazolium bromide (MTT) wereall obtained from Sigma Chemical Co (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Hyclone (Thermo Scientific Inc., Logan, UT, USA). DMEM nutrient mix F12 and antibiotic-antimycotic were obtained from Life Technologies. The water used was of Milli-Q grade purified by a Milli-Q UV Purification System (Sartorius Stedim Biotech S.A., Aubagne Cedex, France). All other solvents and chemicals were of analytical grade or HPLC grade obtained from commercial sources.

Instrumentation. ^{1}H NMR spectra were recorded on a Bruker AMX 500 spectrometer and the chemical shifts (δ) were internally referenced by the residual solvent signals relative to tetramethylsilane. Mass spectra were measured using a Finnigan MAT LCQ ion trap ESI mass spectrometer. UV spectra were recorded on a Shimadzu UV-1800 UV spectrophotometer using 1 cm path-length quartz cuvettes. Platinum concentration determination was performed using Inductively-Coupled Plasma Optical Emission Spectroscopy (ICP-OES) by CMMAC, NUS. Elemental analyses of selected platinum compounds were carried out on the Perkin-Elmer PE 2400 elemental analyzer by CMMAC, NUS. Transmission electron microscopy (TEM) imaging was carried out using the JOEL JEM-3010 electron microscope at an accelerating voltage of 300 kV. The samples were prepared by dispersing them in water by sonication for 30 min before placing on 300 mesh copper grids.

Synthesis of 2. Compound **1** (50 mg, 114 μmol) and succinic anhydride (70 mg, 700 μmol) were stirred at 50ºC in DMF (10 mL) for 24 h. The reaction mixture was lyophilized and washed with DCM (5 x 5 mL) to yield **2** as an off-white product after drying *in vacuo*. Yield: 44 mg (72%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.00 (br, s, 1 H, -COOH), 7.88 (d, 2 H, Ar-H), 7.51 (t, 1 H, Ar-H), 7.42 (t, 2 H, Ar-H), 6.62 (br, 6 H, NH_3), 2.53 (t, 2 H, -CH_2), 2.40 (t, 2 H, -CH_2) ppm. ESI-MS (-ve mode): *m/z* = 537.0 $[M-H]^-$.

Synthesis of 3. Compound **2** (20 mg, 37.2 μmol) and Doxo·HCl (30 mg, 51.7 μmol) were stirred at r.t. in dried DMSO (5 mL) for 24 h. HBTU (21 mg, 55.8 μmol) was added to the solution mixture followed by catalytic amounts of triethylamine. The crude solution mixture was concentrate to 2 mL and precipitated by adding to excess water (20 mL). The residue was washed with water (3 x 5 mL) and acetone (3 x 5 mL). The residue was re-dissolved in DMF (2 mL) and re-precipitated by adding to excess diethyl ether (20 mL). The precipitate was washed with diethyl ether (3 x 5 mL), dried *in vacuo*, to yield **3** as a dark red product. Yield: 27 mg (68%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.85 (br, 3 H, Ar-H), 7.61 (d, 2H, Ar-H), 7.50 (d, 1H, Ar-H), 7.40 (t, 2H, Ar-H), 6.59 (br, 6 H, NH_3), 5.43 (s, 1H, OCHO), 5.21 (s, 1H), 4.91 (s, 1H), 4.87 (t, 1H, C*H*CH_3), 4.74 (d, 1H), 4.58 (d, 2H, C*H*$_2$OH), 4.17 (d, 1H), 3.96 (s, 3H, OCH_3; s, 1H), 2.99 (m, 2 H, CH_2) 2.43 (t, 2H, CH_2), 2.28 (t, 2H, CH_2), 2.20 (d, 1 H), 2.09 (d, 1H), 1.85 (d, 1H), 1.42 (d, 1H), 1.12 (d, 3H, CH_3). ESI-MS (-ve mode): *m/z* = 1061.99 $[M-H]^-$. Anal. Calcd.: C, 42.91; H, 4.07; N, 3.95. Found: C, 42.95; H, 4.20; N, 3.78.

Preparation of MWCNT$_{TEG}$. Pristine MWCNTs were oxidised and purified as described in previous reported procedure.[4] As-prepared MWCNT$_{oxidized}$ (9.7 mg) were dispersed in DMF and sonicated for 5 min. TEG (111 μmol), NHS (45 μmol), EDC·HCl (112 μmol), and DIPEA (112 μmol) were added in the suspension (Fig. 3). The mixture was heated at 60 °C for 24 h and filtered through a hydrophilic PTFE membrane (MWCO 0.22 μm). The residue was washed with DMF and deionized water, and dried *in vacuo* to yield MWCNT$_{TEG}$. The degree of amino-functionalization per gram of MWCNT$_{TEG}$ (amino-loading) was calculated through quantitative Kaiser Test.[5] As-prepared MWCNT$_{TEG}$ (ca. 100 μg) was incubated with 75 μL of 4 g/mL phenol

in EtOH, 100 μL of 0.02 mM KCN in pyridine, and 75 μL of ninhydrin in EtOH at 100°C for 7 min. After heating, the reaction was diluted with 60% EtOH to 5 mL, and the UV absorbance at 570 nm. The amount of $–NH_2$ groups (μmol/g in $MWCNT_{amine}$) was calculated using the following equation:

$$\text{Amino amount (μmol/g)} = \frac{A_{570\,\text{nm}} \times 5 \times 10^6}{\text{W} \times 15000}$$

where $A_{570\,\text{nm}}$ is the absorbance at the wavelength 570 nm; W is the weight of analysed nanotube sample (mg). Using this protocol, $MWCNT_{TEG}$ was ascertained to contain 680 μmol of NH_2 groups/g $MWCNT_{TEG}$.

