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Co-delivery of VP-16 and Bcl-2-targeted antisense on PEG-grafted *o*MWCNTs for synergistic *in vitro* anti-cancer effects in non-small and small cell lung cancer

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ABSTRACT

Present study describes the preparation of a polyethylene glycol-grafted oxidized multi-walled carbon nanotubes (*o*MWCNTs-PEG) hybrid nanosystem as a carrier of etoposide (VP-16) and Bcl-2 phosphorothioate antisense deoxyoligonucleotides (Aso) to achieve a superior cytostastic efficacy in non-small and small cell lung cancer *in vitro*. We have demonstrated that the adsorption of hydrophobic VP-16 and Bcl-2 Aso results in a stable nanotransporter exhibiting good dispersion with excellent release profiles (both, in pH 7.4 and 4.8) and negligible hemolytic activity (up to 6.5%). The evaluation of cytotoxicity was carried out in *in vitro* using small cell (SCLC; DMS53) and non-small cell lung cancer (NSCLC; NCIH2135) cell lines. It was found that Bcl-2 interference significantly increased the anti-cancer efficiency of VP-16 in the chemoresistant NSCLC cells. This was further supported using a flow-cytometry (Annexin V/propidium iodide assay), which revealed a significant increase in apoptotic cells in both the cell lines after the co-administration of VP-16 and Bcl-2 Aso using *o*MWCNTs-PEG hybrid, and fluorescence microscopy, which showed an increase in reactive oxygen species identified after Bcl-2 knock-down. Overall, *o*MWCNTs-PEG provided an exceptional biocompatible vehicle enabling the internalization of negatively charged nucleic acids and pH-sensitive release of cargoes in a hypoxic environment of the most of solid tumors. Moreover, Aso specifically binding to the first six codons of the Bcl-2 mRNA gave a satisfactorily decrease in Bcl-2 translation and an increase in NCIH2135 chemosensitivity towards VP-16.

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1. Introduction

Currently, lung cancer is one of the most serious diseases threatening people's health in the world [1]. Based on its histologic appearance, lung cancer can generally be classified into two major types: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [2]. In comparison with the SCLC, the NSCLC is relatively insensitive to chemotherapy and accounts for between 85% and 90% of lung-cancer deaths [3,4]. Presently, the main clinical therapeutic options in NSCLC management include surgery, radiotherapy and chemotherapy. However, surgery and radiotherapy are considered to be loco-regional tumor treatments. Randomized studies comparing chemotherapy with the "best supportive care" have evidenced a reduction of the symptoms and improvement of a quality of life [5]. Hence, chemotherapy is still necessary for the NSCLC patient in either the pre-operative or post-operative period [6].

Over the past decades, a number of agents have become available for the treatment of lung cancer, including the taxanes, gemcitabine, vinorelbine, and etoposide, whose response is enhanced by co-administration with platinum-based cytostatics [7]. Etoposide or VP-16 is an analogue of 4′-demethylepipodophyllin benzylidene glucoside isolated from podophyllotoxin in 1966 [8]. It belongs to the topoisomerase II inhibitors, which convert topoisomerases into physiological toxins, introducing high levels of transient protein-associated breaks in the genome of treated cells and their subsequent death [9]. The low white blood cell and platelet counts, hair loss, peripheral neuropathy and slight risk of developing secondary leukaemia belong among the most common side effects for the patients taking VP-16 [10]. The side effects and their sever-

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ity depend on the dose of drug administered, thus a suitable nanotransporter could decrease the VP-16 exposure in unwanted bodily sites and provide more effective delivery of the drug to tumor cells.

An ideal nanotransporter should possess high loading capacity and should remain stable in blood circulation for prolonged period of time so that it can avail an enhanced permeability and retention effect [11]. Carbon nanotubes (CNTs) possess high specific surface area which allows efficient drug loading and the possibility of further functionalization [12]. Their great biocompatibility can be fundamentally enhanced by the modification using polymers, such as polyethylene glycol (PEG) – widely used grafting agent, which provides a better solubility, avoids aggregation and prevents the reticuloendothelial system-mediated clearance during blood circulation [13].

Besides cytostatics, the CNTs can be modified using various bioactive compounds, such as antibodies, peptides or antisense deoxyoligonucleotides [14]. The CNTs modified with antisense deoxyoligonucleotides (Aso) act as mRNA silencers and down-regulate the expression of target molecules [15]. In the fight against lung cancers, the antisense therapy can interfere with cancer genes, producing malicious proteins, such as well-known inhibitor of apoptosis (IAP) B-cell lymphoma 2 (Bcl-2) that seems to be a great target for overcoming the chemoresistance in both, SCLC and NSCLC [16,17]. Bcl-2 targeted antisense deoxyoligonucleotides belong to the therapeutic family of pro-apoptotic agents, firstly introduced by Reed et al. [18] and further evaluated in the form of G3139 (Oblimersen sodium, Genasense, Genta, Inc., Berkeley Heights, NJ, USA) having the favorable safety profile, however, with a limited efficiency when administered as a single agent.

