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Enhanced docetaxel-mediated cytotoxicity in human prostate cancer cells through knockdown of cofilin-1 by carbon nanohorn delivered siRNA

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ABSTRACT

We synthesized a non-viral delivery system (***f*-CNH3**) for small interfering RNA (siRNA) by anchoring a fourth-generation polyamidoamine dendrimer (G4-PAMAM) to carbon nanohorns (CNHs). Using this new compound, we delivered a specific siRNA designed to knockdown cofilin-1, a key protein in the regulation of cellular cytoskeleton, to human prostate cancer (PCa) cells. The carbon nanohorn (CNH) derivative was able to bind siRNA and release it in the presence of an excess of the polyanion heparin. Moreover, this hybrid nanomaterial protected the siRNA from RNAse-mediated degradation. Synthetic siRNA delivered to PCa cells by ***f*-CNH3** decreased the cofilin-1 mRNA and protein levels to about 20% of control values. Docetaxel, the drug of choice for the treatment of PCa, produced a concentration-dependent activation of caspase-3, an increase in cell death assessed by lactate dehydrogenase release to the culture medium, cell cycle arrest and inhibition of tumor cell proliferation. All of these toxic effects were potentiated when cofilin-1 was down regulated in these cells by a siRNA delivered by the nanoparticle. This suggests that knocking down certain proteins involved in cancer cell survival and/or proliferation may potentiate the cytotoxic actions of anticancer drugs and it might be a new therapeutic approach to treat tumors.

1. Introduction

Prostate cancer (PCa) is one of the main causes of death among men in the Western world. Autopsy series have revealed that small prostatic carcinomas are present in up to 64 percent of men from 60 to 70 years of age [1]. Moreover, the risk of death due to metastatic PCa is 1 in 30 [2]. Current treatments attempt to block PCa cell growth and induce cell death [3], but the progression of advanced PCa to hormone independence is only temporally disrupted by therapeutic interventions including androgen ablation therapy or chemotherapy [4]. Docetaxel, an antimitotic cytotoxic drug belonging to the taxoid family, is used as the gold-standard therapy for patients with advanced PCa [5]. Docetaxel's anticancer effects are associated with its ability to induce the polymerization of tubulin, which impairs cell proliferation, which in turn leads to mitotic arrest and apoptosis [6,7]. However, docetaxel-based chemotherapy has undesirable side effects that limit the tolerated dose and may reduce the therapeutic efficacy [8]. This result suggests that coadjuvant therapy leading to a reduction of the required dose of docetaxel would be beneficial to PCa therapy. One possible approach is to knockdown proteins involved in cancer cell survival or proliferation using specific RNA interference (RNAi) [9].

Dynamic changes in the actin cytoskeleton, such as depolymerization and severing of actin filaments, are essential for several cellular processes including cell survival, shaping, cytokinesis, migration and chemotaxis [10] suggesting that the cytoskeleton is a potential target for cancer therapies [11,12]. The actin depolymerizing factor (ADF)/cofilin has emerged as one of the protein families that regulates actin and cytoskeleton dynamics [13]. Furthermore, cofilin type 1 (cofilin-1) is an ubiquitous protein regulating various cell functions, such as cell cycle control and proliferation [14], apoptosis [15] and excitotoxic neurodegeneration [16]. In addition, enhanced amounts of cofilin-1 are related to the progression of some types of tumors and metastases [17–19] suggesting that cofilin-1 knockdown may have therapeutic benefits when combined with antitumoral drugs in cancer therapy.

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Small interfering RNAs (siRNAs) are double-stranded RNA molecules that induce sequence-specific degradation of homologous single-stranded RNA [20]. The efficiency of siRNA and its limited side effects have made this technique an attractive alternative to the use of antisense oligonucleotides for therapies based on the inhibition of target genes [21]. Synthetic siRNAs are easy to deliver, require only small doses to produce their silencing effects, and can inactivate a gene at almost any stage in development [22]. It has been proposed that the efficiency of transfection for siRNA at the cellular level depends on cellular uptake, stability and endosomal escape. Various systems including polyethyleneimine, inorganic/metals, cationic lipids and dendrimers have been used for siRNA delivery to several cell types [23–25]. However, many of these systems do not achieve good transfection efficiency. Therefore, new, more efficient siRNA delivery systems that increase transfection efficiency are needed.