Preparation of $MWCNT_{TEG\text{-}COOH}$. $MWCNT_{TEG}$ (10.0 mg) were dispersed in DMSO (3 mL) under sonication (5 min). Succinic anhydride (1.0 g) was then added to the suspension and the reaction mixture was allowed to stir at r.t. for 3 d. The reaction mixture was filtered, washed with DMSO (3 x 5 mL) and dialysed in water for 24 h to remove any unreacted succinic anhydride. Quantitative Kaiser Test (see preparation of $MWCNT_{TEG}$) on $MWCNT_{TEG\text{-}COOH}$ yielded a negative result indicating complete reaction of the amine groups.

Preparation of $MWCNT_{c(RGDfK)}$. $MWCNT_{TEG\text{-}COOH}$ (5.0 mg) were dispersed in dried DMF (3 mL) under sonication (5 min). c(RGDfk) (30.0 mg, 50 μmol) was then added to the suspension followed by the addition of HBTU (19.0 mg, 50 μmol) and catalytic amounts of triethylamine. The reaction mixture was allowed to stir for 2 d at r.t., filtered, and washed with DMF (3 x 5 mL). The residue was then washed with diethyl ether (3 x 5 mL) and dried *in vacuo*. Final weight of the $MWCNT_{c(RGDfK)}$ synthesized was found to be 7.7 mg.

Entrapment of Doxo in $MWCNT_{TEG}$ [Doxo·$MWCNT_{TEG}$]. 4.5 mg of Doxo and 2.2 mg of $MWCNT_{TEG}$ were mixed in 1.5 mL of $CHCl_3$ under a brief sonication. The mixture was stirred at room temperature for 5 d, filtered through PTFE membrane, and washed with deionized H_2O to remove unbound Doxo. The product was recovered from PTFE membrane by dissolving in

diethyl ether, and the suspension was centrifuged to discard the supernatant. The precipitating product $MWCNT_{TEG}$-DOX was dried *in vacuo* and found to be 2.9 mg. However, the exact loading of [Doxo·$MWCNT_{TEG}$] could not be determined due to possible loss of materials through washing and presence of solvent molecules.

Entrapment of 3 in $MWCNT_{TEG}$ [3·$MWCNT_{TEG}$]. Complex **3** (5.0 mg) and $MWCNT_{TEG}$ (1.9 mg) were suspended in H_2O (1.5 mL) under a brief sonication. The mixture was stirred at r.t. for 5 d, filtered through PTFE membrane, washed with $CHCl_3$:MeOH:H_2O (2:2.5:1 v/v). The product formed [**3**·$MWCNT_{TEG}$] was dried *in vacuo*. The Pt content was ascertained to be 24.3 ± 0.8% w/w as analysed using TGA and ICP-OES.

Entrapment of 3 in $MWCNT_{c(RGDfK)}$ [3·$MWCNT_{c(RGDfK)}$]. Complex **3** (3.0 mg) and $MWCNT_{TEG}$ (2.0 mg) were suspended in H_2O (1.5 mL) under a brief sonication. The mixture was stirred at r.t. for 5 d, filtered through PTFE membrane, washed with $CHCl_3$:MeOH:H_2O (2:2.5:1 v/v). The product formed [**3**·$MWCNT_{c(RGDfK)}$] was dried *in vacuo*. The Pt content was ascertained to be 24.5 ± 1.0% w/w as analysed using TGA and ICP-OES.

Quantification of entrapped platinum complexes by TGA and ICP-OES. The samples were heat in air at a heating rate of 10°C/min until a final temperature of 1000°C was reached. The remaining residue was dissolved in *aqua regia* and diluted with 2% HNO_3 for ICP-OES determination of Pt levels.

***Doxo* Release Experiment**. [Doxo·$MWCNT_{TEG}$] (< 0.1 mg) was suspended in deionized H_2O (150 μL), transferred to a dialysis button, and immersed in deionized H_2O (10 mL) to evaluate its stability in H_2O. [**3**·$MWCNT_{TEG}$] (1.5 mg) was suspended in deionized H_2O (300 μL), transferred equally to two release buttons (150 μL each), followed by immersion in 10 deionized H_2O (10 mL) and aq. ascorbic acid solution (3 mM, 10 mL), separately. For each experiment, the dialysis media was replaced at 20 min, 40 min, 1 h, 2 h, 4 h, 6 h, 9 h, 12 h, 24 h, 48 h, 72 h, 96 h after the immersion and their UV absorbance determined at 500 nm (corresponding to Doxo λ_{max}).

Because loading of [Doxo·$MWCNT_{TEG}$] could not be determined, the time-point at 96 h was approximated as terminal release.

Tissue Culture. A2780 and A2780/Cis were cultured in complete RPMI 1640 medium containing 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS). In order to maintain resistance, 1 μM cisplatin need to be added to the media for A2780/Cis every 2-3 passages. The endometrial carcinoma cells Ishikawa (ATCC) was cultured in complete DMEM F12 medium containing 10% FBS pretreated with dextran coated charcoal and 1% antimycotic (100x). The three cell lines were grown at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Experiments were performed on cells within 20 passages. Viable cells were counted using the Trypan Blue exclusion method.

