Targeted Killing of Cancer *In Vivo* and *In Vitro* with EGF-directed Carbon Nanotube-based Drug Delivery

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Detailed Methods

Functionalization of SWNTs

HiPCO single-walled carbon nanotubes (SWNTs) were obtained from Carbon Nanotechnologies, Inc. (Texas, USA). These nanotubes were first oxidized by sonication in 3:1 HNO_3–H_2SO_4 for 4 h at 70 ºC, then filtered, washed with water, and dried. Covalent bioconjugation of protein ligand EGF to these SWNTs was achieved by EDC-promoted amidization to link amine groups of the protein to carboxylates of the nanotubes. Briefly, SWNTs (0.5 mg/ml) were uniformly dispersed in PBS by sonication for 30-45 min. Next, equal volumes (100 µl) of EDC and EGF solutions, both at 2 mg/ml, were added to 100 µl of SWNT dispersion, followed by incubation at 37°C for 1 hr on a shaker. Excess or nonspecifically bound EGF was removed by centrifugation of the samples in Centricon YM-50 tubes (Millipore Corporation, MA, USA) at 3200 rpm for 5 min and the bioconjugate was resuspended in 1 ml of PBS. In every case, control samples (without EDC) were prepared concurrently and, both sets of samples underwent washes (2x for 5 min) with 0.05% Tween-20 in PBS to remove unconjugated proteins. In parallel, a similar rational as described above was used to conjugate carboxyl-fluorescein (100 µl at 3 mg/ml) to EGF (100 µl at 2 mg/ml) using EDC (100 µl). After incubation, excess label was removed in Centricon YM-3 tubes at 3200 rpm for 15 min. The fluorescein-labeled EGF complexes were bioconjugated to SWNTs following the procedure described above. For further characterization, 100 µl (2 mg/ml in PBS) of anti-human EGF antibody (Anogen/Yes Biotech Lab, ON, Canada; clone S-134), was similarly labeled with carboxyl-fluorescein, and the resulting complex was reacted with SWNT-EGF and control samples, followed by removal of unbound antibody (Centricon YM50 tubes at 3200 rpm for 5 min).

Cell lines and culture conditions

Culture conditions of the HNSCC HN13 and HN12 cell lines used is described in detail elsewhere.[1] Briefly cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37°C in 95% air/5% CO2. SAA and control NIH3T3 mouse fibroblast cell lines, a gift from Dr. Stan Lipkowitz (National Cancer Institute) were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and cultured as described above. Freshly plated cells were grown overnight, to 40-50% confluency prior to incubation for 10 min with 100 µL DMEM containing SCE/SQE complex and after 3 washes with PBS, the treated cells were incubated further in fresh DMEM containing serum and carefully monitored (1-24 h) microscopically (Axiovert 200m; Carl Zeiss, NY, USA) for any adverse morphological changes as well as for analysis as detailed below.

Pre-designed siRNA oligonucleotides were utilized to target EGFR for silencing in HN13 cell. EGFR siRNA (Qiagen, CA, USA; catalogue# S100074053; sense: r(CGU CGC UAU CAA GGA AUU A)dTdT ; antisense: r(UAA UUC CUU GAU AGC GAC G)dGdG) together with control siRNA (Qiagen; catalogue# 1022076) were used to transfect (10 nM) into exponentially growing

cells using HiPerFect Transfection reagent (Qiagen), which were than incubated for an additional 48 h prior to use for experiments as detailed below.

Animal studies

All animal studies were carried out according to NIH-approved protocols, in compliance with the Guide for the Care and Use of Laboratory Animals. Female athymic (*nu/nu*) nude mice (Harlan Sprague-Dawley, Indianapolis, IN, USA), 4-6 weeks old and weighing 18 to 20 g were used in the study and housed in appropriate sterile filter-capped cages, and fed and given water ad libitum. HN12 cells maintained as described (see above) were transplanted subcutaneously into the flanks of mice to induce HNSCC tumor xenografts as previously described.[2]

Western Blot

For protein expression analysis by western blot, cells were washed 3X in ice-cold PBS and lysed in NP40-lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 20 mM EDTA; 0.5% Nonidet P-40; 1 mM PMSF; 5 μg/ml aprotinin; 5 μg/ml leupeptin) on ice for 10 min. Cleared lysates were quantified spectrophotometrically (BCA, Bio-Rad, Hercules, CA) and 50 μg resolved by SDS-polyacrylamide gel electrophoresis followed by transfer to PVDF membrane (Immobilon-P, Millipore), and blocking in 5% skimmed milk in TTBS (10mM Tris-HCl pH 7.6, 0.5% Tween 20, 150mM NaCl) for 1 h at RT. Blots were than incubated overnight at 4°C with primary antibodies (against EGFR and β-Tubulin; BD Biosciences, CA, USA), diluted 1:1000 in blocking buffer washed 3x in TTBS, incubated with horseradish peroxidase-conjugated secondary antibodies, and detected by SuperSignal West Pico (Pierce, IL, USA).