Previous results from our group have shown that CNHs can be used as a non-viral carrier to deliver siRNA [26]. In the present study, we synthesized and characterized a new CNH derivative using fourth-generation polyamidoamine (G4-PAMAM) dendrimers anchored to the CNH [27] surface and studied its efficiency to silence key proteins involved in the proliferation of prostate cancer cells and the effect of this silencing on the toxic actions of the antitumoral drug docetaxel.

2. Material and methods

2.1. Techniques

Microwave irradiations were carried out in a CEM DISCOVER S-Class reactor, with infrared pyrometer, pressure control system, stirring and an air-cooling option. The thermogravimetric analyses were performed with a TGA Q50 (TA Instruments) at 10 °C/min under N_2. For the TEM analyses a small amount of the functionalized CNHs was suspended in water and a drop of the suspension was placed on a copper grid (3.00 mm, 200 mesh, coated with carbon film). After air-drying, the sample was investigated by TEM, Philips EM 208, accelerating voltage of 100 kV.

2.2. Materials

Solvents were purchased from SDS and Fluka (Buchs, Switzerland). All dry solvents were freshly distilled under argon over an appropriate drying agent before use. Chemicals were purchased from Sigma–Aldrich (Barcelona, Spain) or Acros Organics (Geel, Belgium) and used as received without further purification. Tert-butyl 6-(4-aminophenoxy)hexylcarbamate **1** was synthesized following the standard procedure found in the literature [28]. CNHs were produced by Carbonium s.r.l., Padova (Italy) by direct graphite evaporation in Ar flow, according to a patented method [29] and used without purification.

2.3. Synthesis of CNH derivatives

2.3.1. Boc-protected CNH intermediate

In a typical experiment, 20 mg of pristine CNHs (***p*-CNHs**) were sonicated in deionized water together with **1** (2.05 g, 6.68 mmol) for 10 min in a microwave glass vessel. After that time, isoamyl nitrite (0.44 mL, 3.34 mmol) was added, and a condenser was put in place. The mixture was irradiated for 90 min at 80 °C at 100 W for 30 min, and after the addition of a new aliquot of isoamyl nitrite (0.44 mL, 3.34 mmol), at 30 W for 60 min at 80 °C. After cooling to room temperature, the crude was filtered on a Millipore membrane (PTFE, 0.2 mm). The collected black solid was washed using cycles of sonication and filtration with methanol and acetone until the filtrate was clear and ultimately dried under high vacuum, yielding 16.2 mg of **Boc-protected CNH intermediate**.

2.3.2. f-CNH1

HCl gas was bubbled for 5 min through a suspension of **Boc-protected CNH intermediate** (16.2 mg) in methanol (20 mL). The reaction mixture was stirred at room temperature for 14h, filtered on a Millipore membrane (GTTP, 0.2 μm) and washed by cycles of sonication and filtration using 50 mL of a mixture water/methanol (1:1). The collected black solid was dried under high vacuum, yielding 16 mg of ***f*-CNH1**.

2.3.3. f-CNH2

Twenty milligrams of ***f*-CNH1** were sonicated in methanol for 5 min in a microwave glass vessel. Sonication was followed by the addition of *N*-ethyldiisopropylamine (1 mL, 4.83 mmol) and methyl acrylate (3 mL, 0.047 mmol), and the mixture was sonicated for 2 min. Finally, a condenser was put in place and the mixture was irradiated at 60 °C at 10 W for 60 min. After cooling to room temperature, the crude was filtered on a Millipore membrane (GTTP, 0.2 μm) and washed by cycles of sonication and filtration using 75 mL of a water/methanol mixture (1:1). The collected black solid was finally dried under high vacuum, yielding 20 mg of ***f*-CNH2**.