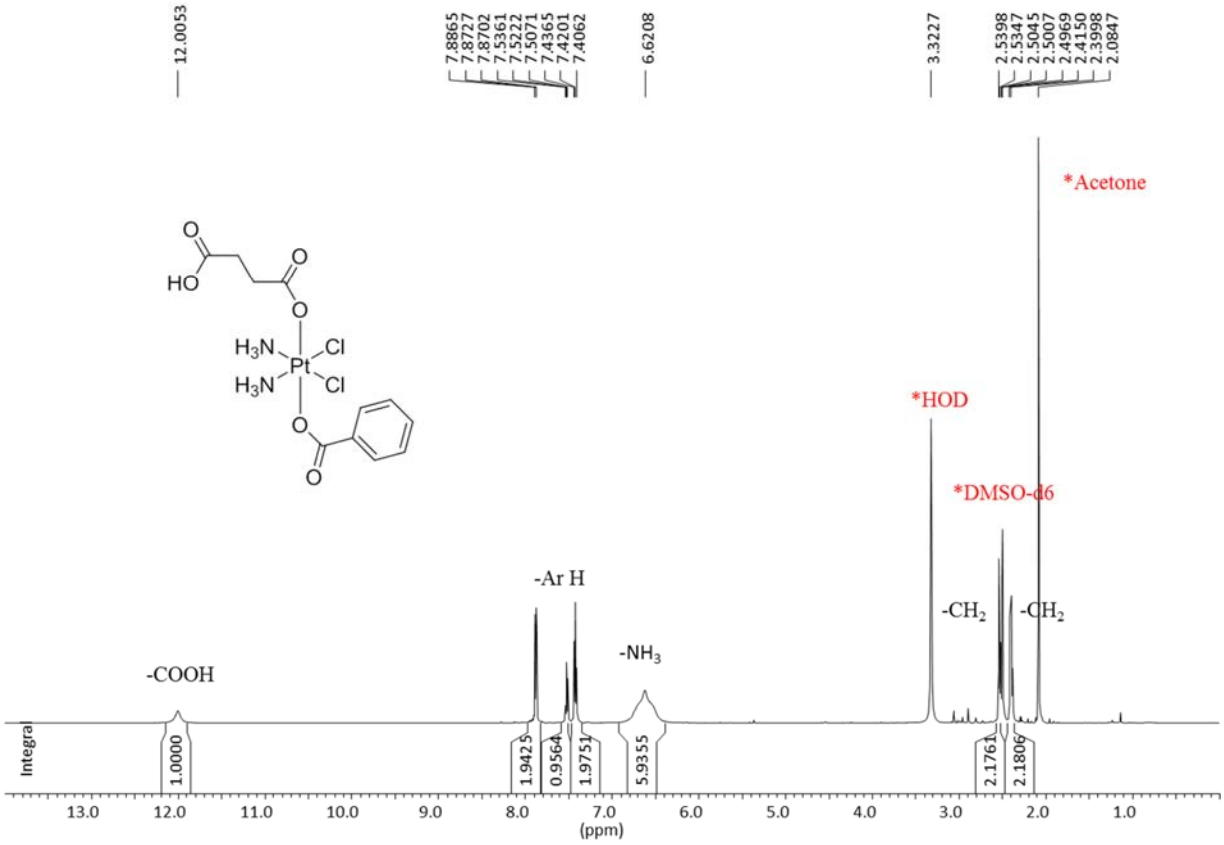
Inhibition of cell viability assay. Drug effects on exponentially growing tumor cells were determined using MTT assay as described previously.[6] A2780, A2780/Cis and Ishikawa cells were seeded at a density of 6,000 cells per well in 96-well plates and incubated for 24 h. Cells were exposed to compounds at different concentrations in their respective medium without FBS and antibiotics. These compounds were prepared as DMSO stock solutions and serially diluted with DMSO to a series of decreasing concentrations, before diluting to the required concentrations in culture media. In this manner, all cells were exposed to 1% v/v DMSO, at which negligible cytotoxicity were observed. The cells were incubated with the test compounds for 6 h, replaced with fresh compound-free complete medium (with FBS and antibiotics) and incubated for 66 h. After 72 h following compound administration, the medium was aspirated, replaced with MTT assay solution (100 μL, 0.5 mg/mL in PBS) and incubated for a further 4 h at 37°C. The medium was aspirated and the purple formazan precipitate dissolved in DMSO (100 μL). Absorbance was measured at a 570 nm using a microplate reader (BioTek). Experiments were performed in triplicates for each concentration and carried out independently at least three times. Cytotoxicity was evaluated with reference to the IC_{50} value which was defined as the concentration needed for a 50% reduction of survival based on the survival curves. IC_{50} values were calculated from dose-response curves (cell survival *vs.* drug concentration) obtained in

repeated experiments and adjusted to actual [Pt] administered which was separately determined using ICP-OES.

Pt uptake in Ishikawa cells. Ishikawa carcinoma cells were cultured DMEM F12 medium supplemented with 10% FBS and 1% antibiotics. Two samples were prepared namely, [**3**·MWCNT$_{c(RGDfK)}$] and **3**, both of which are adjusted to 33.0 μM [Pt] concentrations determined from ICP-OES. Ishikawa cells were seeded in 30 mm cell culture dishes at a density of 3.5×10^6 cells per dish and the dishes were incubated at 37°C with 5% CO_2 for 48 h prior to treatment with the samples. Cells were exposed to [**3**·MWCNT$_{c(RGDfK)}$] or **3** in serum-free media for 3 h and replaced with fresh complete media for a further 3 h incubation. Control plates were also carried out during which cells were exposed to serum-free media without addition of the test compounds. After 6 h of incubation, the dishes were placed on ice and cell monolayer was washed with ice-cold PBS (2 x 5 mL). PBS (1 mL) was added to each dish, and cells were collected with a scraper and counted. The cells were pelleted by centrifugation for 15 min at 4°C. Concentrated HNO_3 (65% v/v, 150 μL) was added to the cells in an uncapped sample vial and heated at 90°C overnight to digest the cells. The digested solution was diluted with MilliQ water (3 mL) prior to ICP-MS analysis.

