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Cancer targeting propensity of folate conjugated surface engineered multi-walled carbon nanotubes

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ABSTRACT

Our main aim in the present investigation was to investigate the cancer targeting potential of docetaxel (DTX) loaded, folic acid (FA) terminated, poly (ethylene glycol) (PEG) conjugated, surface engineered multi walled carbon nanotubes (DTX/FA-PEG-MWCNTs) in tumor bearing Balb/c mice. The percent loading efficiency of DTX/FA-PEG-MWCNTs and DTX loaded MWCNTS (DTX/MWCNTs) was calculated to be 93.40 ± 3.82% and 76.30 ± 2.62%, respectively. Flow cytometry analysis suggested that the DTX/FA-PEG-MWCNTs arrested MCF-7 cells' cycle in the G2 phase and was more cytotoxic as compared to DTX/MWCNTs as well as free drug solution. The obtained pharmacokinetic parameters clearly describe the biocompatibility of engineered nanotubes to degree of functionalization and ability for prolonged residence inside the body. DTX/FA-PEG-MWCNTs was found to be significantly more efficient in tumor suppression as compared with plain MWCNTs (non-targeted) as well as drug solution owing to the enhanced drug release from endosomes after internalization. The DTX/FA-PEG-MWCNTs showed highly significant prolonged survival span (40 days) as compared to DTX/MWCNTs (24 days), free DTX (19 days) and control group (12 days). Overall, we can conclude that the DTX/FA-PEG-MWCNTs shows higher cancer targeting propensity *vis a vis* minimal side effects in tumor bearing Balb/c mice.

1. Introduction

The development of 'safe and effective' nanomedicines for the treatment of diseases including diabetes, acquired immune deficiency syndrome, tuberculosis and cancer still remains the foremost challenging task to researchers, scientists and academicians, worldwide. Cancer accounted for 7.6 million deaths (approximately 13% of all deaths) in 2008 according to recent Fact Sheet of World Health Organization (WHO) wherein approximately 70% deaths occurred in low- and middle-income countries. In 2030, cancer deaths are projected to rise over 13.1 million, worldwide [1]. The failure of chemotherapy is due to the non-selectivity as well as inability to target the anticancer agent(s) to the cancerous cells. The various available nano-sized carrier systems including dendrimers [2], nanoparticles [2] and carbon nanotubes [3–8] are being continuously explored for improved specificity and targeting as well as realizing the attributes of the 'magic bullet' concept.

In the past two decades, surface engineered carbon nanotubes (CNTs) have been explored designed and considered as valuable, promising, 'safe and effective' alternative nano-architecture for pharmaceutical and biomedical applications due to their unique physicochemical properties. CNTs comprise of thin graphite sheets of condensed benzene rings rolled upon into the nanoneedle, seamless tubular hollow cylinder. CNTs can be distinguished on the basis of their lengths, diameters, and most importantly, presence of walls and are categorized into single-, double-, triple-, and multi-walled carbon nanotubes [3,9–11]. The pristine CNTs (first generation; untreated CNTs) are not suitable for drug delivery on account of their hydrophobicity and toxicity due to the presence of impurities, which can fortunately be overcome by surface functionalization. Higher degree of functionalization (hence lower toxicity) makes nanotubes better, safer and effective drug delivery system [12].

The surface alterations of CNTs can be performed either by covalent or non-covalent interactions depending on the intermolecular interaction. The non-covalent modifications, based on the extended π-system (p-orbital) of the nanotubes sidewall, interact with the guest chemical moieties through π–π stacking interactions. Currently, surface engineered CNTs are being explored for targeted

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delivery and have been claimed to be non-toxic to human cells [9,12,13].

Docetaxel (N-debenzoyl-N-tert-butoxycarbonyl-10-deacetyl-paclitaxel) used in the present investigation is a semi-synthetic taxane, derived from the precursor 10-deacetyl baccatin III and extracted from the European yew tree *Taxus baccata* for targeting to β-subunit of tubulin. It entered into clinical trials in 1990 and demonstrates the efficacy in the treatment of several malignancies including prostate, small and non-small cell lung cancer and breast cancer *etc.* [14].

In the present investigation we intended to explore the cancer targeting potential of the docetaxel (DTX) bearing surface engineered MWCNTs. The developed DTX bearing surface engineered MWCNTs nanoconjugates were characterized for loading efficiency, *in vitro* release, hemocompatibility and toxicity in tumor bearing Balb/c mice.

2. Experimental

2.1. Materials

Multi Walled Carbon Nanotubes (MWCNTs) produced by chemical vapor deposition (CVD) with 99.3% purity, were purchased from Sigma Aldrich Pvt. Ltd. (St. Louis, Missouri, USA). Docetaxel was received as a benevolent gift from M/s Fresenius Kabi Oncology Ltd; (FKOL) (formerly Dabur Research foundation), Sahibabad, India. All reagents and solvents were used as received.