TUNEL assay

The APO-BrdU TUNEL (Terminal transferase dUTP nick end labeling) assay kit (Invitrogen) was used to detect cells undergoing apoptosis. Briefly, cells were grown onto 24 mm diameter coverslips until 50-60% confluent followed by treatment with SCE and control complexes as described above. After treatment, the cells were washed in PBS (x3 for 2 min each) and incubated in fresh media for 1 hr. Next, cells were fixed in 3.5 % PBS-formaldehyde for 15 min at RT, rinsed in PBS (x3) and permeabilized in 0.5 % PBS-Tween 20 for 5 min at RT followed by DNA end-labeling for 1 h at 37 °C. After, the labeled cells were washed, and incubated further with 95 μL antibody staining solution (AlexaFluor 488-conjugated anti-BrdU) for 30 min at 37 °C, followed by additional washes (x3) and mounting of the coverslips onto SuperFrost microslides with Vectashield mounting medium (Vector Lab Inc, Burlingame, CA). Fluorescence image acquisitions were performed with Axio plan 2 (Carl Zeiss).

TEM imaging of SWNTs for the presence of Qdots and Cisplatin

A specimen of SWNTs for TEM imaging was first prepared by dispersing nanotubes in dimethylformamide:methanol (1:10 v/v). A 3 μL droplet from the resulting solution was then deposited onto a Quantifoil grid and left to dry in air. A specimen of SWNT-Qdot-EGF bioconjugate was prepared by first dispersing nanotubes in PBS and depositing a 3 μL droplet from this solution onto a Quantifoil grid. After adsorption for 3 min, the excess solution was blotted with filter paper, washed with a few 3-μL droplets of de-ionized water in order to remove buffer salts, and left to dry. Images were recorded in a Tecnai TF30 TEM (FEI, Hillsboro, OR, USA) equipped with a Gatan Ultrascan 1000 CCD camera (Gatan, Pleasaton, CA, USA).

For STEM analysis of SWNTs for presence of cisplatin, samples were first dispersed in dimethylformamide:methanol (1:10 v/v), and a 3 μl droplet was deposited onto a 3 nm thick carbon support film, and allowed to air dry. SWNT-Cisplatin-EGF was dispersed in PBS and 3 μl was placed onto a 3 nm-thick carbon support film to adsorb for 3 min after which the droplet was blotted and the carbon film washed with distilled water in order to remove buffer salts. STEM imaging of SWNTs was performed with a FEI TF30 Tecnai electron microscope operating at 300 kV SWNT-Cisplatin bioconjugates were characterized using Z-contrast dark-field STEM imaging. We use Multislice simulations to numerically generate images of single Pt atoms situated on carbon substrates up to 10 nm in thickness. X-ray microanalysis for the presence of Pt on SWNTs was performed using a VG HB501 dedicated scanning transmission electron microscope operating at 100 kV and equipped with an Oxford energy-dispersive X-ray microanalysis system.

***In vitro* and *In vivo* transmission electron microscope imaging of SWNT bioconjugates**

HN13 and siRNA transfected cells were grown 50-60% confluent on thermonax coverslips, after which they were treated with nanotube bioconjugates for 10 min, followed by washes in PBS and additional incubation in fresh media for 1 h. Next, cells were fixed in a mixture of 2.5% paraformaldehyde and 2.0% glutaraldehyde in PBS for 30 min followed by extensive washes in PBS. Then the cells were rinsed in 0.1 M sodium cacodylate buffer (SB; pH =7.4) and postfixed in 1.0% osmium tetroxide in the above buffer for 30 min. After several rinses in the SB, the samples were dehydrated in a series of ethanol solutions (20%, 40%, 60%, 75%, 95% for 5 min and 100% for 30 min with 3 changes) and infiltrated with Epon-Aradite (Ted Pella, Redding, CA, USA) for 2 days (25% Epon-Aradite and ethanol for 2 h, 50% for 4 h, 75% for overnight and 100% for 1 day with 2 changes). Samples were polymerized at 60°C for 2 days. Next, ultrathin sections (~80 nm) were cut on a Reichert Ultracut E Microtome (American Optical, Buffalo, NY, USA) and collected on film supported slot grids. Sections were lightly counter-stained with uranyl acetate and lead citrate, and examined under a FEI CM120 transmission electron microscope (TEM; equipped with a Gatan GIF100 image filter) operating at a beam energy of 120 keV.