Cell culture experiment for confocal studies. Ishikawa cells were cultured in MEM medium supplemented with 2mM glutamine, 1% non-essential amino acids (NEAA) and 5% FBS at 37°C with 5% CO_2. Cells were seeded on cover slip in 6-well plates at a density of 12 x 10^4 cells per mL and incubated 12 h before treatment. During treatment, the medium was replaced with incubation media containing either blank MWCNT$_{c(RGDfK)}$, (b) Doxo·HCl, (c) **3**, (d) [**3**·MWCNT$_{c(RGDfK)}$] (3 μM [Pt]) in MEM at 37°C with 5% CO_2 for 6 h, as well as untreated controls. After 6 h incubation, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, stained with Hoechst 33342 nuclear stain (1.3 μg/mL) and then washed twice with PBS.

(a)



(b)

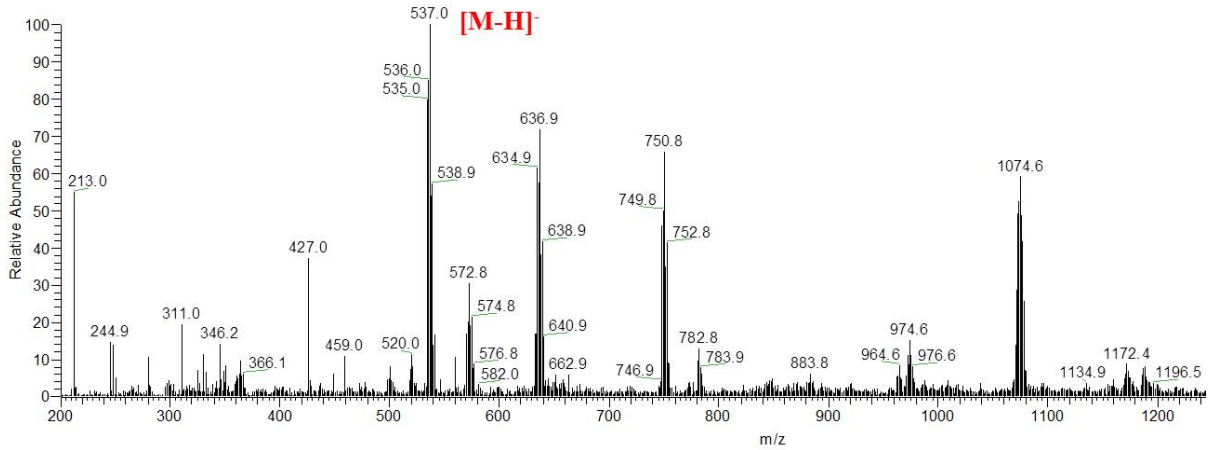
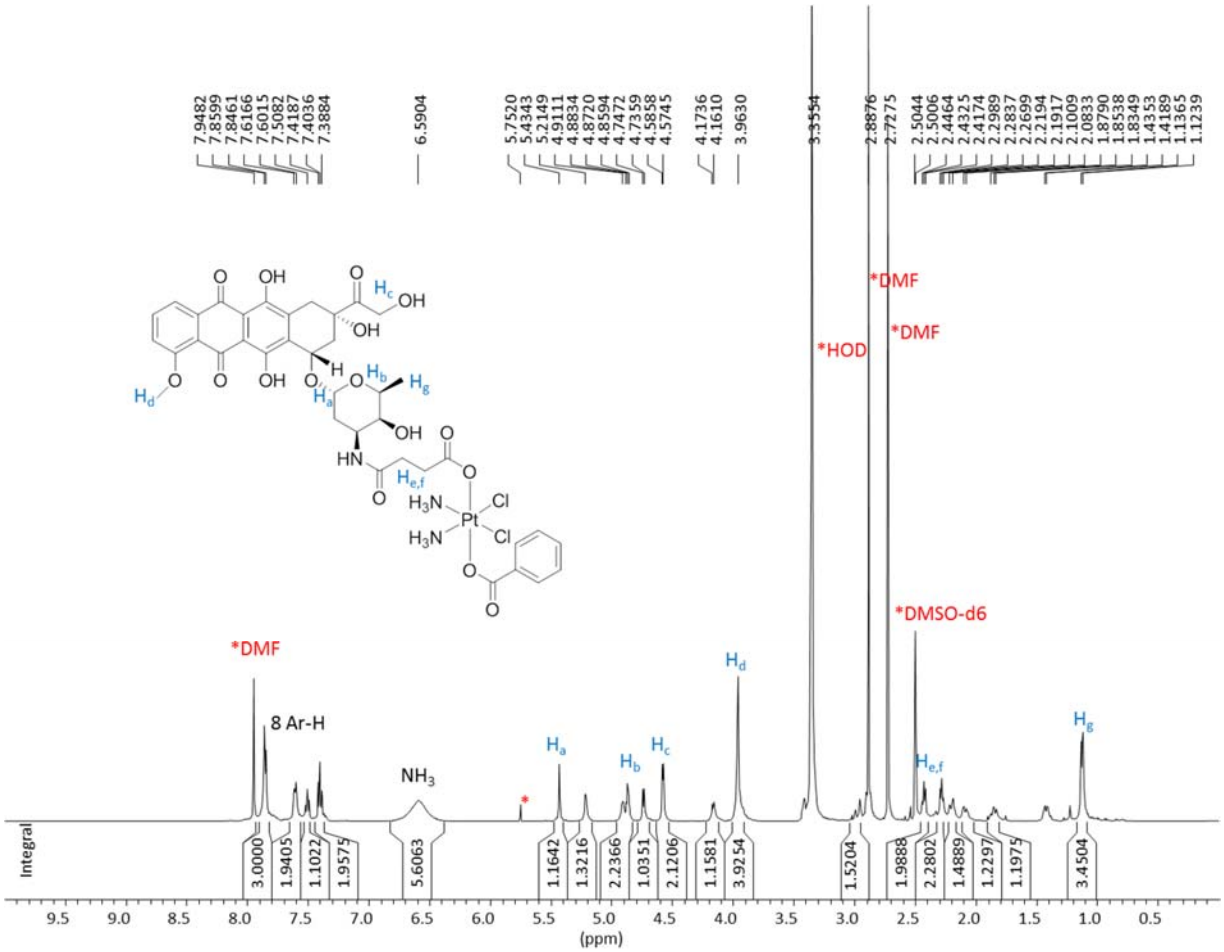


Figure S1. (a) ^{1}H NMR in DMSO-d$_6$; (b) ESI-MS spectrum (-ve mode) of **2**.

