DESIGN AND CELLULAR STUDIES OF A CARBON NANOTUBE-BASED DELIVERY SYSTEM FOR A HYBRID PLATINUM-ACRIDINE ANTICANCER AGENT

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ABSTRACT

A three-component drug-delivery system has been developed consisting of multi-walled carbon nanotubes (MWCNTs) coated with a non-classical platinum chemotherapeutic agent ($[\text{PtCl}(\text{NH}_3)_2(\text{L})]\text{Cl}$ (**P3A1**; $\text{L} = N$ -(2-(acridin-9-ylamino)ethyl)- N -methylpropionimidamide) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine- N -[amino(polyethylene glycol)-5000] (DSPE-mPEG). The optimized **P3A1**-MWCNTs are colloidally stable in physiological solution and deliver more **P3A1** into breast cancer cells than treatment with the free drug. Furthermore, **P3A1**-MWCNTs are cytotoxic to several cell models of breast cancer and induce S-phase cell cycle arrest and non-apoptotic cell death in breast cancer cells. By contrast, free **P3A1** induces apoptosis and allows progression to G2/M phase. Photothermal activation of **P3A1**-MWCNTs to generate mild hyperthermia potentiates their cytotoxicity. These findings suggest that delivery of **P3A1** to cancer cells using MWCNTs as a drug carrier may be beneficial for combination cancer chemotherapy and photothermal therapy.

KEY WORDS: nanoparticle; chemotherapy; triple negative breast cancer; cytotoxicity; photothermal therapy; drug delivery

INTRODUCTION

Platinum-based chemotherapeutic drugs such as cisplatin are among the most effective agents available to clinicians for treatment of testicular and ovarian cancer, but have limited ability to prolong survival of breast, colorectal, prostate, or lung cancer patients.[1] This is in large part due to the development of drug resistance and significant toxic side effects, which limit the ability to give patients the drugs at sufficient doses.[2] Second- and third-generation platinum chemotherapeutics such as carboplatin and oxaliplatin show reduced toxic side effects, but intrinsic resistance in many types of cancer and acquired cross-resistance to therapy still limit their efficacy.[1] Resistance to these platinum agents occurs through tolerance of drug-induced DNA damage, insufficient DNA binding, poor drug uptake and increased activity of cellular detoxification pathways.[2] Cisplatin, carboplatin, and oxaliplatin all induce the same type of DNA damage and suffer from cross-resistance.[1, 2]

Recently developed platinum–acridine (PA) hybrid anticancer agents show a potential for cancer cell killing that is far superior to cisplatin.[3, 4] The acridine group functions as a DNA intercalator that when combined with a platinum moiety, leads to rapid mono-adduct formation with nucleotides near the intercalation site resulting in a more severe form of DNA damage than the cross-links induced by cisplatin.[3-9] PA also form permanent adducts at significantly higher frequency in genomic DNA than cisplatin resulting in effective inhibition of DNA replication and transcription,[6] which results in submicromolar activity against intrinsically platinum-resistant cancers, including breast cancer, *in vitro*. [3, 10] Second-generation platinum-acridines are less reactive with non-DNA intracellular nucleophiles, which may contribute to their high potency.[11] The non-crosslinking DNA damage differs greatly from current clinical agents and is able to circumvent resistance.[3]

Nanoparticles such as liposomes or polymer-based products are being tested in clinical trials to improve tumor delivery and reduce off-target toxicity of platinum drugs (reviewed in ref. [12]). Carbon nanotubes (CNTs), which consist of sheets of sp^2 hybridized carbon rolled into single (SWCNT) or multi-walled (MWCNT) tubes, also show promise for the delivery of cisplatin to cancer cells both *in vitro* and *in vivo*. [13-19] CNTs have the capacity to cross biological barriers like the cell membrane, [20] improve the blood stability and tumor targeting of encapsulated or conjugated drugs and small molecules, [21-23] and overcome drug resistance. [24] In addition, the unique combination of electrical, thermal, and spectroscopic properties of CNTs offers further opportunities for advances in the detection, monitoring and therapy of cancer that are not available with other drug carriers. [25] CNTs absorb near-infrared radiation (NIR) emitted from lasers, generating intense heat that can be localized to tumors after single [25-27] or multiple rounds of NIR exposure. [28] This type of laser-based heat treatment represents a promising approach for the management of recurrent breast cancer. [29-33] Functionalization of CNTs by acid-oxidation and polymer-coating can render CNTs safe for *in vivo* use [23, 34, 35] and addition of targeting ligands may increase tumor-specific uptake. [14, 21-23]

Use of CNTs to deliver chemotherapeutic compounds such as doxorubicin or ruthenium-polypyridyl complexes adsorbed onto CNTs by non-covalent π -stacking results in enhanced cancer cell killing in comparison to the free drug. [24, 36] Acridine also strongly adsorbs onto the surface of CNTs and is released allowing intercalation into DNA. [37, 38] CNTs have a large surface area-to-volume ratio which allows high capacity drug loading. [39] Inspired by the well-documented ability of CNTs to act as a strong adsorbent of cationic dyes, [40] we aimed to generate a CNT-based delivery system for a specific platinum-acridine, **P3A1**, [4, 41] that could

combine the high cytotoxic activity of **P3A1** with the drug delivery and photothermaltherapy capability of CNTs.

EXPERIMENTAL

Cell lines and reagents. The human breast cancer cell lines MDA-MB-231, MDA-MB-468, MDA-MB-436, SUM159, and BT20 were obtained from American Type Culture Collection (ATCC). All cell lines were authenticated by ATCC and tested to be pathogen-free (including *Mycoplasma*, bacteria, and fungi). All cells were used within 6 months of resuscitation. MDA-MB-231, MDA-MB-468, SUM159, and BT20 were maintained as previously described.[42] MDA-MB-436 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Sigma Aldrich), 100 IU/ml penicillin (Life Technologies), and 100 µg/ml streptomycin (Life Technologies).

Synthesis. Compound **P3A1** ($[\text{PtCl}(\text{NH}_3)_2(\text{L})]\text{Cl}$ ($\text{L} = N\text{-(2-(acridin-9-ylamino)ethyl)-}N\text{-methylpropionimidamide}$) was synthesized according to a previously reported procedure [41] and isolated as the monochloride salt with an analytical purity of 96% (based on LC-MS; see the Supplementary Figure S1).

*Preparation of **P3A1**-loaded carbon nanotubes.* Multi-walled carbon nanotubes (Nanostructured and Amorphous Materials Inc.) were acid-oxidized using a sulfuric acid and nitric acid treatment (3:1 mixture) for 3 hours at 75 °C. The MWCNTs were collected on an Omnipore JV filter (0.1 µm, Millipore) and washed with distilled water. To remove carbonaceous debris formed during the acid-oxidation process, the surfaceacidized MWCNTs were resuspended in sodium hydroxide (1.0 M), sonicated for 10 minutes, and stirred at room temperature for 30 minutes.[23] MWCNTs were again collected on a filter as above, washed with distilled water and then

resuspended in hydrochloric acid (0.1 M) to protonate the carboxyl groups introduced during the oxidation process. After a final water washing and collection step, acid-oxidized MWCNTs were dried overnight at 80°C. Stocks were prepared by dispersion of MWCNTs in water (4.5 mg/mL) by bath sonication. For drug loading experiments, **P3A1**-MWCNT[1] was prepared in a single step by adding acid-oxidized MWCNTs (225 µg) to a glass vial containing saline, **P3A1** (0-1.7 µmol/mg MWCNT) and DSPE-mPEG (8.55 mg/mL). Similarly, **P3A1**-MWCNT[2] was prepared in two steps by adding acid-oxidized MWCNTs (225 µg) to a glass vial containing **P3A1** (0-1.11 µmol/mg MWCNT) in saline. Finally, DSPE-mPEG was added to the dispersion at a final concentration of 8.55 mg/mL. Each reaction mixture was bath sonicated twice for 15 minutes at 4°C to prevent overheating, and unbound **P3A1** and DSPE-mPEG were separated using spin columns (Vivaspin 100 kDa molecular weight cut-off columns; EMD Millipore). For subsequent experiments, **P3A1**-MWCNT[1] or **P3A1**-MWCNT[2] were prepared by mixing the appropriate amounts of stock solutions of MWCNTs (400 µL, 1.8 mg), DSPE-mPEG(5000) (500 µL, 20 mg/mL in water, Nanocs), **P3A1** (400 µL, 5 mM in saline), and saline (10x, 124 µL), and mixtures were then sonicated as above. **P3A1**-containing materials and platinum-free controls were incubated for 2 hours at 4°C. Unassociated reagents were removed using a spin column as above. The final product was aliquoted and stored in saline at -80°C until use.