Athymic nude mice bearing HN12 xenografts treated with SWNT-Cisplatin-EGF or control bioconjugates and monitored for 2 weeks were euthanized with CO_2 and tissue sections were carefully dissected (3 mm sections) of various vital organs along with the tumor . Next, cells were fixed in a mixture of 2.5% paraformaldehyde and 2.0% glutaraldehyde in PBS for 30 min followed by extensive washes in PBS. Then the cells were rinsed in 0.1 M sodium cacodylate buffer (SB; pH =7.4) and postfixed in 1.0% osmium tetroxide in the above buffer for 30 min. After several rinses in the SB, the samples were dehydrated in a series of ethanol solutions (20%, 40%, 60%, 75%, 95% for 5 min and 100% for 30 min with 3 changes) and infiltrated with Epon-Aradite (Ted Pella, Redding, CA) for 2 days (25% Epon-Aradite and ethanol for 2h, 50% for 4h, 75% for overnight and 100% for 1 day with 2 changes). Samples were polymerized at 60 ^{o}C for 2 days. Next, ultrathin sections (~80 nm) were cut on a Reichert Ultracut E Microtome (American Optical, Buffalo, NY) and collected on film supported slot grids. Sections were slightly counter-stained with uranyl acetate and lead citrate, and examined under a FEI CM120 transmission electron microscope (TEM; equipped with a Gatan GIF100 image filter) operating at a beam energy of 120keV.

Bright field Optical microscopy

HNSCC cells cultured as mentioned above were treated with SWNT-Cisplatin-EGF bioconjugates (0.6 mg in 500 μl PBS) and were observed under bright field fluorescence imaging microscope for

any morphological changes (Axiovert 200M from Carl Zeiss Microimaging Inc). Images were acquired at 20x and 40x zoom lens.

Raman spectroscopy

Raman characterization for detecting nanotubes in mice tumor tissues using characteristic SWNTs Raman signatures. Five micron cryosections of the frozen xenografts retrieved from mice that had been injected intravenously with SWNT-Cisplatin-EGF and SWNT-Cisplatin bioconjugates were prepared, fixed (in 90% ethanol for ~30 sec) mounted and analyzed directly under Renishaw raman instrument.

Supplementary Figures

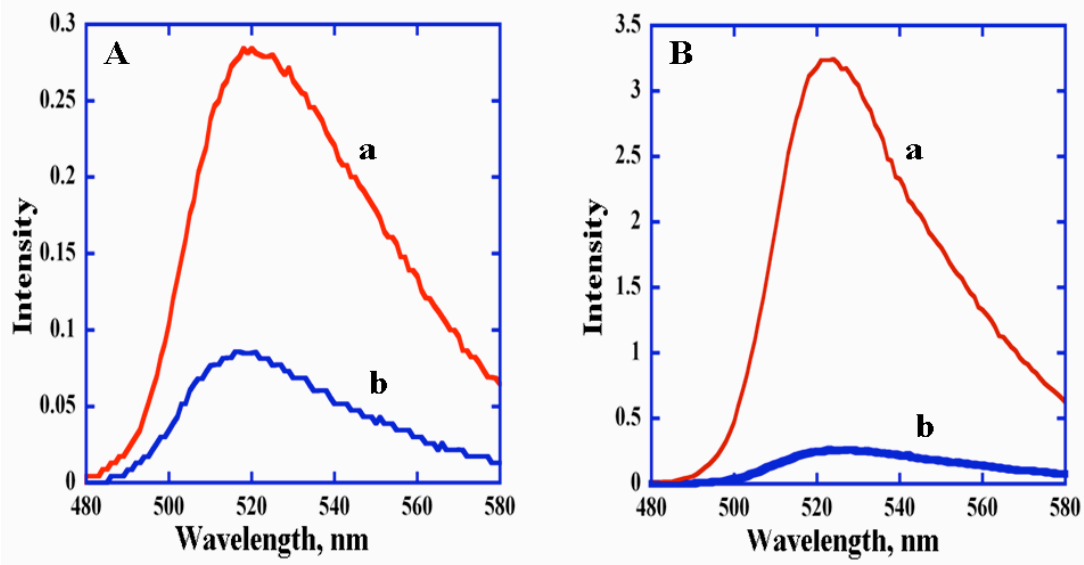


Figure S1. Detection of EGF bound to SWNTs using fluorescent labels with emission at ~520 nm. (A) Emission spectra using fluorescein-EGF: (a) SWNTs derivatized with fluorescein-EGF using EDC protocol; (b) SWNTs treated with fluorescein-EGF with no EDC. (B) Emission spectra using a fluorescein labeled antibody to EGF: (a) SWNTs derivatized with EGF using EDC protocol, then treated with fluorescein-antiEGF; (b) SWNTs derivatized with EGF with no EDC, then treated with fluorescein-antiEGF. All samples were washed exhaustively with 0.05% Tween 20 detergent to remove adsorbed biomaterials from the nanotubes.

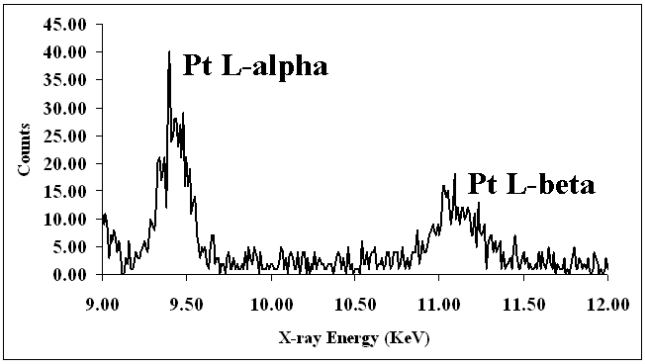


Figure S2. Energy dispersive X-ray emission spectrum collected from bundles of cisplatin-SWNTs on a thin carbon support. The spectrum shows two peaks characteristic of platinum atoms, namely the Pt L-alpha and the Pt L-beta transitions. Results confirm that cisplatin is attached to the SWNTs.