(a)



(b)

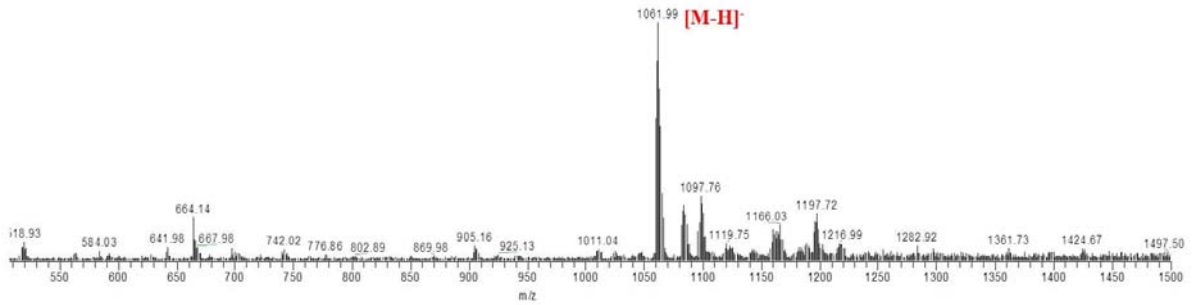


Figure S2. (a) ^{1}H NMR in DMSO-d_6; (b) ESI-MS spectrum (-ve mode) of **3**.

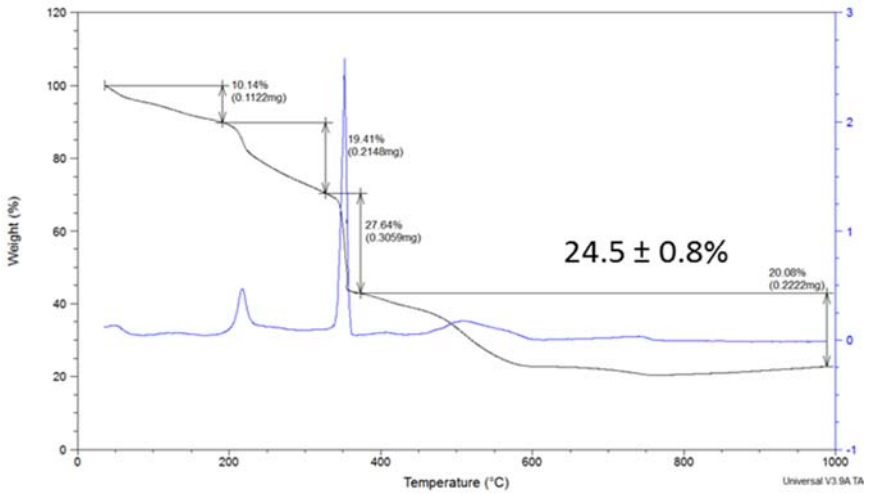


Figure S3. TGA graph of [**3**·MWCNT$_{c(RGDfk)}$] using water as solvent. TGA graph is labeled with the wt% of metal residue after ramping the sample up to 1000 °C at a rate of 10°C/min under nitrogen.