2.3.4. f-CNH3

10 mg of ***f*-CNH2** were suspended in 10 mL of methanol and sonicated for 10 min, followed by the addition of the corresponding dendrimer: (0.015 mL of PAMAM dendrimer G4-NH_2 (10% w/v solution in methanol)). Next, a condenser was put in place and the reaction mixture was heated at 40 °C for 1 day. Subsequently, the crude was filtered on a Millipore membrane (GTTP, 0.2 μm) and washed by cycles of sonication and filtration using 75 mL of a water/methanol mixture (1:1). The collected black solid was dried under high vacuum, yielding 10 mg of ***f*-CNH3.**

2.4. Agarose gel retardation assay

Agarose gel electrophoresis was performed to analyze the siRNA (Qiagen, Valencia, CA) binding ability of ***f*-CNH3** as previously described [30]. The ***f*-CNH3**/siRNA complexes were prepared at increasing N/P ratios (the molar ratio between the ***f*-CNH3** amine groups and the siRNA phosphate groups) and incubated for 30 min at room temperature. For the N/P ratio calculation, we considered that only the outer primary amines of the G4-PAMAM dendrimers were protonated at the pH used in the experiments (5.5). Each sample was then loaded onto a 1.2% agarose gel containing ethidium bromide (0.05 mg/mL). Electrophoresis was performed at 60 mV for 15 min, and the resulting gels were photographed under UV-illumination. The binding capacity was evaluated based on the relative intensity of the free siRNA band in each gel lane with respect to the lane with naked siRNA.

2.5. siRNA release by polyanion competition

The ability of complexes to release siRNA after a challenge with the competing polyanionic heparin was determined as a measure of the ability of the dendriplex to be dissociated in an environment with an excess of negatively charged compounds, such as the cell interior [30]. Complexes were prepared as indicated above at a ***f*-CNH3**/siRNA N/P ratio of 7.5 to ensure complete binding of siRNA by the CNH derivative, and then incubated with increasing heparin sulfate concentrations (0, 1, 2, 4, 6, 8, and 10 μg/μg***f*-CNH3**). The samples were run on an agarose gel as described above.

2.6. siRNA protection against RNAses

To study the protective actions of the dendriplex against siRNA cleavage by RNAses, ***f*-CNH3**/siRNA complexes, prepared at an N/P ratio of 7.5 as indicated above, or naked siRNA were incubated with 0.25% RNAase A for 30 min at 37 °C. Afterwards, RNAse was inactivated and the samples were incubated with an excess of heparin sulfate to ensure a complete siRNA release from the dendriplex as previously described [9]. Then, samples were loaded onto an agarose gel, under the same experimental conditions as the experiments described above.

2.7. ***f*-CNH3**/siRNA complex formation

Predesigned siRNA targeting human cofilin-1 (Hs_CFL1_3), and a control scrambled (SCR) siRNA targeting a sequence not sharing homology with the human genome (AllStars Negative Control) were purchased from Qiagen (Crawley, UK). LNCaP cells were transfected with siRNAs using the ***f*-CNH3** as non-viral vector. Briefly, 15 μg/mL of ***f*-CNH3** together with 100 nmol/L siRNA were incubated for 30 min at room temperature (15–25 °C) to form the transfection complexes. Cells, seeded on either 6- or 24-well plates, were 70–80% confluent at the time of transfection. RPMI-1640 medium was replaced with fresh medium and the ***f*-CNH3**/siRNA suspension was added to cells and incubated at 37 °C in a humidified atmosphere with 5% CO_2. For some experiments, after ***f*-CNH3**/siRNA transfection for 72 h, LNCaP cells were exposed to varying doses of docetaxel (1–50 nmol/L) for 24 h. Experiments were carried out three times unless otherwise specified.

2.8. Cell line and culture conditions

The human androgen-sensitive human PCa (LNCaP) cells were obtained from the American Type Culture and Collection (ATCC). LNCaP cells were routinely cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 2 mmol/L L-glutamine (Invitrogen, Carlsbad, CA), 100 μg/ml streptomycin (Sigma, St. Louis, MO) and 100 IU/ml penicillin (Sigma, St. Louis, MO) in a 5% CO_2-humidified incubator at 37 °C.

2.9. siRNA uptake and toxicity

A fluorescein-labeled siRNA (siRNA-FAM, Qiagen, Crawley, UK) and propidium iodide (PI, Sigma, St. Louis, MO) were used to study the uptake of siRNA into LNCaP