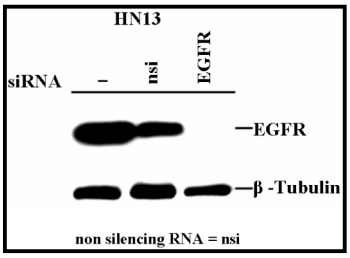


Figure S3. Western blot analysis of EGFR in the HN13 cells after knockdown by siRNAs. The blots were subsequently assessed for the presence of protein EGFR with β-tubulin serving as loading control.

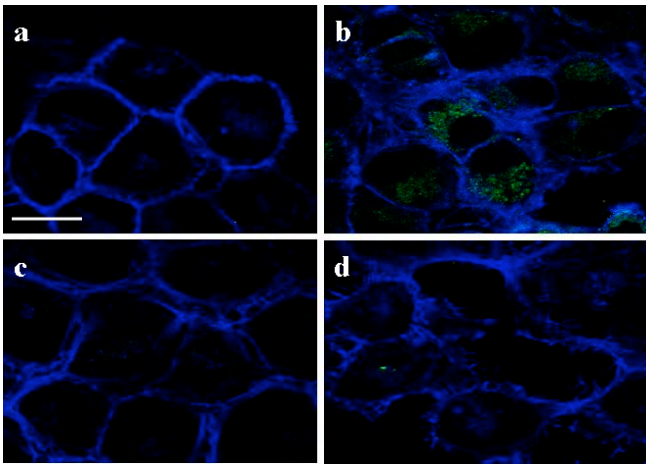
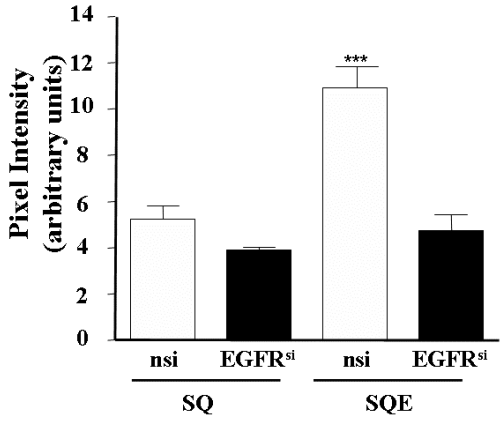


Figure S4. Confocal images of HN13 cells treated with SWNT-Qdot525-EGF (SQE) or SWNT-Qdot525 (SQ): **(a)** Marginal difference in the internalization of SQ complexes into cells treated with control siRNA that does not knockdown EGFR and **(c)** cells treated with siRNA to knockdown EGFR, no Qdots (green) within the cell membrane (blue) after treatment with SQE were detectable; **(b)** In cells treated with bioconjugates with EGF (SQE), uptake was achieved in cells treated with control siRNA as observed by the green fluorescence within the limits of the membrane; and **(d)** siRNA-treated HN13 cells with knocked down EGFR, no green fluorescence was observed after treatment with SQE. Scale bar 20 μm.

Figure S5. Pixel Intensity of fluorescence in HN13 cells. Micrographs of cells treated with siRNA (nsi and $EGFR^{si}$) followed by exposure to the bioconjugates (SWNT-Qdot: SQ; SWNT-Qdot-EGF: SQE) were analyzed for pixel intensities of the green fluorescence of the Qdots (525). Cells pretreated with non silencing siRNA exhibit the greatest internalization only when bioconjugates include the targeting ligand EGF. By contrast, when the receptor for the ligand is diminished (EGFR siRNA) a marginal difference is detected irrespective of the presence of EGF. Difference in the levels of internalization of the SWNT-Qdot-EGF bioconjugate was statistically significant at $p<0.05$ using ANOVA (***).