Hence, the aim of this study was to prepare oxidized PEGylated multi-walled carbon nanotubes (*o*MWCNTs-PEG) functionalized with VP-16 and phosphorothioated Aso targeted against Bcl-2 (denoted below as *o*MWCNTs-PEG-VP-16-Aso). The oxidized *o*MWCNTs, used as the core, can serve as a drug nanotransporter and the PEG shell, can load the drug as much as possible. Further, we demonstrated the co-administration of VP-16 and Aso conjugated on *o*MWCNTs-PEG in SCLC and NSCLC cell lines (DMS53 and NCIH2135, respectively) and examined the potential of the designated nanostructure to enhance the treatment efficacy of VP-16 in terms of growth properties of lung cancer cells, their effects on mRNA expression and protein levels and capability to induce the apoptosis.

2. Materials and methods

2.1. Chemicals

VP-16 (etoposide), all reagents for the syntheses, native oligonucleotides, standards and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity, unless noted otherwise.

2.2. Synthesis of oMWCNTs-PEG and conjugation with VP-16 and Bcl-2 antisense

The conjugates were prepared as described in our previous study [19]. Briefly, 2 mg of MWCNTs (Sigma Aldrich, St. Louis, MO, USA) were oxidized using 1 mL of 68% HNO_3 (Sigma Aldrich, MO, USA) in aqueous solution (*w/w*). After heating (1 h, 80 °C, 5000 × g), the sample was sonicated for 15 min and centrifuged (10 000 × *g* at 20 °C for 10 min). The supernatant was discarded and the resulting oxidized MWCNTs (hereinafter *o*MWCNTs) were centrifuged (10 000 × *g* at 20 °C for 10 min) in the presence of MiliQ water until the pH became 7. Finally, the volume was made up to 2 mL of MiliQ water. The grafting with PEG was carried out with 1 mL of crude PEG solution (40%, *v/v*) mixed with *o*MWCNTs (ratio 1:1) for 20 min at 25 °C. The solution was further centrifuged (10 000 × *g* at 20 °C for 10 min) and the supernatant was discarded to remove unbound PEG. The PEGylated-*o*MWCNTs (hereinafter denoted *o*MWCNTs-PEG) was re-dissolved in 1 mL of H_2O.

The conjugation of VP-16 with *o*MWCNTs-PEG was performed by mixing (1 h, 25 °C) the solutions in a ratio of 1:1 (*v/v*) to obtain the final concentration of 100 μM of the conjugated VP-16. The obtained complex was further aliquoted and modified using Aso targeting of Bcl-2, comprising 100% phosphorothioate linkages (5′-TCTCCCAGCGTGCGCCAT-3′), its reverse (rev) (5′-TACCGCGTGCGACCCTCT-3′) and scrambled (scr) sequence (5′-GCCATTGCATGCTCCGCC-3′), both 100% phosphorothioated deoxyoligonucleotides, which were utilized to monitor the specificity of the Aso. The modification with deoxyoligonucleotides was performed by using their aqueous solutions (final concentrations 10 μM) for direct mixing with *o*MWCNTs-PEG with VP-16 (mixed in ratio 1:1 *v/v*) in a thermo-mixer for 24 h at 25 °C and 5000 × g. The resulting solution of *o*MWCNT-PEG-VP-16-Aso was centrifuged (5000 × *g* at 20 °C for 10 min) and the supernatant was discarded to remove the unbound deoxyoligonucleotides. Re-dissolution was done using MiliQ water and the complexes were stored in 4 °C prior to application.

2.3. Scanning electron microscopy

The morphology of *o*MWCNTs-PEG-VP-16-Aso was examined using MIRA3 LMU (Tescan a.s., Brno, Czech Republic) with an accelerating voltage of 15 kV and a beam current of about 1 nA.

2.4. Evaluation of drug and deoxyoligonucleotides loading on oMWCNTs-PEG

After synthesis, the supernatant was discarded by centrifugation (10 000 × *g* at 20 °C for 10 min) and the amount of the present VP-16 or phoshorothioate deoxyoligonucleotides was examined using multifunctional microplate reader Tecan Infinite 200 PRO (Tecan, Männedorf, Switzerland) at λ_{exc} 250 nm and λ_{em} 320 nm and square wave voltammetry according to our previous study [19]. The drug loading was calculated according to Datir et al. [20].

2.5. VP-16 and Bcl-2 Aso release kinetics

To determine the *in vitro* release profiles of VP-16 and Aso, the complexes were stored in 1 mL PBS (pH 7.4, 0.01 M) and acetate buffer (pH 4.8, 0.2 M) in a 37 °C tempered water bath. At chosen time points (0; 1; 3; 6; 12; 24 h) aliquots of the eluted medium (50 μL) was removed for quantification; this volume was replaced with fresh buffer to prevent sink conditions. The quantification of VP-16 was carried out using multifunctional microplate reader Tecan Infinite 200 PRO (Tecan) at λ_{exc} 250 nm and λ_{em} 320 nm. The release of Aso was quantified by square wave voltammetry.

2.6. Analysis of DNA genotoxicity by the single cell gel electrophoresis (SCGE)

The cells were plated at a density of 10^5 cells/well in six-well dishes and treated with VP-16, *o*MWCNTs, *o*MWCNTs-VP-16, and the entire nanotransporter *o*MWCNTs-PEG-VP-16-Aso, *o*MWCNTs-PEG-VP-16-Rev and *o*MWCNTs-PEG-VP-16-Scr for 24 h. The applied concentrations of VP-16 and deoxyoligonucleotides were 10 μM and 1 μM, respectively, in all cases. As a control, 150 μM H_2O_2 was employed. After harvesting, about 15 μL of the cell suspension was mixed with 75 μL of 0.5% low melting point agarose (CLP, San Diego, CA, USA) and layered on one end of a

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