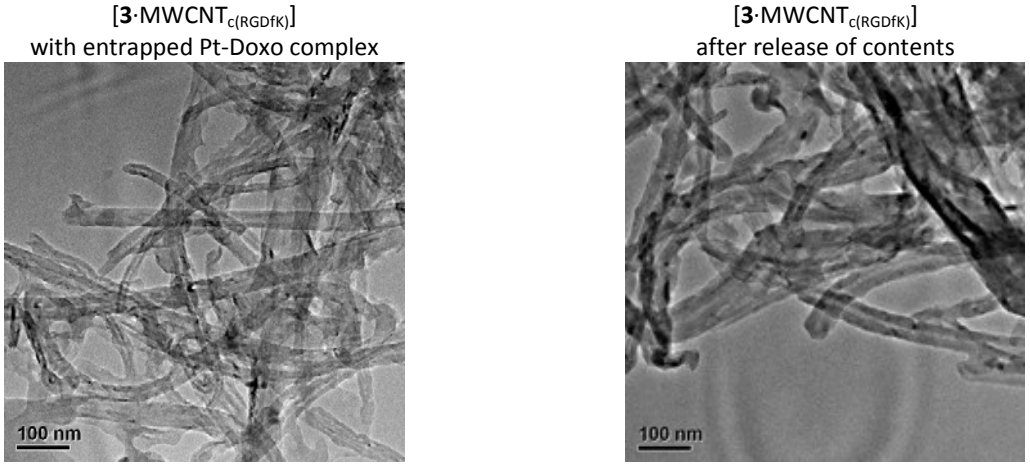
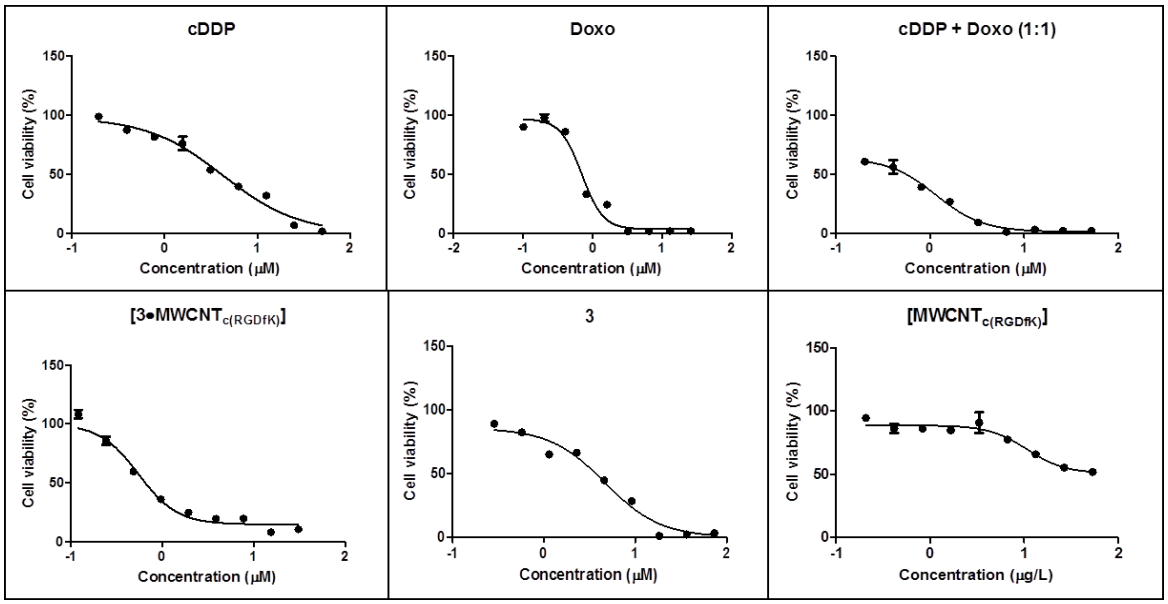
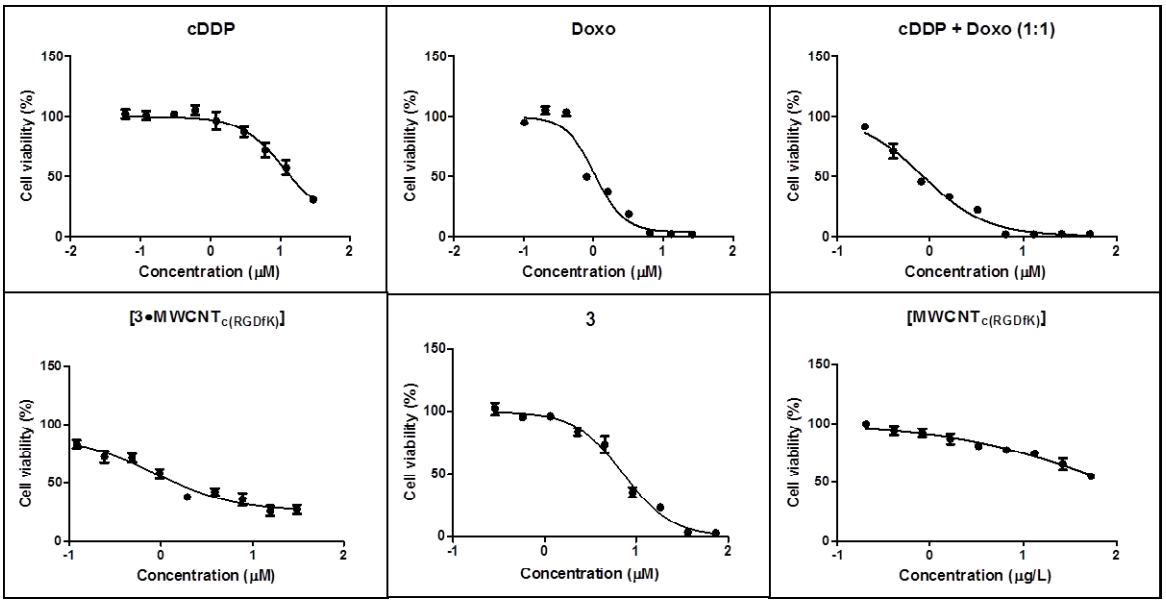


Figure S4. TEM images of [**3**·MWCNT$_{TEG}$] prior to (left) and after (right) release of contents via treatment with 3 mM ascorbic acid after 4 d.