non silencing RNA = nsi ; EGFR siRNA = $EGFR^{si}$

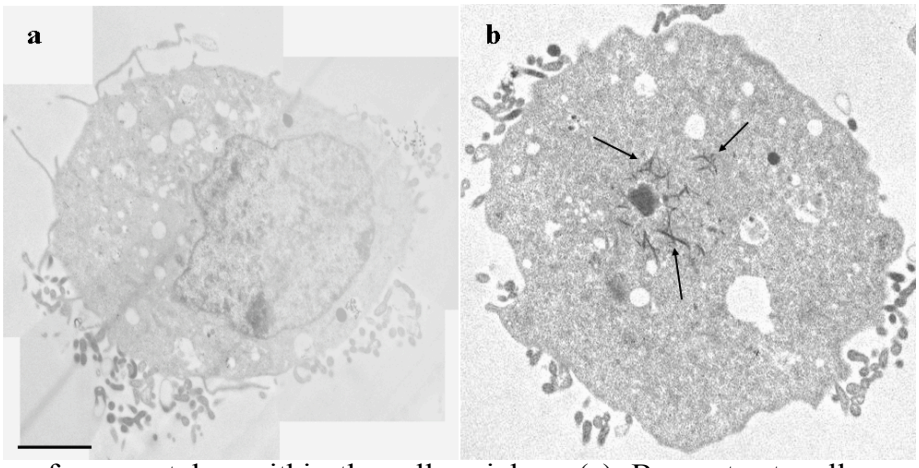


Figure S6. Transmission Electron Micrograph of HN13 cells treated with bioconjugates. Cells treated with control bioconjugates without EGF and consequently lacking the ligand-receptor interaction show very few nanotubes within the cell periphery (a). By contrast, cells exposed to bioconjugates with EGF show structures resembling internalized nanotubes (indicated by white arrows) accumulated around the perinuclear region (b). Scale is 2 μm.

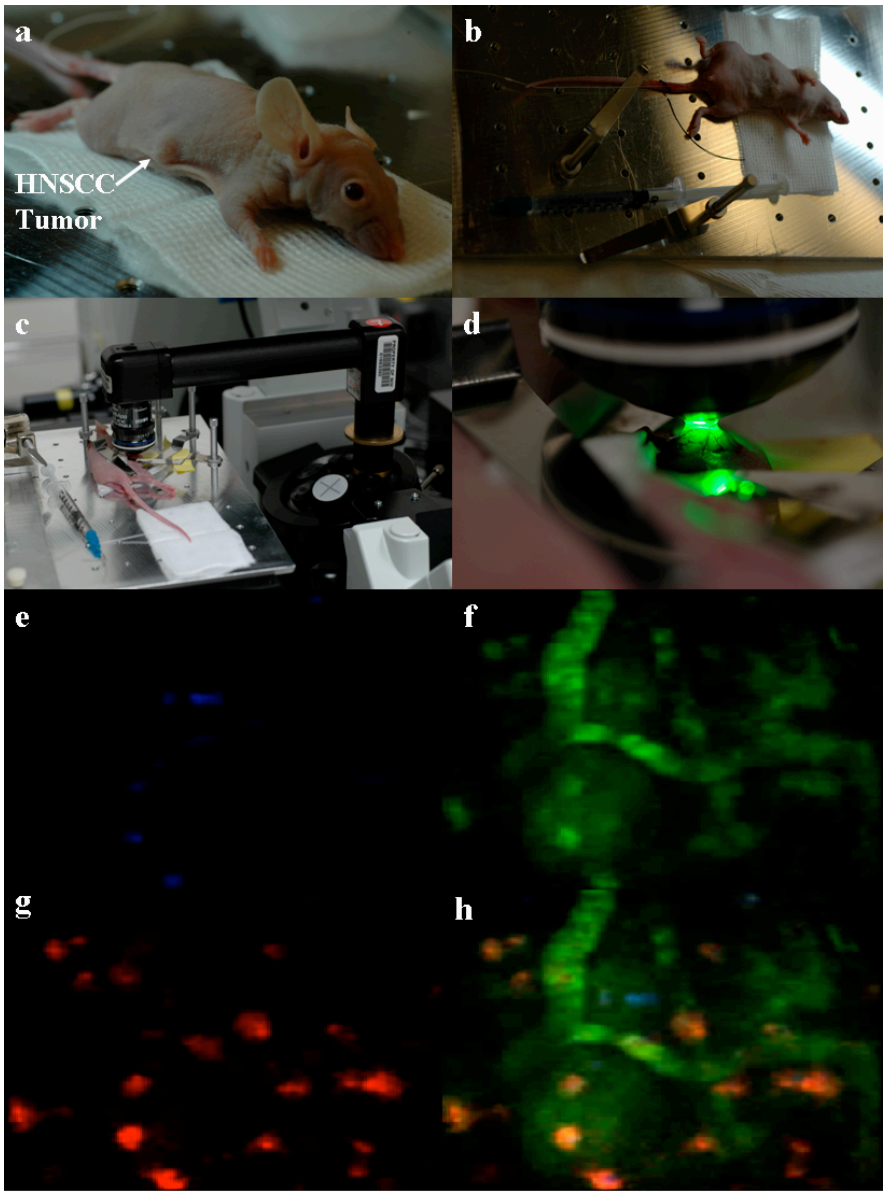


Figure S7. Visualization of bioconjugates *in vivo* using intravital two-photon microscopy. HN12 cells were used to induce xenografts in athymic mice (a). Animals were anesthetized and silicon catheters were inserted into the tail artery to allow for multiple intravenous injections (b). Mice were positioned whereby the tumor was easily accessible and directly below the lens of the two-photon microscope (c-d). Systemic injection of Höechst DNA dye was immediately followed by injection of FITC-labeled 500 kDa dextran thereby labeling the nuclei (blue) and the blood vessels (green), respectively in the animal (e-h). In the example shown, the red of SWNT-Qdot605-EGF bioconjugates (red) are observed (g) the merged image shows that the bioconjugates appear to be internalized by the tumor cells within the xenograft (h).