Cytotoxicity Assays. $3-5 \times 10^4$ breast cancer cells were plated in 96-well tissue culture plates (BD Falcon) and allowed to attach overnight. Cells were treated at doses indicated in the figures based on loaded **P3A1** and MWCNT concentration determined by UV-Vis spectroscopy as described in the supplementary information. An average of 0.84 ± 0.06 and 0.54 ± 0.06 µmol/mg MWCNT was loaded for **P3A1**-MWCNT[1] and **P3A1**-MWCNT[2], respectively.

Cells were incubated for the specified times and survival was assessed using the CellTiterGlo assay kit (Promega) according to the manufacturer's protocol.

Nanoparticle Tracking Analysis. DSPE-mPEG coated MWCNTs, **P3A1**-MWCNT[1] and **P3A1**-MWCNT[2] were diluted 1:5000 in degassed Type I water. Hydrodynamic diameters were obtained in quintuplicate using the Nanosight NS500 (Malvern Instruments; software version 3.1, camera level: 16, duration: seconds, threshold: 3).

Dynamic Light Scattering (DLS). Hydrodynamic diameter and ζ -potential were measured using the Zetasizer Nano ZS90 (Malvern Instruments; software version 6.34) at 25°C with automatic settings. MWCNTs were diluted to ~15 ng/mL for DLS in water or saline for measurements.

Confocal Microscopy. MDA-MB-231 cells (3.5×10^4) were plated in 4-well chamber slides (Nunc), allowed to attach for 48 hours, and then treated with vehicle, MWCNT, **P3A1**-MWCNT, or **P3A1** at 7.5 μ M **P3A1** or equivalent MWCNT for 6 hours. Cells were washed twice in PBS, fixed in 4% paraformaldehyde, mounted with hardset mounting medium (Vector Labs) and coverslips, and assessed by confocal microscopy (Excitation 372 nm, Emission 449-549 nm; direct interference contrast (DIC)).

Transmission Electron Microscopy. **P3A1**-MWCNT[1], **P3A1**-MWCNT[2] or control DSPE-mPEG-coated MWCNT in water were pipetted onto copper-coated formvar grids and then imaged using a Tecnai Spirit transmission electron microscope. MDA-MB-231 cells (3.0×10^6) were plated in 100-mm tissue culture dishes and allowed to attach overnight. Cells were treated with **P3A1**-MWCNT[1] (20 μ g/mL MWCNT) and incubated for the times indicated. Cells were prepared as previously described.[23]

Flow Cytometry. MDA-MB-231 cells ($0.75\text{--}2 \times 10^6$) were plated in 100-mm tissue culture plates (BD Falcon) and allowed to adhere overnight. Cells were then treated with **P3A1**-MWCNT[1],

P3A1, DSPE-mPEG coated MWCNT, **P3A1**-DSPE-mPEG, DSPE-mPEG, DSPE-mPEG coated MWCNT, **P3A1**-DSPE-mPEG, or saline at the specified concentrations for 48 and 72 hours.

Cells were washed with PBS and co-stained with Annexin V (APC) and propidium iodide (BD Pharmingen) per the manufacturer's protocol. Briefly, cells were trypsinized, pelleted, washed twice with cold PBS, and then suspended in $1 \times$ Annexin V binding buffer at a concentration of 1×10^6 /mL. 1×10^5 cells were then mixed with Annexin V and incubated for 15 minutes at room temperature in the dark then 400 μ L of $1 \times$ Binding Buffer with or without propidium iodide (2.0 μ g/mL) was added, mixed, and samples were analyzed on the Accuri6 Flow Cytometer (BD Biosciences). Analysis of data was performed using FCS Express version 3 (De Novo Software). For cell cycle analysis, cells were treated as indicated, fixed in 50% ice-cold ethanol, washed once in PBS, and then were treated with FxCycle PI/RNase staining solution (Life Technologies) per the manufacturer's protocol. Analysis was performed using ModFit software.

Inductively coupled plasma mass spectrometry. MDA-MB-231 (2.0×10^6) cells were plated in 60-mm tissue culture plates and allowed to attach overnight. Cells were then treated with saline, **P3A1**-MWCNT[1], or **P3A1** (1 μ M) for 6 hours and then trypsinized, washed twice in PBS, pelleted, and stored at -20°C . Samples were digested with a mixture of 5 % HCl and 5 % HNO₃ using a microwave-assisted digestion system (Ethos UP, Milestone, Sorisole, Italy). The digested samples were then diluted to a final acid concentration of 1 % v/v before Pt determination by inductively coupled plasma mass spectrometry (ICP-MS). Trace metal grade HCl and HNO₃ (Fisher, Pittsburgh, PA, USA), and distilled-deionized water (18 M Ω cm, Milli-Q®, Millipore, Bedford, MA, USA) were used to digest the samples and prepare all solutions. Standard reference solutions used for calibration were prepared in 1 % v/v acid (0.5% HCl plus 0.5% HNO₃) from a 1000 mg/L Pt stock (SPEX CertPrep, Metuchen, NJ, USA). A tandem ICP-MS

(8800 Triple Quadrupole, Agilent, Tokyo, Japan) equipped with a SPS 4 automatic sampler, a Scott-type double pass spray chamber operated at 2 °C, and a Micromist concentric nebulizer was used in all determinations. Helium gas ($\geq 99.999\%$ purity, Airgas, Colfax, NC, USA) was used in the ICP-MS's collision/reaction cell to minimize potential spectral interferences while monitoring the isotope ^{195}Pt . Other relevant instrument operating conditions such as radio frequency applied power, sample depth, carrier gas flow rate, reaction gas flow rate, and the number of sweeps per replicate were 1550 W, 10.0 mm, 1.05 L/min, 4.0 mL/min, and 100, respectively.

Western Blots. MDA-MB-231 cells were seeded ($2.5\text{-}5.0 \times 10^5$) in 60-mm tissue culture dishes (BD Falcon) and allowed to attach overnight. The following day cells were treated for the indicated times. Plates were washed two times with ice cold PBS and lysates harvested in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) supplemented with protease inhibitors (Thermo Scientific). Protein content was quantified by BCA protein assay (Pierce). Protein (40 μg) was separated using a 12% SDS-PAGE gel (Biorad) and transferred to nitrocellulose membrane (Biorad). Membranes were probed with primary antibodies for cleaved-PARP (Cell Signaling Technologies, D64E10, diluted 1:1000 in 5% milk in tris-buffered saline containing 0.1% Tween-20 (TBS-T) and GAPDH (Cell Signaling Technology, D16H11, diluted 1:10,000 in 5% milk in TBS-Tween). Then membranes were then probed with appropriate horse-radish peroxidase conjugated secondary antibodies (Cell Signaling Technology, diluted 1:1,000-5,000 in 5% milk in TBS-Tween) and developed using an enhanced chemiluminescence detection system (Pierce) according to the instructions provided by the manufacturer.