(a) A2780 ovarian carcinoma



(b) A2780/Cis ovarian carcinoma



(c) Ishikawa endometrial adenocarcinoma

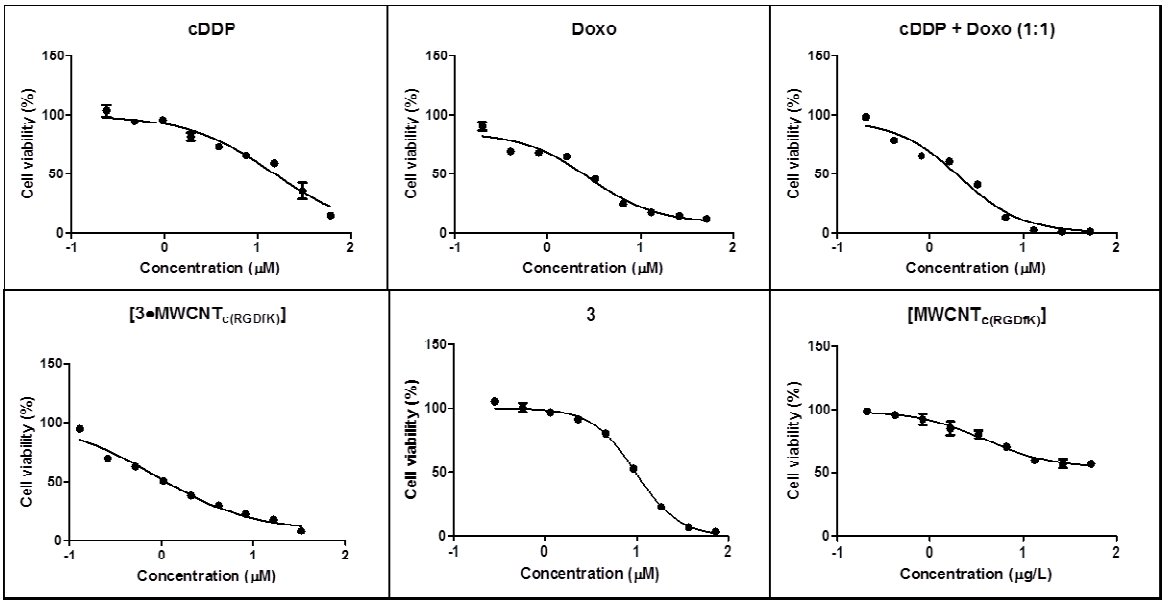


Figure S5. Dose-dependent efficacy studies for cDDP, Doxo, cDDP+Doxo, **3**, [$MWCNT_{c(RGDfK}$] and [**3**·$MWCNT_{c(RGDfK)}$] on (a) A2780, (b) A2780/Cis cells and, (c) Ishikawa cells.

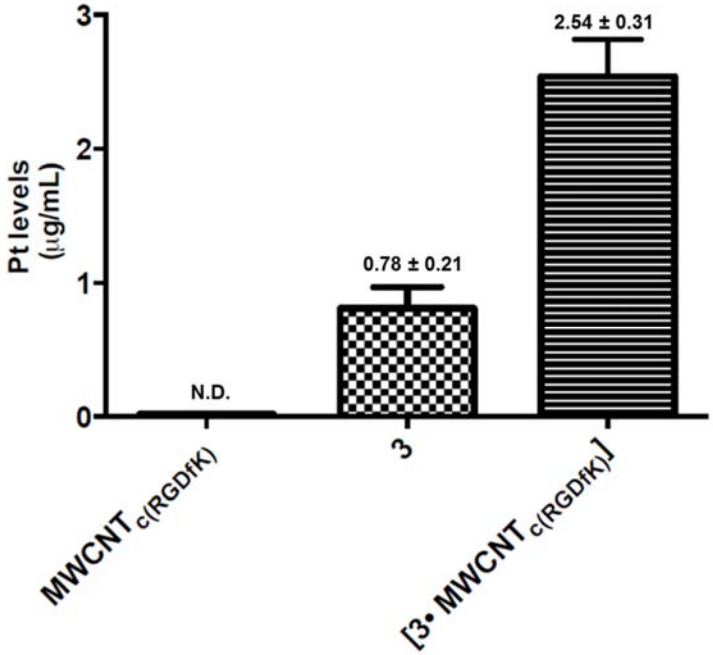


Figure S6. Pt content of Ishikawa cell extracts after treatment with $MWCNT_{c(RGDfK)}$, **3** and [**3**·$MWCNT_{c(RGDfK)}$].