Figure S8. (Web Enhanced Object) Intravital two-photon microscopy. Time-lapse showing systemic injection of SWNT-Qdot605 bioconjugate. Ten minute time-lapse showing an area within the tumor mass during injection of SWNT-Qdot525 bioconjugates without EGF (red). Cell nuclei were pre-labeled with Höechst vital DNA stain (blue) and blood volume was pre-labeled 500 kDa FITC-dextran (green). Upon systemic injection rapid influx of the bioconjugate in the tumor micro-environment is observed. The polydispersed SWNT bioconjugates appears to be confined within the vasculature and is carried by the blood stream. Time is displayed in minutes and seconds.

Figure S9. (Web Enhanced Object) Intravital two-photon microscopy of SWNT-Qdot605-EGF bioconjugate distribution 45 minutes after injection. Two minute time-lapse showing an area within the tumor mass 45 minutes after injection of the SWNT-Qdot525-EGF bioconjugates. Cell nuclei were pre-labeled with Höechst vital DNA stain (blue) and blood volume was pre-labeled 500 kDa FITC-dextran (green). Most of the SWNT-Qdot525-EGF (red) bioconjugate appears to be located outside of the vasculature. The bioconjugates appear to localize in close proximity to nuclei of cells within the tumor mass suggesting its internalization by the tumor cells. Time is displayed in minutes and seconds.

Figure S10. (Web Enhanced Object) Intravital two-photon microscopy of internalized SWNT-Qdot605-EGF bioconjugate. Eighteen minute time-lapse showing an area within the tumor mass 50 minutes after injection of the SWNT-Qdot525-EGF bioconjugates. Cell nuclei were pre-labeled with systemic injection of Höechst vital DNA stain (blue). A group of cells dynamically trafficking the internalized bioconjugates can be seen. Notably, a large number of cells, possibly macrophages, can be observed rapidly moving through the tumor micro-environment. Time is displayed in minutes and seconds.

Figure S11. (Web Enhanced Object) Intravital two-photon microscopy of SWNT-Qdot605 control bioconjugate distribution 45 minutes after injection. Twelve minute time-lapse showing an area within the tumor mass 45 minutes after injection of the SWNT-Qdot605 bioconjugate. Cell nuclei were pre-labeled with Höechst vital DNA stain (blue) and blood volume was pre-labeled 500 kDa FITC-dextran (green). Unlike FITC-dextran, no SWNT-Qdot525 (red) bioconjugate could be detected traveling within the vasculature. Very little of bioconjugate lacking the EGF targeting ligand was observed within tumor micro-environment in close proximity to the blood vessels. Notably, a large number of cells, possibly macrophages, can be observed rapidly moving within the tumor mass. Time is displayed in minutes and seconds.

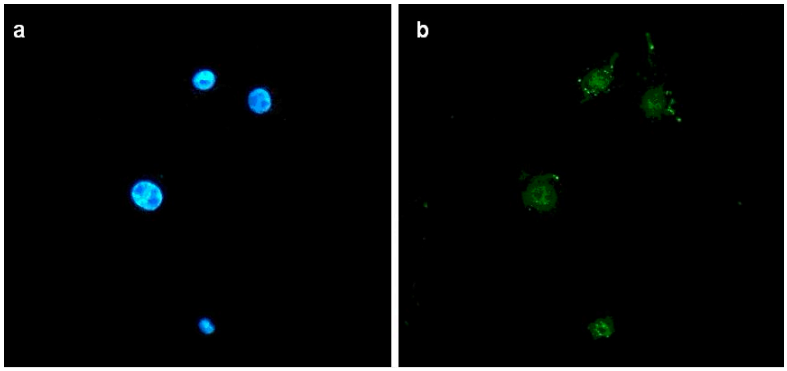


Figure S12. TUNEL assay results showing apoptosis of HN13 cells after 12 hr incubation with SWNT-EGF-cisplatin using (kit A-23210, Molecular Probes): (left) Nuclear DAPI (blue) staining, which is also seen for untreated cells; (right) FITC staining of cells showing green fluorescence from dye attached to fragmented DNA formed due to apoptosis.

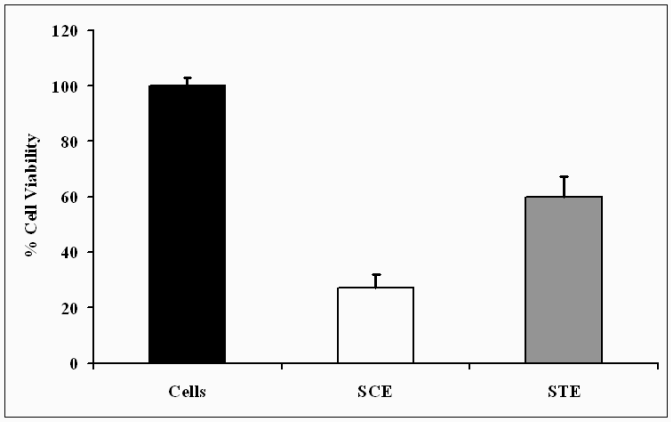


Figure S13. Cell proliferation assay results on HN13 cells with the less active anticancer drug transplatin using the MMT assay discussed in the main text. SCE = SWNT-Cisplatin-EGF;STE = SWNT-Transplatin-EGF; Cells = control, HN13 cells only. Concentrations of drugs in the dispersions were 1.3 μM.