2.2. Surface engineering of the pristine MWCNTs

Firstly, the procured pristine MWCNTs were purified by treating in a microwave oven (GEM Insta Cook, Gurgaon, India) at 400 ± 2 °C for 2 h. The microwave treated MWCNTs (500 mg) were refluxed with a mixture of concentrated Nitric and Sulphuric acid (HNO_3:H_2SO_4::1:3 ratio) in a flat bottom flask (equipped with the reflux condenser and thermometer) with continuous magnetic stirring (100 RPM; Remi, Mumbai, India) at 120 ± 5 °C for 6 h; washed, ultra centrifuged (20,000 rpm for 15 min; Z36HK, HERMLE LaborTchnik GmbH, Germany), vacuum dried (Jyoti Scientific Industries, Gwalior, India), lyophilized (Heto dry Winner, Denmark, Germany), and collected [9,13,15].

2.3. Folic acid (FA) conjugation with surface engineered MWCNTs

The MWCNTs were conjugated with folic acid (FA) using PEG spacer and characterized following the method reported by us earlier [9].

2.4. Loading efficiency

The MWCNTs: DTX in optimized ratio (1:2) were added to anhydrous ethanol (0.5 mL) in an ultrasonic bath for about 15 min with drop-wise addition of PBS (pH 7.4) solution and ultrasonicated using an ultrasonic probe (400 W) for approximately 10 min (Lark, Chennai, India). The resultant suspension was ultracentrifuged at 10,000 rpm for 10 min until the MWCNTs were fully separated, and the obtained supernatants were discarded. The remaining solids were thoroughly rinsed with anhydrous ethanol and deionized water to remove excess docetaxel. The amount of unbound DTX in the solution was determined by measuring the absorbance at λ_{max} 230 nm in a spectrophotometer (Shimadzu 1601, UV–Visible Spectrophotometer, Shimadzu, Japan) and the DTX loading efficiency was calculated (n = 3). The product was collected, dried, lyophilized (Heto dry winner, Denmark, Germany) and stored at 5 ± 3 °C for further studies. Finally, the DTX loaded MWCNTs formulations *i.e.* DTX loaded MWCNTs (DTX/MWCNTs) and DTX loaded FA-PEG-MWCNTs (DTX/FA-PEG-MWCNTs) were prepared.

2.5. Characterization of pristine and functionalized MWCNTs

The pristine and surface engineered MWCNTs were extensively characterized using different analytical characterization tools. The surface topography of the pristine and surface engineered MWCNTs was determined through transmission electron microscopy (TEM; Morgagni 268-D, Fei Electron Optics, Holland) after drying on carbon-coated copper grid and negative staining with 1% phosphotungstic acid (PTA) [13].

The surface fracture of the nanotubes nanoformulations was studied using atomic force microscopy (AFM) in a tapping mode with Digital Nanoscope IV Bioscope (Veeco Innova Instruments, Santa Barbara, CA, USA) after drying in air.

The average particle size and size distribution were determined by photon correlation spectroscopy in a Malvern Zetasizer nano ZS90 (Malvern Instruments, Ltd, Malvern, UK) at room temperature (RT) after addition of surfactant.

The Raman spectra of the pristine and *f*-MWCNTs for order-disorder hexagonal carbon were recorded using Raman micro-spectroscopy RINSHAW, *inVia* Raman Spectrophotometer (RENISHAW, Gloucestershire, UK). The microspectrophotometer was operated with 532 nm laser radiation under objective lens of 20× magnification (Olympus BX 41, USA) with a slit of 1 × 6 mm whereas the incident power was approximately 1 mW with 30 s exposure time [13].

The X-ray diffractograms (XRD) were recorded (X-ray diffractometer, PW 1710 Rigaku, San Jose, CA) by adjusting X-ray power of 40 kV and 40 mA of MWCNTs formulations [13].

2.5.1. In vitro *release studies*

The release of docetaxel from the developed MWCNTs formulations (DTX/FA-PEG-MWCNTs and DTX/MWCNT) was monitored separately in sodium acetate buffer saline pH 5.3 (lysosomal pH), and phosphate buffer saline pH 7.4 (physiological pH) through a modified dialysis diffusion technique while maintaining the physiological temperature 37 ± 0.5 °C throughout the study (n = 3) [13,14,16–19]. The known amount of DTX loaded MWCNTs formulations (10 mL) was added in the dialysis sac (MWCO, 12 kDa), hermetically tied and placed into the receptor compartment (ethanol: phosphate buffer pH 5.3:7.4::3:7 containing Tween 80) with slow and continuous magnetic stirring at 37 ± 0.5 °C under strict sink condition. Tween 80 was used in the release medium to solubilize the DTX and to facilitate the passage across the dialysis membrane. Aliquots were withdrawn at definite time points from the mixture and immediately replenished with an equal volume of fresh medium for estimation of the concentration of DTX using UV/Visible spectrophotometer at λ_{max} 230.0 nm (UV/Vis, Shimadzu 1601, Kyoto, Japan).

2.5.2. Accelerated stability study

The DTX/FA-PEG-MWCNTs and DTX/MWCNTs were stored in tightly closed glass vials separately in dark as well as in amber colored and colorless glass vials at 5 ± 3, 25 ± 2 and 40 ± 2 °C for a period of six months in stability chambers (Remi CHM-6S, India) (n = 3) [13,20]. The MWCNTs formulations were analyzed initially and periodically up to six months for any change in particle size, drug content and organoleptic features like aggregation, precipitation, color and odor, if any.

2.6. Comparison of hemolytic toxicity

The hemolytic toxicity of the administered MWCNTs formulations was assessed *in vitro* [13,19]. Briefly, fresh whole human blood