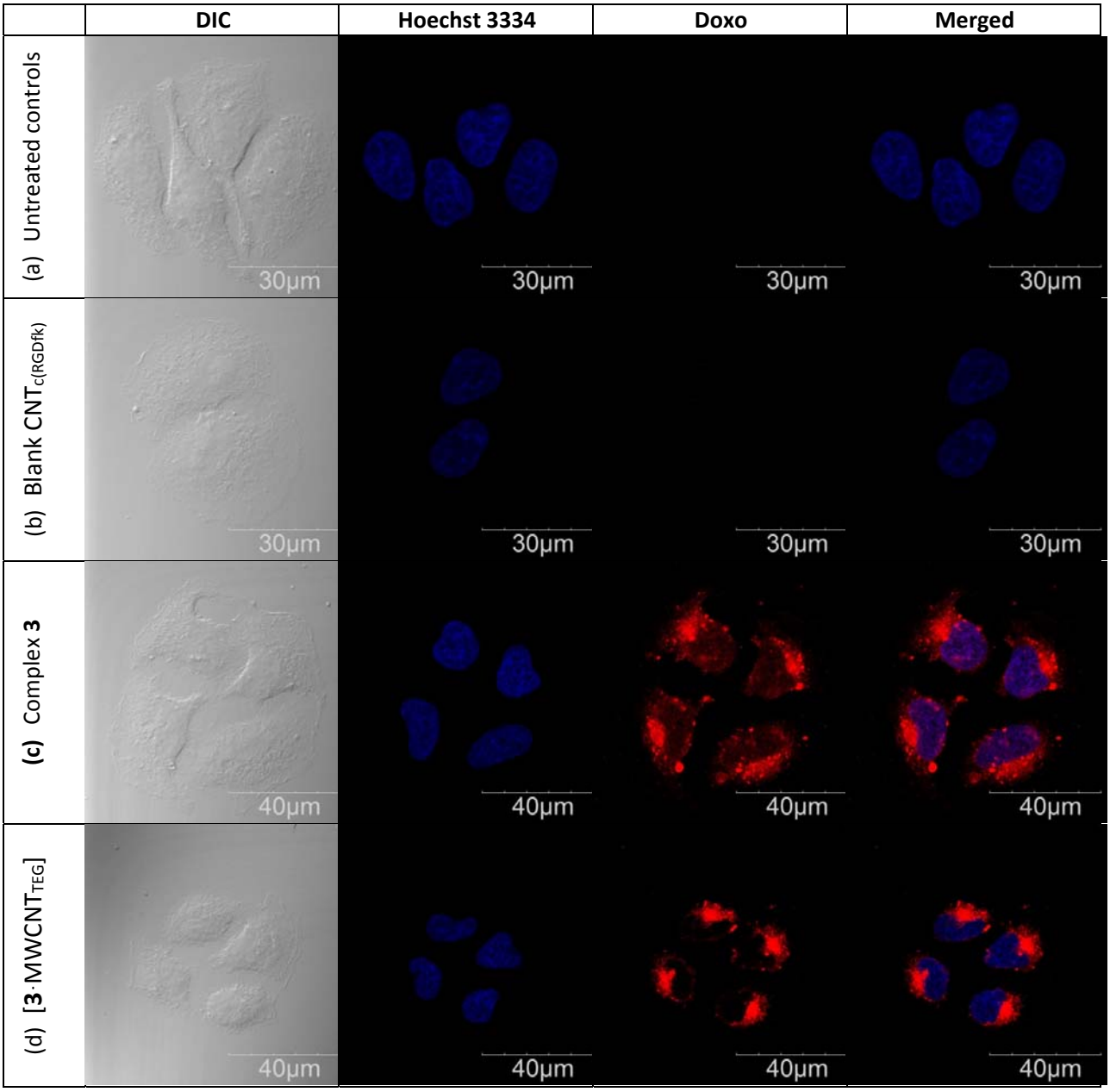


Figure S7. Fluorescence image of Ishikawa cells (a) untreated (control), and exposed to (b) blank $MWCNT_{c(RGDfk)}$, (c) **3** and, (d) [**3**·$MWCNT_{TEG}$] (adjusted to 3 µM [Pt]) for 6 h at 37°C, fixed with 4% paraformaldehyde and stained with and Hoechst 33342 (1.3 µg/mL).

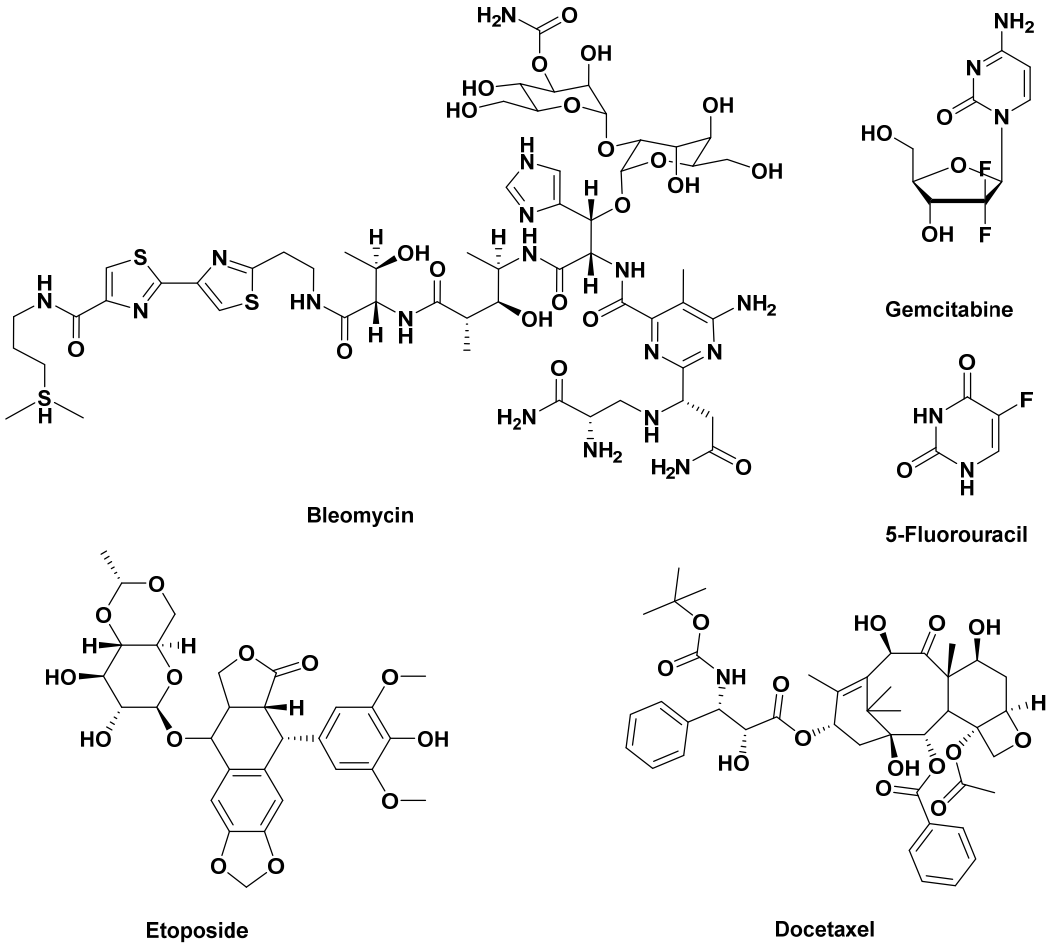


Chart S1. Molecular structures of anticancer agents used in combination with cDDP.

References

1. C. F. Chin, Q. Tian, M. I. Setyawati, W. Fang, E. S. Q. Tan, D. T. Leong and W. H. Ang, *J. Med. Chem.* **2012**, *55*, 7571-7582.
2. S. Dhara, *Indian J. Chem.* **1970**, *8*, 193-194.
3. M. Galanski and B. K. Keppler, *Inorg. Chem.* **1996**, *35*, 1709-1711.
4. T. R. Nayak, P. C. Leow, P.-L. R. Ee, T. Arockiadoss, S. Ramaprabhu and G. Pastorin, *Curr. Nanosci.* **2010**, *6*, 141-154.
5. V. K. Sarin, S. B. H. Kent, J. P. Tam and R. B. Merrifield, *Anal. Biochem.* **1981**, *117*, 147-157.
6. J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Minna and J. B. Mitchell, *Cancer Res.* **1987**, *47*, 936-942.