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A precision-guided MWNT mediated reawakening the sunk synergy in RAS for anti-angiogenesis lung cancer therapy

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ABSTRACT

Multi-walled carbon nanotube (MWNT) with its versatility has exhibited tremendous superiority in drug delivery. Despite plenty of researches on MWNT based delivery systems, precision-guided assistances to maximize their profitable properties are still lacking in substantive progress. We developed here a dual-targeting and co-delivery system based on MWNT for antiangiogenesis therapy in lung cancer which aimed at renin-angiotensin system (RAS) dysregulation by synergistically conducting angiotensin II type 1 receptor (AT_1R) and type 2 receptor (AT_2R) pathway. In this work, iRGD peptide connected to polyethyleneimine (PEI) was linked to MWNT skeleton, accompanying with candesartan (CD) conjugated to MWNT mediated by cystamine (SS). The functionalized MWNT is assembled with plasmid AT_2 (pAT_2) to form iRGD-PEI-MWNT-SS-CD/pAT_2 complexes. iRGD and CD act as pilots for complexes to dually target symbolic αvβ3-integrin and AT_1R both overexpressed on tumor angiogenic endothelium and lung cancer cell. CD as chemotherapy showed synergistic downregulation of VEGF when combining of pAT_2 and efficiently inhibited angiogenesis. iRGD-PEI-MWNT-SS-CD/pAT_2 complexes greatly appreciated drug activities by changing drug distribution and exhibited remarkable tumor growth suppression in A549 xenograft nude mice. Our work presents that such dual-targeting strategy highly improves the delivery performance of MWNT and open a new avenue for RAS related lung cancer therapy.

1. Introduction

Lung cancer has listed as malignant cancer with highest morbidity and mortality, yet the horrific data have not been effectively controlled [1,2]. Due to the lack of typical symptoms and signs [3], most patients with lung cancer present with metastatic disease at diagnosis [4] which results a poor benefit from surgery or chemotherapy [5–7]. Traditional therapies have reached a plateau in this sense since their incompetence in improving unfavorable prognosis [6,8,9], associating with extensive toxic effects. Lung cancer with angiogenesis throughout its occurrence, development and migration is a representative vessels dependence lesion [10–12]. Several studies have shown that the presence of neo-angiogenesis is a significantly negative prognostic factor for both overall and disease-free survival in lung cancer [13,14]. Therefore, anti-angiogenesis therapy with molecular targeting shows a potential strategy for lung cancer management.

Angiotensin II (Ang II) playing extensive physiological functions *via* angiotensin II type 1 receptor (AT_1R) and type 2 receptor (AT_2R) is a key biological peptide in renin-angiotensin system (RAS). Expanding cognition in RAS revealed that Ang II is frequently dysregulation in malignancy, and this correlates with poor patient outcomes [15,16]. Multiple studies now showed us that Ang II not only plays principal role in blood pressure regulation, but also involved deeply in controlling of cell proliferation and migration,

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and angiogenesis [17,18], which associated with the development and progress of a wide variety of tumors. It was generally realized there is an antagonism between AT_1R and AT_2R. Activating AT_1R accelerates angiogenesis by waterfall cytokine invoking and expedites tumor metastasis and malignant transformation [19,20]. While activating AT_2R inhibits angiogenesis and promotes cell apoptosis to neutralize the aforementioned effects [20–22]. Abnormally raising of Ang II level in lung cancer [23,24] leads to the hypothesis that simultaneously controlling of two receptors will harmony the dual directional regulation, further enhance anti-angiogenesis efficiency for NSCLC therapy.

To appreciate this synergetics therapy hegemony, two therapeutic agents, Candesartan (CD) and plasmid AT_2 (pAT_2), were employed as model drugs targeting to AT_1R and AT_2R, respectively. CD is one of the strongest member in angiotensin receptor blockers (ARBs) family which specifically binds to AT_1R, preventing AT_1R from activation by Ang II. Previous researches showed administrating with ARBs can prolong survival period of patients with advanced cancer [25,26] and reduce tumor vascular density [27–29]. With overexpression of AT_1R in lung cancer, CD is the solid choice to act as both of therapeutic and targeting ligand. pAT_2 is an exogenous gene coding AT_2R. Site-specific transfection with pAT_2 cause increasing expression of AT_2R in tumor which could enhance the activation effect of AT_2R, thus an AT_2R related antiangiogenesis and apoptosis could be achieved.