Carbon nanotube-mediated photothermal therapy. MDA-MB-231 cells were plated in 48-well plates in non-neighboring wells at a cell density of 2.5×10^4 per well and allowed to attach overnight. Medium was aspirated, replaced with phenol-red free RPMI supplemented with 10% FBS, and treated as indicated. Cells were irradiated at a wavelength of 970 nm and an irradiance of 3 W/cm^2 using a K-Cube veterinary laser (K-Laser USA). Twenty-four hours later, relative viability was assessed using CellTiterGlo reagent according to the manufacturer's protocol.

RESULTS

Preparation and physicochemical characterization of P3A1-loaded nanotubes.

To test if MWCNTs can be coated with a platinum-acridine (PA) agent, we used the derivative **P3A1** (Fig. 1a, **i**) $[(\text{PtCl}(\text{NH}_3)_2(\text{L}))\text{Cl}]$ ($\text{L} = N$ -(2-(acridin-9-ylamino)ethyl)- N -methylpropionimidamide). MWCNTs were acid oxidized to generate carboxyl groups (approximately 15-17% as a percentage of overall MWCNT weight (Supplementary Fig. S2)) on their surface, and then base washed to remove adsorbed carbonaceous debris (Fig. 1a, **ii**). All MWCNTs used in this study were coated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine- N -[amino(polyethylene glycol)-5000] (DSPE-mPEG) (Fig. 1a, **iii**) to further improve dispersibility and biocompatibility. Thermogravimetric analysis indicated that DSPE-mPEG coated MWCNT contained approximately 75% DSPE-mPEG by weight after purification from excess coating material (Supplementary Fig. S2).

We assessed two methods for preparing **P3A1**-loaded carbon nanotubes. For the first method, we used a one-step synthesis, in which **P3A1**, MWCNT, and DSPE-mPEG were mixed simultaneously. The resulting drug-loaded nanoparticles are referred to as **P3A1**-MWCNT[1].

To determine optimal drug loading conditions, **P3A1**-MWCNT[1] was prepared with fixed quantities of MWCNT and DSPE-mPEG in the presence of increasing concentrations of **P3A1**. After adsorption, **P3A1** loaded onto MWCNTs was separated from unbound **P3A1** and DSPE-mPEG using size-exclusion columns. **P3A1** that was not bound to nanoparticles remained in the filtrate, and the unbound drug concentration was determined spectrophotometrically at an acridine-specific wavelength ($\lambda_{\text{max}} = 415 \text{ nm}$) to determine the amount of MWCNT-associated **P3A1** (Supplementary Fig. S3). The amount of **P3A1** loaded onto **P3A1**-MWCNT[1] relative to the total **P3A1** added to the dispersion is shown in Fig. 1b. The highest level of **P3A1** was achieved at the highest input of **P3A1** ($1.7 \text{ } \mu\text{mol/mg MWCNT}$). The ratio of drug input to drug loaded on MWCNTs (**P3A1** loaded/**P3A1** input) gradually decreased from 100% at a **P3A1** input of $0.25 \text{ } \mu\text{mol/mg MWCNT}$ to less than 50% at an input greater than $1.5 \text{ } \mu\text{mol/mg MWCNT}$, defining a saturation point.

We used a two-step procedure to prepare a second type of **P3A1**-coated carbon nanotubes, termed **P3A1**-MWCNT[2]. **P3A1** was first mixed with MWCNTs and then washed using size-exclusion columns to remove unassociated **P3A1**; in a second step, aqueous DSPE-mPEG was added to the **P3A1** and MWCNT mixture. Notably, mixing **P3A1** and MWCNTs in the absence of DSPE-mPEG resulted in substantial aggregation and sedimentation of the MWCNTs. However, after coating with DSPE-mPEG in the second step, **P3A1**-MWCNT[2] readily dispersed in water following sonication. We found a bimodal loading profile for **P3A1**-MWCNT[2] without apparent saturation at **P3A1** to MWCNT ratios of up to $1 \text{ } \mu\text{mol/mg}$ (Fig. 1c).

We next examined the size distribution and ζ -potential of uncoated MWCNTs, DSPE-mPEG coated MWCNTs, **P3A1**-MWCNT[1], and **P3A1**-MWCNT[2] using nanoparticle tracking

analysis (NTA) and dynamic light scattering (DLS). The results are summarized in Table 1 and representative NTA data are shown in Fig. 1d-f. NTA indicated that **P3A1**-MWCNT[1] exhibited a monomodal size distribution with uniform light scattering intensity that is nearly identical to DSPE-mPEG coated MWCNT prepared without **P3A1**. However, **P3A1**-MWCNT[2] exhibited a broader size distribution with multiple peaks and a more heterogeneous light scattering intensity. DLS also showed that **P3A1**-MWCNT[2] possessed the largest hydrodynamic diameter in water. Significantly, no increase in the hydrodynamic diameter of **P3A1**-MWCNT[1] was observed when measured in saline instead of water, indicating that the nanoparticles did not aggregate at this ionic strength. No change in hydrodynamic diameter was seen after storage of **P3A1**-MWCNT[1] in saline for 10 days at room temperature (Supplementary Fig. S4) indicating that **P3A1**-MWCNT[1] resisted aggregation under these conditions. In contrast, **P3A1**-MWCNT[2] tended to aggregate in normal saline as noted by an increase in the hydrodynamic diameter within a few minutes of incubation, though they were more stable in comparison to the uncoated MWCNT (Table 1). The ζ -potentials in water at pH 6 for **P3A1**-MWCNT[1], **P3A1**-MWCNT[2], and DSPE-mPEG coated MWCNT were considerably less negative than uncoated MWCNTs due to the expected partial charge shielding effects of DSPE-mPEG and **P3A1**.

We next imaged **P3A1**-MWCNT[1], **P3A1**-MWCNT[2], and drug free DSPE-mPEG coated MWCNT by transmission electron microscopy (TEM). Electron-dense regions indicative of the platinum in **P3A1** were observed near the MWCNT surface of both **P3A1**-MWCNT[1] and **P3A1**-MWCNT[2] but not for DSPE-mPEG-coated MWCNT (Fig. 2a-f). For **P3A1**-MWCNT[1], platinum was evenly distributed along the nanotube in single or multiple layers, indicating that **P3A1** may both stack on the nanotube surface and self-aggregate within

the DSPE-mPEG layer. By contrast, for **P3A1**-MWCNT[2] areas of platinum-related high electron density were found in concentrated pockets. Numerous drug-free and drug-encapsulating micellar vesicles were seen around **P3A1**-MWCNT[2] but none were found around **P3A1**-MWCNT[1]. The methods of preparation and structural characteristics of thus obtained nanoparticles are shown schematically in Fig. 2g. Because **P3A1**-MWCNT[1] exhibited superior colloidal stability and more homogenous drug loading as compared to **P3A1**-MWCNT[2], we selected this formulation as our lead material for subsequent studies, with other constructs used only as comparative controls. Fourier-Transform Infrared Spectroscopy (FTIR) was performed to further characterize **P3A1**-MWCNT[1] and determine if association of P1A1 with the MWCNTs involves coordination of platinum to the surface carboxylates. However, peaks corresponding to bond stretching characteristic of both DSPE-mPEG and **P3A1** were undetectable when the sample contained MWCNT (Supplementary Fig. S5). Further analysis using inductively-coupled plasma mass spectrometry (ICP-MS) confirmed the presence of platinum in **P3A1**-MWCNT[1] (Supplementary Fig. S6).

P3A1-loaded nanotubes release drug after being taken up by breast cancer cells

We next quantified the stability of the coating of **P3A1** on **P3A1**-MWCNT[1] overtime. When stored in saline at room temperature, 90% of the **P3A1** that was initially associated with the MWCNTs (representing 100% of drug input) remained associated with **P3A1**-MWCNT[1] and only 10% of the amount of drug loaded onto the MWCNTs was released after 10 days (Supplementary Fig. S7). Although the fraction of the drug released from the MWCNTs (relative to the total amount initially bound to the MWCNTs) remained a constant 10% over time, this may be reflective of a dynamic equilibrium between MWCNT-associated and free

P3A1 in saline. Both sodium dodecyl sulfate (SDS) or ethanol increase the release of **P3A1** from MWCNTs following a brief incubation (Supplementary Fig. S7). For all subsequent experiments, **P3A1** constructs were stored frozen at -80 °C in saline to avoid undesired dissociation of the pharmacophore from the carrier. No changes in nanoparticle size or **P3A1** content were detected after multiple freeze-thaw cycles (data not shown).

