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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 ([PtCl(NH_3)$_2$(L)]Cl (**P3A1**; L = *N*-(2-(acridin-9-ylamino)ethyl)-*N*-methylproprionimidamide) 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.

1. 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 monoadduct 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]. PAs 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

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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 photothermal therapy capability of CNTs.

2. Experimental

2.1. 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).

2.2. Synthesis

Compound **P3A1** ($[PtCl(NH_3)_2(L)]Cl$ (L = *N*-(2-(acridin-9-ylamino)ethyl)-*N*-methylproprionimidamide) 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 Fig. S1).

2.3. 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 h 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 surface acidized MWCNTs were resuspended in sodium hydroxide (1.0 M), sonicated for 10 min, and stirred at room temperature for 30 min [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 min at 4 °C to prevent overheating, and unbound **P3A1** and DSPE-mPEG were separated using spin columns (Vivaspin 100 k 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 (10×, 124 μL), and mixtures were then sonicated as above. **P3A1**-containing materials and platinum-free controls were incubated for 2 h 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.

2.4. Cytotoxicity assays

3–5 × 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 CellTiter Glo assay kit (Promega) according to the manufacturers protocol.

2.5. 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).

2.6. 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.

2.7. Confocal microscopy

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

2.8. 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 μg/mL MWCNT) and incubated for the times indicated. Cells were prepared as previously described [23].

2.9. Flow cytometry

MDA-MB-231 cells (0.75–2 × 10^6) were plated in 100-mm tissue culture plates (BD Falcon) and allowed to adhere overnight. Cells were

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