A crucial requirement for the above statement is developing a targeting vector to effectively co-deliver drug and gene to lung cancer. The versatile multi-walled carbon nanotube (MWNT), an inorganic one-dimensional carbon material with nested and cylindrical structure [30], is considered as potential candidate for this role. The needle-like architecture and predominant surface binding energy gift MWNT the ability to ship therapeutics crossing many biological and biophysical barriers with minimal cytotoxic effects [31] which make it a hotspot in nano-delivery systems [32]. Aside the great achievements in nanomedicine, tumor therapeutic efficacy is still far from perfect by absolutely targeting [33,34]. General active targeting strategy often failed since expression of targeting antigens in tumors are not high enough [35] or targeting antigens are not highly specific in tumor tissues [36,37]. This fact also empathizes with MWNT, regrettably burying its talents. Inspired by Kazuki et al. [38], a tumor-homing peptide (iRGD) which guided compounds bounding to tumor vessels and spreading into the extravascular tumor parenchyma was utilized as bullet, whereas conventional RGD peptides only delivered the cargo to the blood vessels. It would perform as targeting ligand cooperating with therapeutic CD which specifically recognizes AT_1R abundant in lung cancer tissues. Such dual-targeting concept providing the MWNT higher odds for both tumor angiogenic endothelium and lung cancer cell capture may break the foregoing dilemmas, and amplify anti-tumor response by reducing nonspecific distribution. In addition, for assisting to construct the novel vector, proficient polyethyleneimine 1.8k (PEI 1.8k) as water-soluble modifier to improve biocompatibility and complexation ability of MWNT, cystamine (SS) as linkage of CD to achieve smart release in tumor reducing environment, were employed just like intermediary to integrate all mentioned resources.

Herein, we successfully developed a novel MWNT based dual-targeting and co-delivery system by conjugating iRGD linked PEI and SS modified CD to the backbone of MWNT to complex with pAT_2 (iRGD-PEI-MWNT-SS-CD/pAT_2). Subsequently, the chemical structure and physicochemical properties of iRGD-PEI-MWNT-SS-CD/pAT_2 complexes were investigated in detail. The loading and releasing capacities of drug and gene were also evaluated. Further to this, cellular uptake and gene transfection with different complexes or conditions were performed to investigate influence of targeting ligands in cellular affinity, as well as intracellular trafficking. A series pharmacodynamics and pharmacology assays were carried out on A549 and HUVEC cell lines for preliminary evaluation of synergetic effects of both therapeutic. *In vivo*, the selective targeting behavior and biodistribution of Cy7 labeled iRGD-PEI-MWNT-SS-CD/pAT_2 were studied in A549 bearing nude mice using a near-infrared fluorescence imaging system. The tumor suppression and anti-angiogenic effect were finally evaluated after i.v. administration of iRGD-PEI-MWNT-SS-CD/pAT_2 in A549 bearing nude mice.

2. Materials and methods

2.1. Materials

Multi-walled carbon nanotube (MWNT, 5–15 μm in length, 10–20 nm in diameter) were purchased from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China). Branched polyethyleneimine (PEI, 1.8K and 25K) and candesartan (CD) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Astra Zeneca (Hamburg, Germany), respectively. *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), cystamine dihydrochloride (SS·2HCl), glutathione (GSH), concentrated sulphuric acid (98%) and concentrated nitric acid (65%) were offered by Aladdin Reagent Database Inc. (Shanghai, China). iRGD tumor-homing peptides (iRGD, 90%), and reporter plasmid (pEGFP-C3) encoding enhanced green fluorescent protein (EGFP) and plasmid AT_2 (pAT_2) encoding Angiotensin II type 2 receptor (AT_2R) both propagated in DH-5a *Escherichia coli* were purchased from Biogottechnology, co, Ltd (Nanjing, China). The plasmids were driven by CMV promoter purchased from Addgene and purified by Endo Free Plasmid Maxi Kit (Qiagen, Germany). DNase I, fluorescein isothiocyanate (FITC), LysoTracker Red and 4′, 6-diamidino-2-phenylindole (DAPI) were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Primary antibodies and Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA). All other reagents were of analytical grade and used without further purification.

2.2. Preparation of functionalized MWNT (f-MWNT, including PEI-MWNT, PEI-MWNT-SS-CD, iRGD-PEI-MWNT and iRGD-PEI-MWNT-SS-CD)

Pristine MWNT (200 mg) was suspended in mixed acid (concentrate $H_2SO_4 + HNO_3$, v/v = 3/1, 120 mL) by ultrasonic water bath at room temperature (R.T.) for different time (6 h, 12 h, 18 h, 24 h, 30 h, and 36 h). The suspension was then diluted with deionized water and filtered through a MCE membrane (0.22 μm), followed by washing repeatedly until pH of the filtrate was neutral. The resulting solid was redispersed in deionized water and lyophilized to receive oxidized MWNT (MWNT-COOH).

For synthesis of SS-CD, NHS (103.6 mg, 0.9 mmol) and EDC (172.5 mg, 0.9 mmol) were added into CD (132.1 mg, 0.3 mmol) dissolved in DMSO with 30 min of agitation at R.T. to receive activated carboxyl groups. Then the solution was reacted with SS (185.1 mg, 1.2 mmol) for 24 h, which was prepared by removing hydrochloride from cystamine dihydrochloride (SS·2HCl) according to the method described by Hamid, Z. A. et al. [39]. Reaction products in DMSO were extracted into dichloromethane followed by vacuum rotary evaporation to enrich target compounds, then developed on silica G thin layer plate with DMC-MeOH (v/v = 10/1). Monosubstituted SS-CD was obtained by redissolving the target spot on plate into dichloromethane and vacuum rotary evaporation.

PEI-MWNT was synthesized by an amidation reaction between