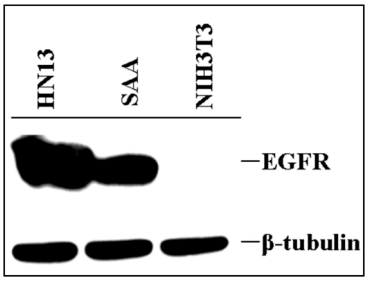


Figure S14. Western blot assay of EGFR in cell lines. HN13 cells are compared against SAA and NIH3T3 mouse fibroblasts, expressing high EGFR and marginal levels, respectively. The blots were subsequently assessed for the presence of protein EGFR with β-tubulin serving as loading control.

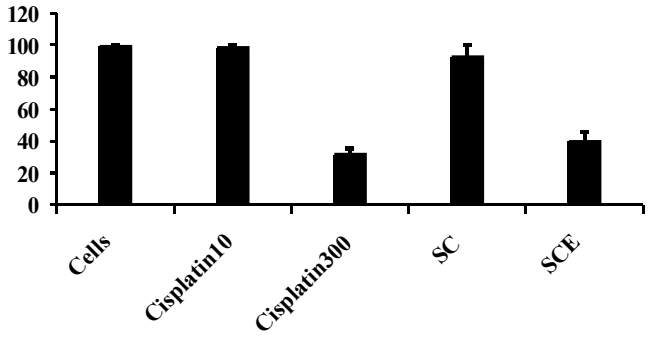


Figure S15. Cell viability assay with normal HN12 cells **Cells**–normal growth untreated. **Cisplatin10** – cells treated with 10 µM free cisplatin. **Cisplatin300** – incubated with 300 µM free cisplatin. **SC** - incubated with SWNT-Cisplatin and washed with PBS after 10 min. **SCE**– incubated with SWNT-Cisplatin-EGF (1.3 µM cisplatin) then washed with PBS after 10 min.