We next evaluated the release of **P3A1** from MWCNTs following uptake by cancer cells. MDA-MB-231 breast cancer cells were treated with **P3A1**, **P3A1**-MWCNT[1], DSPE-mPEG coated MWCNT or normal saline at equivalent doses of **P3A1** or MWCNT. **P3A1**-MWCNT[1] were taken up by cancer cells and could be detected within cells by electron microscopy following treatment (Fig. 3a). The intrinsic blue fluorescence of the 9-aminoacridine chromophore of **P3A1** allowed detection of intracellular **P3A1** using confocal fluorescence microscopy.[43] Photomicrographs show blue fluorescence indicative of the presence of **P3A1** in both **P3A1** only and **P3A1**-MWCNT[1] treated cells (Fig. 3b). Despite exposure to an equivalent drug dose, **P3A1**-MWCNT[1] treated cells exhibited a lower fluorescence intensity compared to **P3A1**-treated cells, which is most likely a consequence of partial quenching of acridine fluorescence in the formulation. By contrast, detection of intracellular platinum by ICP-MS unequivocally confirmed that **P3A1**-MWCNT[1] had accumulated at a significantly higher level in cells than **P3A1** (Fig. 3c).

P3A1-loaded nanotubes are cytotoxic to breast cancer cells

An initial cytotoxicity assay was performed to assess the effect of dose and incubation time (24, 48, 72 h) on the cytotoxicity of **P3A1**-MWCNT[1] using MDA-MB-231 cells (Fig. 4a). **P3A1**-MWCNT[1] was slightly, but significantly ($p < 0.05$; student's t-test) less cytotoxic than free **P3A1** in the concentration range 5-20 μM after 24 hours and at all concentrations tested at 48 and

72 hours. Both **P3A1** and **P3A1-MWCNT[1]** were significantly ($p < 0.05$; student's t-test) more cytotoxic than drug-free, DSPE-mPEG coated MWCNTs at all concentrations and incubation times. Finally, **P3A1-MWCNT[1]** proved to be significantly more cytotoxic than **P3A1-MWCNT[2]** (Supplementary Fig. S8).

P3A1-MWCNT[1] consists of multiple components, each of which potentially may contribute to the overall cytotoxicity of the construct. In order to determine which components played a role in the cytotoxicity of our **P3A1** drug delivery system, MDA-MB-231 breast cancer cells were treated for 48 hours with **P3A1** alone, **P3A1-MWCNT[1]**, **P3A1-DSPE-mPEG**, or a mixture of **P3A1-DSPE-mPEG** and drug-free DSPE-mPEG coated MWCNTs at equivalent **P3A1** and MWCNT concentrations (Supplementary Fig. S8). As a control, cells also were treated with dispersions of drug-free DSPE-mPEG or DSPE-mPEG coated MWCNTs prepared using processing conditions identical to the drug loaded constructs. Drug-free DSPE-mPEG did not cause a cytotoxic effect, and DSPE-mPEG coated MWCNTs killed less than 25% of cells at the highest concentration (26 $\mu\text{g/mL}$ of MWCNTs). **P3A1-MWCNT[1]** was slightly less cytotoxic than respective free **P3A1** at all concentrations tested, though substantially more cytotoxic than all other treatments. Importantly, **P3A1-DSPE-mPEG** was substantially less cytotoxic than **P3A1-MWCNT[1]**, and the addition of drug free, DSPE-mPEG coated MWCNT did not alter the cytotoxicity of **P3A1-DSPE-mPEG**, indicating that intact **P3A1-MWCNT** complex, rather than a treatment with a mixture of the individual components, was important for the cytotoxicity of **P3A1-MWCNT[1]**.

To further explore the potential use of **P3A1-MWCNT[1]** for cancer therapy, we exposed four additional breast cancer cell lines (MDA-MB-436, MDA-MB-468, SUM159 and BT20) to **P3A1** alone, **P3A1-MWCNT[1]**, or drug-free DSPE-mPEG coated MWCNT at equivalent doses

of **P3A1** or MWCNTs for 48 hours and quantified cell viability. **P3A1**-MWCNT[1] was similarly or slightly less cytotoxic than free **P3A1** toward all cell lines tested (Fig. 4b). The cytotoxicity of drug-free DSPE-mPEG coated MWCNT varied across cell lines tested, but was substantially lower than the levels observed for **P3A1** and **P3A1**-MWCNT[1]. The key finding in here is that substantial cytotoxic activity of **P3A1** was retained after coating and release from MWCNTs.

P3A1-MWCNTs induce a form of cell death that is distinct from the free drug

Nanoparticle-based drug delivery systems can alter the way drugs are taken up by cells, how drugs are released within cells, and how drugs distribute within cells. This in turn can affect the underlying pathways and mechanisms of cancer cell death induced by the nanoparticle-drug conjugate, which may differ substantially from the free drug.[44] Therefore, we examined the mechanism of cell death induced by **P3A1**-MWCNT[1] and free **P3A1**. MDA-MB-231 cells were treated with a fixed dose of **P3A1**-MWCNT[1] (12.5 µg/mL MWCNT; 10 µM **P3A1**), **P3A1** alone (10 µM), DSPE-mPEG coated MWCNTs (12.5 µg/mL MWCNT), or normal saline for 48 or 72 hours. Propidium iodide (PI) and Annexin V (AnnV) co-staining followed by flow cytometric analysis was performed to allow discrimination between necrosis (PI+; AnnV-), early apoptosis (PI-; AnnV+), and late apoptosis (PI+; AnnV+). The data indicate that free **P3A1** and **P3A1**-MWCNT[1] induced distinctly different mechanisms of cell death (Fig. 5a). **P3A1**-induced apoptosis was apparent at 48 hours, with increasing cell death (both early and late stage) at 72 hours compared to controls. By contrast, **P3A1**-MWCNT[1] showed a marked increase in necrosis at 48 and 72 hours without indications of early apoptosis. To confirm this finding we extracted protein from MDA-MB-231 cells treated with **P3A1** or **P3A1**-MWCNT[1] as above

for 24, 48 or 72 hours and quantified cleaved poly(ADP-ribose) polymerase 1 (PARP) by immunoblotanalysis (Fig. 5b). Cleavage of PARP is a hallmark of apoptosis and can also indicate necrosis.[45] Consistent with the PI/AnnV staining results, cleaved PARP was greatly increased in cells treated with **P3A1** alone for 48 hours, but little was detected in cells treated with **P3A1**-MWCNT[1] for 48 hours. After 72 hours, a high level of cleaved PARP was detected in cells following either treatment.

To gain insight into the cell cycle effects, additional flow cytometry experiments were performed. Cells treated with **P3A1**-MWCNT[1] induced marked S-phase cell cycle arrest (Fig. 5c). Increasing concentrations of **P3A1**-MWCNT[1] resulted in a loss of replicating cells, with the majority of the cells remaining in G1-phase after 48 hours of treatment. On the other hand, equivalent concentrations of **P3A1** did not show a similar S-phase cell cycle arrest. Even when tested at high concentrations (80 μ M), **P3A1** did not show major alterations in cell cycle distribution. Together, these data suggest that **P3A1**-MWCNTs caused S-phase arrest and induced cell death by a non-apoptotic mechanism rather than an apoptotic mechanism, both of which are effects that are distinct from those induced by the free drug.

P3A1-loaded nanotubes are effective for multimodal photothermal therapy and drug delivery.

Carbon nanotubes produce intense heat when stimulated with tissue-penetrating near-infrared (NIR) radiation[26, 27]. Therefore we examined combined **P3A1**-MWCNT[1] treatment with photothermal therapy. MDA-MB-231 cells were treated with **P3A1**-MWCNT[1], control DSPE-mPEG coated MWCNT, or vehicle, then exposed to 980 nm laser irradiation for thermal therapy. We found that **P3A1**-MWCNT[1] treatment sensitized cells to laser irradiation (3 W/cm²) (Fig. 6a). Control DSPE-mPEG MWCNT treatment did not induce a loss in cell viability after 60

seconds of laser exposure, whereas **P3A1**-MWCNT[1] treated cells showed a significant reduction in viability under the same laser conditions. Importantly, this enhanced cytotoxicity occurred after only a brief exposure to mild, non-ablative hyperthermia. The maximum temperature reached after 60 seconds of exposure in the presence of **P3A1**-MWCNT[1] (or DSPE-mPEG coated MWCNTs) was only 44.2 °C (Fig. 6b). Following longer laser irradiation, the heat generated by the nanotubes alone was sufficient to kill most of the treated cells and was not further enhanced by loaded **P3A1**. By contrast, free **P3A1** did not show any increase in cytotoxicity after laser irradiation (Supplementary Fig. S9). To assess whether laser irradiation and/or heat altered the release of **P3A1** from **P3A1**-MWCNT[1], a sample of **P3A1**-MWCNT[1] was laser irradiated as above (46°C). Laser irradiation marginally increased the proportion of **P3A1** released from **P3A1**-MWCNT[1] to 11.9±0.2 of the total amount of **P3A1** originally loaded onto the tube as compared to 11.1±0.4 percent released from a non-irradiated sample of **P3A1**-MWCNT[1] processed in parallel.

DISCUSSION

The platinum-acridine derivative **P3A1** shows promising results for the treatment of intractable cancers both *in vitro* and in rodent cancer models.[4] The key to further development of this and similar agents for clinical use will be to improve drug-like properties and reduce dose-limiting toxicities, while retaining anticancer activity. As a step toward the development of a more effective and selective therapeutic agent, we generated a MWCNT-based drug delivery system for **P3A1**. We showed that **P3A1** readily coats MWCNTs in the presence of DSPE-mPEG to form a colloidal stable nanoparticle complex. **P3A1**-MWCNT[1] delivered more **P3A1** to breast cancer cells than treatment with the free drug, were cytotoxic to several models of breast cancer,

and induced a form of cytotoxicity that was distinct from the free drug and involved both S-phase cell cycle arrest and non-apoptotic cell death. Owing to the optical-thermal properties of the MWCNT backbone, **P3A1**-MWCNT[1] also enabled photothermal cancer therapy.

Multiple groups have reported the use of CNTs and other fullerenes,[13-19] as well as liposomal and polymeric nanoparticles,[12, 46] for delivery of conventional platinum chemotherapeutics. In general, these previous studies focused on encapsulation of cisplatin or cisplatin derivatives within the lumen of CNTs as a strategy to prevent premature drug activation and for slow drug release following cell uptake.[13, 15-17] Alternatively, other CNT-based platinum chemotherapy delivery systems rely on covalent attachment to tether the functional platinum group to the CNT surface via a cleavable linker.[14, 18, 19] While these studies clearly demonstrate the capacity of using CNT for targeted drug delivery and controlled release, further clinical development may be hampered due to reliance on intracellular cleavage and modification of the platinum complex for activation, complicated linker synthesis, or inherent problems common to classical platinum pharmacophores.[1] Our system does not rely on activation of a prodrug and requires no modification of the drug for loading onto CNTs. In addition to π -stacking interactions,[37, 38, 47] electrostatic force between the negatively charged MWCNTs and positively charged **P3A1**, which exist as 2^+ charged cations at neutral pH,[4] likely plays a role in the interaction of **P3A1** with CNTs. Furthermore, the carboxylate groups on the MWCNTs may help further stabilize the platinum moieties via direct coordination.[48]

P3A1-MWCNT[1] offers several potential advantages over previously described platinum therapeutic agents. First, we showed that **P3A1**-MWCNT[1] was effective for the treatment of five triple-negative breast cancer (TNBC) cell lines (Fig. 5) at a dose that is less than a tenth of the reported dose of cisplatin required for a similar effect on MDA-MB-231

cells.[3]TNBC patients show the highest levels of tumor recurrence, the lowest five-year survival rates of all breast cancer subtypes,[49] and do not benefit from current molecularly targeted therapies.[50]The high activity we establish for **P3A1** and **P3A1-MWCNT[1]** treatment of TNBC cells offers the possibility of expanding platinum therapy to TNBC patients.

We assessed two methods for preparing **P3A1** loaded carbon nanotubes (**P3A1-MWCNT[1]** and **P3A1-MWCNT[2]**) and noted a difference in **P3A1** loading when assessed by electron microscopy. It appeared that the presence of DSPE-mPEG during loading of **P3A1** onto **P3A1-MWCNT[1]** acted to stabilize the particle suspension against aggregation and allowed for a more even coating of **P3A1**. However, addition of **P3A1** in the absence of DSPE-mPEG induced aggregation of **P3A1-MWCNT[2]** and the high drug concentration present on the surface of the nanotubes may have caused partitioning of **P3A1** into DSPE-mPEG vesicles during the subsequent coating process. The micellar structures that appear on the surface of **P3A1-MWCNT[2]** visualized by TEM may explain the larger hydrodynamic diameter and more heterogeneous size distribution of **P3A1-MWCNT[2]** as compared to **P3A1-MWCNT[1]**.

We found that **P3A1-MWCNT[1]** induced severe replication stress which prevented cells from passing through S-phase and progressing through the cell cycle, leading to cell death without initiation of apoptosis; whereas free **P3A1** induced apoptosis without a significant effect on progression through the cell cycle. **P3A1-MWCNT[1]** may be beneficial compared to free **P3A1** as it immediately prevents breast cancer cells from dividing. Mutations or loss of the tumor suppressor p53, which is common in many cancers including TNBC,[51] can prevent induction of apoptosis.[52] We show that **P3A1-MWCNT[1]** induces non-apoptotic cell death in a p53 mutant TNBC cell line, MDA-MB-231, and **P3A1-MWCNT[1]** was cytotoxic to three additional TNBC cell lines that harbor p53 mutations (BT20, MDA-MB-436, MDA-MB-

468). These data suggest that **P3A1-MWCNT[1]** may be useful for treatment of cancers deficient in p53 or apoptotic machinery.

Serum or other components of the culture media could play a role in the extracellular release of **P3A1**, but the increased platinum delivery by **P3A1-MWCNT** and the different modes of cell death and cell cycle effects induced by free **P3A1** and **P3A1-MWCNT[1]** suggest that the nanoparticle-drug conjugate is delivered intact to cells. Confocal fluorescence microscopy showed that cell-associated **P3A1** fluorescence intensity was less for **P3A1-MWCNT[1]** treated cells compared to cells treated with free **P3A1** at equivalent doses (Fig. 3b); however, cell-associated platinum assessed by ICP-MS was higher in **P3A1-MWCNT[1]**-treated cells compared to **P3A1**-treated cells (Fig. 3c). Because acridine fluorescence is quenched when associated with CNTs[38] and **P3A1-MWCNT[1]** delivers more platinum to cells in comparison to treatment of cells treated with an equivalent amount of free **P3A1** (as shown by ICP-MS), it is reasonable to suggest that at least a portion of the **P3A1** is still CNT-associated following cell uptake. CNTs may serve as a **P3A1** “reservoir” within in the cells. Therefore, the difference between the activity of **P3A1** and **P3A1-MWCNT[1]** possibly lies within the delivery of the chemotherapeutic with regards to drug release, intracellular trafficking and localization, maximal achieved dose, as well as dosing rate.