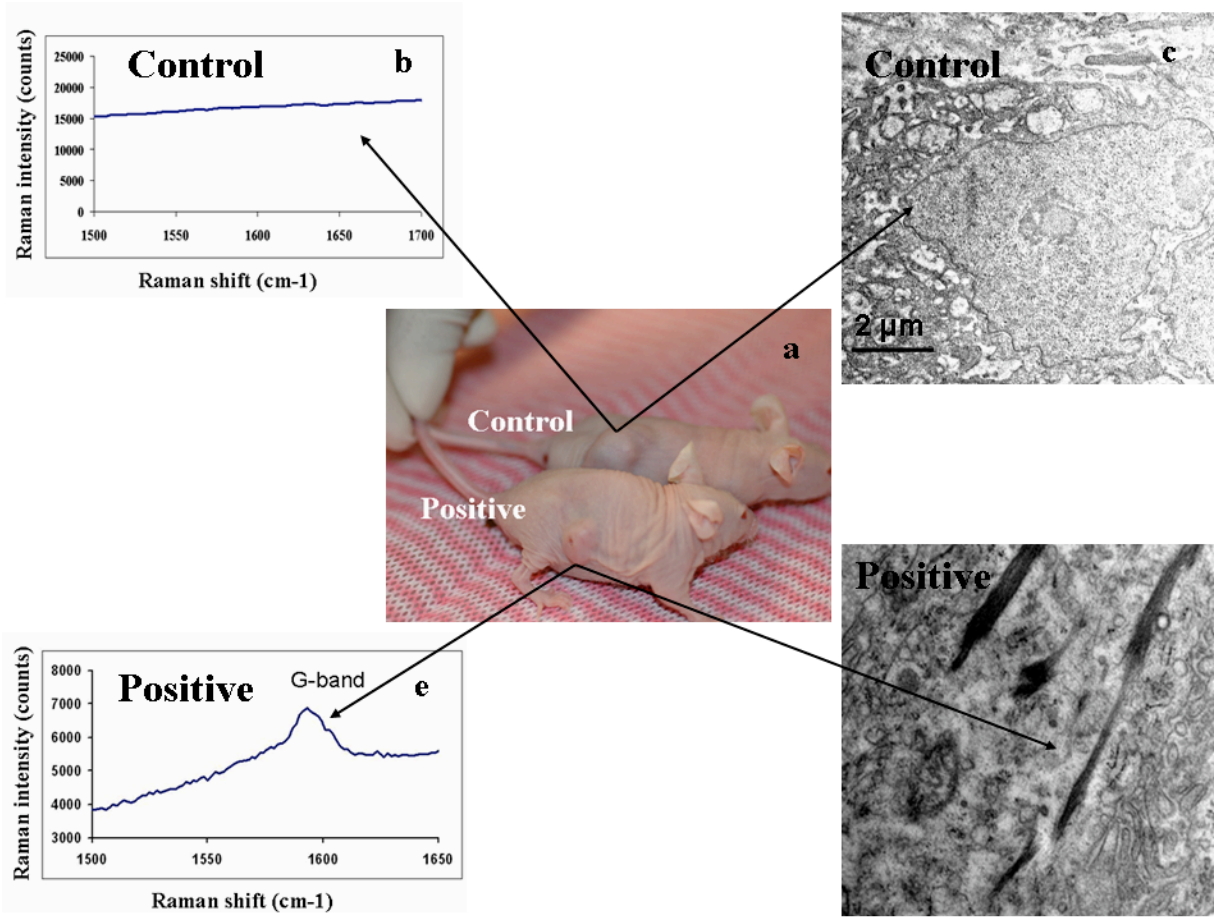


Figure S16. Athymic nude mice treated with SWNT bioconjugates. a. Picture of mice bearing HN12 tumor treated with positive (SWNT-Cispaltin-EGF) and control (SWNT-Cisplatin) just before sacrificing. **(b & c)** Raman and TEM micrographs of control mouse without any peak and no structures resembling nanotubes respectively. **(d & e)** Raman and TEM micrographs of positive mouse showing G-band signature peak for SWNT and structures resembling nanotubes clearly indicating the presence of SWNTs only in the positive mouse which shows the specificity of the ligand mediated targeting. Scale bar = 2 µm

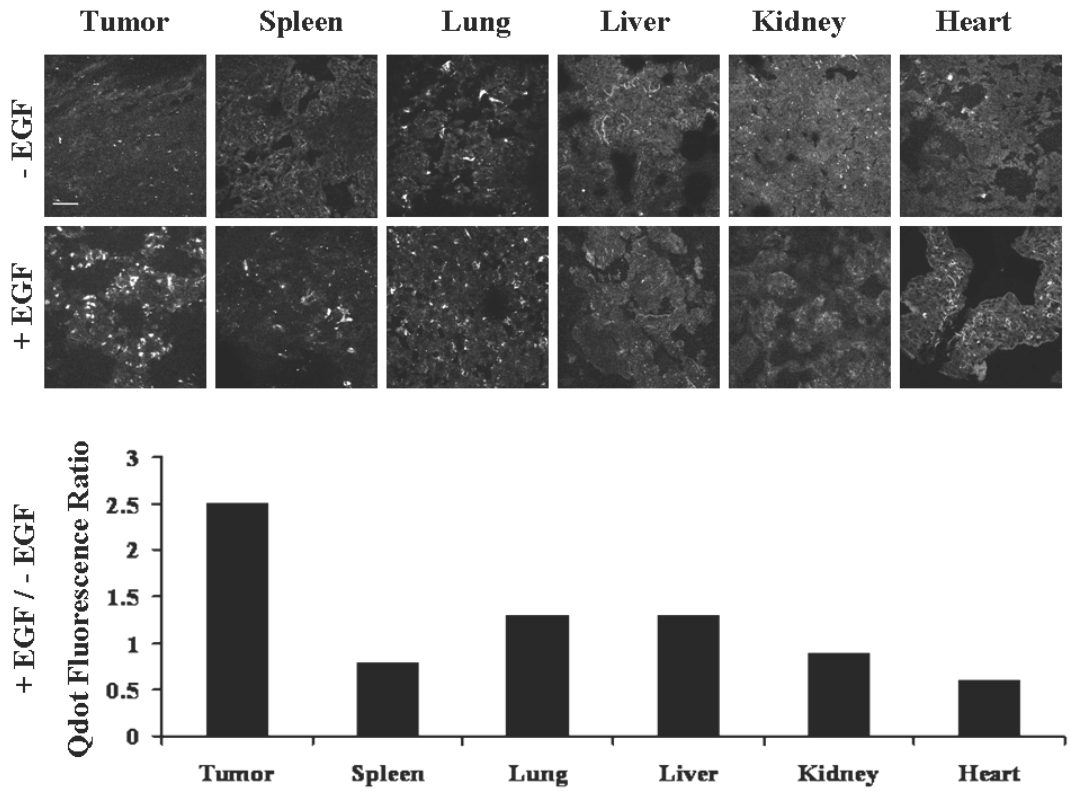


Figure S17. Distribution of SWNT bioconjugates *in vivo*. Tissues removed from tumor bearing mice, which had injections of Höechst stain, FITC-labeled dextran and Qdot labeled nanotube bioconjugates were freshly frozen, and subsequent cryosections were fixed and processed for confocal microscopy. The resulting micrographs were used to analyze the pixels for quantitative intensities of the signal of the Qdot605. Values given are for bioconjugates as indicated (positive: SWNT-Qdot605-EGF; negative: SWNT-Qdot605).

References

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