Thermal therapy shows promise for the treatment of many types of cancer.[29] Due to the MWCNT backbone, our drug delivery system has the inherent capability for combination NIR stimulated photothermal therapy and **P3A1** treatment. We show that photothermal activation of **P3A1-MWCNT[1]** is more effective than photothermal treatment of drug-free MWCNTs or **P3A1-MWCNT[1]** alone (Fig. 6). Importantly, this effect occurs under mild hyperthermia conditions (44 °C) and a brief exposure (60 seconds) to laser irradiation and therefore would be

suitable as part of a treatment regimen designed to spare healthy breast tissue in a clinical setting.[30]We previously found that similar NIR exposure does not affect the cytotoxicity of MWCNT,[27, 28]. The small increase in the amount of **P3A1** released following laser irradiation of **P3A1**-MWCNT[1] compared to drug release from non-irradiated **P3A1**-MWCNT[1] is likely insufficient to account for the increased cytotoxicity. Therefore this increase possibly is caused by chemo-sensitization due to hyperthermia rather than greater drug release.[25]

The long-term rationale behind using nanoparticles, including CNTs, as drug delivery vehicles is the capacity for nanoparticles to accumulate in tumors at a higher rate than small molecules. This is due to the leaky and tortuous nature of tumor blood vessel architecture, a phenomenon known as the enhanced permeability and retention (EPR) effect.[53] Phospholipid-polyethylene glycol coated CNT, like those used in this report, can greatly increase blood residence and tumor selective delivery of small molecules and chemotherapeutics adsorbed onto their surface.[21-23] Acid-oxidation and polymer-coating can mitigate acute CNT toxicity in vivo,[23, 34, 35] and growing evidence suggests in vivo biodegradation mechanisms that could dramatically reduce any potential long-term risks of CNT exposure[54, 55]. CNTs are degraded within a few days by peroxidase enzymes from both plants and animals[56] and inside the cells by myeloperoxidase,[57] eosinophile peroxidase[58] or other reactive oxygen species mediated pathways.[55, 59] Moreover, enzymatically degraded CNTs appear to be less toxic as compared to their pristine counterparts.[57] As more understanding is developed regarding the degradation of CNTs in biological environments, it may be possible to design CNTs that leave no long-term biological footprint.[54]

CONCLUSION

Here we show the development of a new anti-neoplastic agent based upon a versatile MWCNT drug delivery system loaded with the chemotherapeutic agent **P3A1**, a platinum-based antineoplastic agent that is an order of magnitude more effective than current analogs.[3] The **P3A1**-MWCNT complex is easily formed in aqueous solution minimizing processing and purification steps, and enables combined chemo- and photothermal therapy for multimodal cancer treatment regimens. The unique combination of tumor cell replicative stasis and non-apoptotic cell death induced by **P3A1**-MWCNT[1] may lead to improvements in cancer therapy using **P3A1** or similar drugs. Our system offers further possibilities for improved tumor accumulation, selective targeting, cancer imaging enabled by the MWCNT backbone.[25] We therefore believe that this agent warrants further preclinical investigation for the treatment of breast and other cancers.

ABBREVIATIONS

AnnV	annexin V
CNT	carbon nanotube
DLS	dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DSPE-mPEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-5000]
EPR	enhanced permeability and retention
FBS	fetal bovine serum
FTIR	Fourier-transform infrared spectroscopy
GAPDH	glyceraldehyde 3-phosphatase dehydrogenase
ICP-MS	inductively coupled plasma mass spectrometry
MWCNT	multiwalled carbon nanotube
NIR	near-infrared radiation
NTA	nanoparticle tracking analysis
P3A1	([PtCl(NH ₃) ₂ (N-(2-(acridin-9-ylamino)ethyl)-N-methylpropionimidamide)
PA	platinum-acridine
PARP	poly ADP Ribose
PBS	phosphate buffered saline
PI	propidium iodide
RPMI	Roswell Park Memorial Institute medium
SWCNT	single walled carbon nanotube
TBS	tris-buffered saline
TEM	transmission electron microscopy

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AUTHOR CONTRIBUTIONS

C.D.F., U.B. and R.S. conceived the research. C.D.F., S.D., M.Y., X.Y., B.W.B, M.L.W., Y.Z., M.D.G., G.L.D., U.B. and R.S. developed the methodology, carried out the experiments, and performed data analysis. C.D.F, U.B. and R.S. wrote the main manuscript text. All authors reviewed and edited the manuscript.

REFERENCES

- [1] L. Kelland, Nat Rev Cancer, vol. 7, 2007, pp. 573-584.
- [2] L. Galluzzi, L. Senovilla, I. Vitale, J. Michels, I. Martins, O. Kepp, M. Castedo, G. Kroemer, Oncogene, vol. 31, 2012, pp. 1869-1883.
- [3] L.A. Graham, J. Suryadi, T.K. West, G.L. Kucera, U. Bierbach, Journal of medicinal chemistry, vol. 55, 2012, pp. 7817-7827.
- [4] Z. Ma, J.R. Choudhury, M.W. Wright, C.S. Day, G. Saluta, G.L. Kucera, U. Bierbach, Journal of medicinal chemistry, vol. 51, 2008, pp. 7574-7580.
- [5] S. Dutta, M.J. Snyder, D. Rosile, K.L. Binz, E.H. Roll, J. Suryadi, U. Bierbach, M. Guthold, Cell biochemistry and biophysics, vol. 67, 2013, pp. 1103-1113.
- [6] X. Qiao, A.E. Zeitany, M.W. Wright, A.S. Essader, K.E. Levine, G.L. Kucera, U. Bierbach, Metallomics : integrated biometal science, vol. 4, 2012, pp. 645-652.
- [7] C.L. Smyre, G. Saluta, T.E. Kute, G.L. Kucera, U. Bierbach, ACS medicinal chemistry letters, vol. 2, 2011, pp. 870-874.
- [8] L.A. Graham, G.M. Wilson, T.K. West, C.S. Day, G.L. Kucera, U. Bierbach, ACS medicinal chemistry letters, vol. 2, 2011, pp. 687-691.
- [9] J.R. Choudhury, L. Rao, U. Bierbach, Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry, vol. 16, 2011, pp. 373-380.
- [10] S. Ding, X. Qiao, G.L. Kucera, U. Bierbach, Chemical communications, vol. 49, 2013, pp. 2415-2417.
- [11] Z. Ma, L. Rao, U. Bierbach, Journal of medicinal chemistry, vol. 52, 2009, pp. 3424-3427.
- [12] H.S. Oberoi, N.V. Nukolova, A.V. Kabanov, T.K. Bronich, Advanced drug delivery reviews, vol. 65, 2013, pp. 1667-1685.

- [13] K. Ajima, T. Murakami, Y. Mizoguchi, K. Tsuchida, T. Ichihashi, S. Iijima, M. Yudasaka, ACS nano, vol. 2, 2008, pp. 2057-2064.
- [14] S. Dhar, Z. Liu, J. Thomale, H. Dai, S.J. Lippard, Journal of the American Chemical Society, vol. 130, 2008, pp. 11467-11476.
- [15] J. Li, A. Pant, C.F. Chin, W.H. Ang, C. Menard-Moyon, T.R. Nayak, D. Gibson, S. Ramaprabhu, T. Panczyk, A. Bianco, G. Pastorin, Nanomedicine : nanotechnology, biology, and medicine, vol. 10, 2014, pp. 1465-1475.
- [16] L. Muzi, C. Menard-Moyon, J. Russier, J. Li, C.F. Chin, W.H. Ang, G. Pastorin, G. Risuleo, A. Bianco, Nanoscale, vol. 7, 2015, pp. 5383-5394.
- [17] S.L. Yoong, B.S. Wong, Q.L. Zhou, C.F. Chin, J. Li, T. Venkatesan, H.K. Ho, V. Yu, W.H. Ang, G. Pastorin, Biomaterials, vol. 35, 2014, pp. 748-759.
- [18] A.A. Bhirde, V. Patel, J. Gavard, G. Zhang, A.A. Sousa, A. Masedunskas, R.D. Leapman, R. Weigert, J.S. Gutkind, J.F. Rusling, ACS nano, vol. 3, 2009, pp. 307-316.
- [19] R.P. Feazell, N. Nakayama-Ratchford, H. Dai, S.J. Lippard, Journal of the American Chemical Society, vol. 129, 2007, pp. 8438-+.
- [20] L. Lacerda, J. Russier, G. Pastorin, M.A. Herrero, E. Venturelli, H. Dumortier, K.T. Al-Jamal, M. Prato, K. Kostarelos, A. Bianco, Biomaterials, vol. 33, 2012, pp. 3334-3343.
- [21] Z. Liu, K. Chen, C. Davis, S. Sherlock, Q. Cao, X. Chen, H. Dai, Cancer Res, vol. 68, 2008, pp. 6652-6660.
- [22] W. Shao, A. Paul, B. Zhao, C. Lee, L. Rodes, S. Prakash, Biomaterials, vol. 34, 2013, pp. 10109-10119.
- [23] C.D. Fahrenholtz, M. Hadimani, S.B. King, S.V. Torti, R. Singh, Nanomedicine (Lond), vol. 10, 2015, pp. 2481-2497.

- [24] N. Wang, Y. Feng, L. Zeng, Z. Zhao, T. Chen, *ACS Appl Mater Interfaces*, vol. 7, 2015, pp. 14933-14945.
- [25] R. Singh, S.V. Torti, *Advanced drug delivery reviews*, vol. 65, 2013, pp. 2045-2060.
- [26] S.V. Torti, F. Byrne, O. Whelan, N. Levi, B. Ucer, M. Schmid, F.M. Torti, S. Akman, J. Liu, P.M. Ajayan, O. Nalamasu, D.L. Carroll, *Int J Nanomedicine*, vol. 2, 2007, pp. 707-714.
- [27] B.N. Eldridge, B.W. Bernish, C.D. Fahrenholtz, R. Singh, *ACS Biomaterials Science & Engineering*, American Chemical Society, 2016.
- [28] X. Ding, R. Singh, A. Burke, H. Hatcher, J. Olson, R.A. Kraft, M. Schmid, D. Carroll, J.D. Bourland, S. Akman, F.M. Torti, S.V. Torti, *Nanomedicine (Lond)*, vol. 6, 2011, pp. 1341-1352.
- [29] Z. Zhao, F. Wu, *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology*, vol. 36, 2010, pp. 1149-1155.
- [30] K. Dowlatshahi, D.S. Francescatti, K.J. Bloom, *American journal of surgery*, vol. 184, 2002, pp. 359-363.
- [31] J.L.R. Roti, *Int J Hyperther*, vol. 24, 2008, pp. 3-15.
- [32] B. Emami, C.W. Song, *Int J Radiat Oncol Biol Phys*, vol. 10, 1984, pp. 289-295.
- [33] A.R. Burke, R.N. Singh, D.L. Carroll, J.C. Wood, R.B. D'Agostino, Jr., P.M. Ajayan, F.M. Torti, S.V. Torti, *Biomaterials*, vol. 33, 2012, pp. 2961-2970.
- [34] R. Singh, D. Pantarotto, L. Lacerda, G. Pastorin, C. Klumpp, M. Prato, A. Bianco, K. Kostarelos, *Proc Natl Acad Sci U S A*, vol. 103, 2006, pp. 3357-3362.
- [35] A.R. Burke, R.N. Singh, D.L. Carroll, J.D. Owen, N.D. Kock, R. D'Agostino, Jr., F.M. Torti, S.V. Torti, *Biomaterials*, vol. 32, 2011, pp. 5970-5978.

- [36] H. Ali-Boucetta, K.T. Al-Jamal, D. McCarthy, M. Prato, A. Bianco, K. Kostarelos, *Chemical communications*, 2008, pp. 459-461.
- [37] Y. Meng, F.Y. Liu, J.F. Han, S.G. Sun, J.L. Fan, F.L. Song, X.J. Peng, *Mater Sci Eng B-Adv*, vol. 177, 2012, pp. 887-891.
- [38] N. Mackiewicz, J.A. Delaire, A.W. Rutherford, E. Doris, C. Mioskowski, *Chemistry*, vol. 15, 2009, pp. 3882-3888.
- [39] M. Prato, K. Kostarelos, A. Bianco, *Acc Chem Res*, vol. 41, 2008, pp. 60-68.
- [40] J.L. Gong, B. Wang, G.M. Zeng, C.P. Yang, C.G. Niu, Q.Y. Niu, W.J. Zhou, Y. Liang, *J Hazard Mater*, vol. 164, 2009, pp. 1517-1522.
- [41] S. Ding, X. Qiao, G.L. Kucera, U. Bierbach, *Journal of medicinal chemistry*, vol. 55, 2012, pp. 10198-10203.
- [42] J. Swanner, J. Mims, D. Carroll, S. Akman, C. Furdui, S. Torti, R. Singh, *International Journal of Nanomedicine*, vol. 10, 2015, pp. 3937-3953.
- [43] A.J. Pickard, F. Liu, T.F. Bartenstein, L.G. Haines, K.E. Levine, G.L. Kucera, U. Bierbach, *Chemistry*, vol. 20, 2014, pp. 16174-16187.
- [44] X. Li, Q. He, J. Shi, *ACS nano*, vol. 8, 2014, pp. 1309-1320.
- [45] S. Gobeil, C.C. Boucher, D. Nadeau, G.G. Poirier, *Cell Death Differ*, vol. 8, 2001, pp. 588-594.
- [46] S. Dhar, F.X. Gu, R. Langer, O.C. Farokhzad, S.J. Lippard, *Proc Natl Acad Sci U S A*, vol. 105, 2008, pp. 17356-17361.
- [47] C. Roquelet, J.S. Lauret, V. Alain-Rizzo, C. Voisin, R. Fleurier, M. Delarue, D. Garrot, A. Loiseau, P. Roussignol, J.A. Delaire, E. Deleporte, *Chemphyschem*, vol. 11, 2010, pp. 1667-1672.

- [48] H.Y. Lee, K.A. Mohammed, E.P. Goldberg, F. Kaye, N. Nasreen, *Am J Cancer Res*, vol. 5, 2015, pp. 603-615.
- [49] R. Dent, M. Trudeau, K.I. Pritchard, W.M. Hanna, H.K. Kahn, C.A. Sawka, L.A. Lickley, E. Rawlinson, P. Sun, S.A. Narod, *Clin Cancer Res*, vol. 13, 2007, pp. 4429-4434.
- [50] C.A. Hudis, L. Gianni, *Oncologist*, vol. 16 Suppl 1, 2011, pp. 1-11.
- [51] T. Sorlie, C.M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, T. Thorsen, H. Quist, J.C. Matese, P.O. Brown, D. Botstein, P.E. Lonning, A.L. Borresen-Dale, *Proc Natl Acad Sci U S A*, vol. 98, 2001, pp. 10869-10874.
- [52] J.S. Fridman, S.W. Lowe, *Oncogene*, vol. 22, 2003, pp. 9030-9040.
- [53] J. Fang, H. Nakamura, H. Maeda, *Advanced drug delivery reviews*, vol. 63, 2011, pp. 136-151.
- [54] G.P. Kotchey, Y. Zhao, V.E. Kagan, A. Star, *Advanced drug delivery reviews*, vol. 65, 2013, pp. 1921-1932.
- [55] D. Elgrabli, W. Dachraoui, C. Menard-Moyon, X.J. Liu, D. Begin, S. Begin-Colin, A. Bianco, F. Gazeau, D. Alloyeau, *ACS nano*, vol. 9, 2015, pp. 10113-10124.
- [56] B.L. Allen, P.D. Kichambare, P. Gou, I.I. Vlasova, A.A. Kapralov, N. Konduru, V.E. Kagan, A. Star, *Nano Lett*, vol. 8, 2008, pp. 3899-3903.
- [57] V.E. Kagan, N.V. Konduru, W.H. Feng, B.L. Allen, J. Conroy, Y. Volkov, I.I. Vlasova, N.A. Belikova, N. Yanamala, A. Kapralov, Y.Y. Tyurina, J.W. Shi, E.R. Kisin, A.R. Murray, J. Franks, D. Stolz, P.P. Gou, J. Klein-Seetharaman, B. Fadeel, A. Star, A.A. Shvedova, *Nat Nanotechnol*, vol. 5, 2010, pp. 354-359.

- [58] F.T. Andon, A.A. Kapralov, N. Yanamala, W.H. Feng, A. Baygan, B.J. Chambers, K. Hultenby, F. Ye, M.S. Toprak, B.D. Brandner, A. Fornara, J. Klein-Seetharaman, G.P. Kotchey, A. Star, A.A. Shvedova, B. Fadeel, V.E. Kagan, *Small*, vol. 9, 2013, pp. 2721-2729.
- [59] V.E. Kagan, A.A. Kapralov, C.M. St Croix, S.C. Watkins, E.R. Kisin, G.P. Kotchey, K. Balasubramanian, I.I. Vlasova, J. Yu, K. Kim, W. Seo, R.K. Mallampalli, A. Star, A.A. Shvedova, *ACS nano*, vol. 8, 2014, pp. 5610-5621.

TABLES

	DLS ¹ hydrodynamic diameter in water (SD ²)	Polydispersity index in water (SD)	DLS hydrodynamic diameter in saline (SD)	Polydispersity index in saline (SD)	ζ-potential (SD)	NTA ³ hydrodynamic diameter in water (SD)
Uncoated MWCNT	152.0 nm (± 0.6)	0.262 (± 0.024)	2826 nm (± 390)	0.572 (± 0.038)	-58.8 mV (± 4.6)	171.5 nm (± 1.8)
DSPE-PEG coated MWCNT	135.9 nm (± 2.9)	0.228 (± 0.001)	136.4 nm (± 0.7)	0.248 (± 0.008)	-17.4 mV (± 0.5)	132.7 nm (± 3.4)
P3A1- MWCNT[1]	151.3 nm (± 4.4)	0.234 (± 0.032)	138.3 nm (± 0.9)	0.235 (± 0.009)	-12.7 mV (± 0.9)	138.9 nm (± 4.9)
P3A1- MWCNT[2]	187.0 (± 1.4)	0.230 (± 0.003)	226.0 nm (± 1.4)	0.273 (± 0.011)	-18.0 mV (± 0.5)	168.6 nm (± 2.5)

Table 1: Physicochemical characterization of P3A1-MWCNT constructs.

¹ dynamic light scattering (DLS); ² standard deviation (SD); ³ nanoparticle tracking analysis (NTA)

FIGURE LEGENDS

Figure 1. *Chemical Structures, quantification of drug loading onto MWCNTs and nanoparticle size distributions* (A) Chemical structures of (i) platinum-acridine **P3A1**, (ii) acid-oxidized carbon nanotubes, and (iii) 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-5000] (DSPE-mPEG) are shown. (B) **P3A1**-MWCNT[1] or (C) **P3A1**-MWCNT[2] was prepared and unbound **P3A1** was separated by size exclusion columns. Data are displayed as input **P3A1** compared to MWCNT loaded **P3A1** and calculated as $\mu\text{mol P3A1}$ per mg MWCNT. The hydrodynamic size distributions of (D) DSPE-mPEG coated MWCNT, (E) **P3A1**-MWCNT[1] and (F) **P3A1**-MWCNT[2] were assessed by Nanoparticle Tracking Analysis (NTA).

Figure 2. *Representative TEM images of drug loaded nanotubes* Electron micrographs of (A) DSPE-mPEG coated MWCNT, (B-D) **P3A1**-MWCNT[1], and (E-F) **P3A1**-MWCNT[2] (scale bar = 100 nm) are shown. WHITE ARROWS indicate some areas of electron dense platinum (**P3A1**) bound to MWCNTs. BLACK ARROWS indicate aggregates of **P3A1** and DSPE-mPEG not bound to MWCNTs. (G) A schematic summarizing the preparation and structures of the **P3A1** nanoparticles is shown.

Figure 3. *Evaluation of **P3A1**-MWCNT[1] uptake and drug release in cancer cells.* (A) MDA-MB-231 cells were treated with **P3A1**-MWCNT[1] ($20 \mu\text{g/mL MWCNT}$) for 16 hours, fixed with glutaraldehyde, and prepared for TEM. Images of **P3A1**-MWCNT[1] (white arrows) internalized by breast cancer cells are shown. Numbered regions in the lower magnification image (30,000x; scale bar = 500 nm) are shown in higher magnification (90,000x; scale bar =

100 nm) in the adjacent images. **(B)** MDA-MB-231 cells were treated with vehicle, drug-free DSPE-mPEG coated MWCNT, **P3A1**-MWCNT[1] or **P3A1** (7.5 μ M **P3A1** or 9.8 μ g/mL MWCNT) for 6 h, and then assessed for **P3A1** fluorescence by confocal microscopy. **(C)** MDA-MB-231 cells were treated with vehicle, **P3A1**-MWCNT[1] or P3A1 (1.0 μ M) for 6 h, harvested, microwave-assisted digested, analyzed by ICP-MS, and cell-associated platinum content is shown.

Figure 4. *Quantification of **P3A1**-MWCNT[1] induced cytotoxicity in breast cancer cells.* **(A)** MDA-MB-231 were treated for 24, 48, and 72 h, or **(B)** MDA-MB-468, BT20, MDA-MB-436 or SUM159 were treated for 48 h with **P3A1**-MWCNT[1], DSPE-mPEG coated MWCNT (0-26 μ g/mL), **P3A1** (0-20 μ M), or equivalent doses of **P3A1**-MWCNT[1]. Data are shown as relative viability assessed by CellTiterGlo assay compared to untreated control $\pm$ SD.

Figure 5. *Analysis of cell death mechanism and cell cycle of breast cancer cells treated with **P3A1**-MWCNT[1].* **(A)** MDA-MB-231 were treated with drug free DSPE-mPEG coated MWCNT (12.5 μ g/mL), **P3A1** (10 μ M), or equivalent dose of **P3A1**-MWCNT[1] for 48 and 72 hours. Cells were co-stained with propidium iodide (PI) and Annexin V (AnnV), and then staining was assessed by flow cytometry. Data shown are representative of 3 independent experiments. **(B)** Lysates collected from cells treated as in (A) were probed by Western Blot for cleaved PARP (cl-PARP) following treatment for 24, 48 or 72 hours. GAPDH was used as a loading control. **(C)** MDA-MB-231 cells were treated with **P3A1** (0-80 μ M), **P3A1**-MWCNT[1] (0-20 μ M P3A1, 0-25 μ g/mL MWCNT), or vehicle (saline) for 48 h. DNA content based upon PI staining was assessed by flow cytometry.

Figure 6. *Evaluation of P3A1-MWCNT[1] for multimodal drug delivery and photothermal therapy.* (A) MDA-MB-231 were treated with DSPE-mPEG coated MWCNT (12.5 $\mu\text{g/mL}$), **P3A1-MWCNT[1]** (12.5 $\mu\text{g/mL}$, 10 μM **P3A1**) or vehicle in dye free medium, then laser irradiated (970nm, 3W/cm²) for the indicated times. Treatment efficacy was assessed by CellTiterGlo assay 24 hours later. Data are expressed as relative viability compared to untreated control $\pm$ SD (** $p < 0.01$, Two-tailed Student's t-test). (B) Temperature was assessed by thermocouple immediately after laser irradiation in parallel samples treated as in (A).