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Internalization of C60 fullerenes into cancer cells with accumulation in the nucleus via the nuclear pore complex

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ABSTRACT

A highly water-soluble, non-ionic, and non-cytotoxic fullerene malonodiserinolamide-derivatized fullerene C_{60} (C_{60}-ser) is under investigation as a potential nanovector to deliver biologic and cancer drugs across biological barriers. Using laser-scanning confocal microscopy and flow cytometry, we find that PF-633 fluorophore conjugated C_{60}-ser nanoparticles (C_{60}-serPF) are internalized within living cancer cells in association with serum proteins through multiple energy-dependent pathways, and escape endocytotic vesicles to eventually localize and accumulate in the nucleus of the cells through the nuclear pore complex. Furthermore, in a mouse model of liver cancer, the C_{60}-serPF conjugate is detected in most tissues, permeating through the altered vasculature of the tumor and the tightly-regulated blood brain barrier while evading the reticulo-endothelial system.

1. Introduction

Fullerenes are carbon cages with nanosized dimensions and an esthetic symmetry that has fascinated scientists since their discovery in 1985 [1]. Biological use of pristine C_{60} is limited by its insolubility in aqueous environments. The motivation to exploit size, geometry and molecular topology for therapeutic applications has resulted in the synthesis and characterization of a number of water-soluble fullerene derivatives. It is not surprising to note that amidst concerns of toxicity, the list of potential biological application of these derivatized carbon-structures continues to grow to include drug delivery agents [2,3], gene therapy agents [4,5], photodynamic [6–9] and photoacoustic [10] cancer therapy agents, as antioxidants [11], radioprotection agents [12,13] and diagnostic contrast agents [14,15].

The use of derivatized, water-soluble, non-toxic fullerenes is of particular interest to us in designing nanovectors for cancer therapeutics. The fullerene cage offers a unique three-dimensional scaffold for covalent attachment of multiple drugs for single-dose combination therapy and thus, has a clear advantage over many other nanovectors that utilize non-covalent loading, embedding, or encapsulation strategies. For instance, in separate studies, C_{60}-paclitaxel and C_{60}-doxorubicin conjugates were designed as a slow-release formulation that significantly enhanced the bioavailability and therapeutic efficacy of paclitaxel and doxorubicin against lung cancer cells and melanoma tumors, respectively [2,3]. Despite their immense potential as nanovectors, studies on transport of derivatized fullerenes in cancer cells and in relevant pre-clinical tumor models are limited. One of the main challenges to the study of biodistribution and subcellular localization is the difficulty in detecting individual fullerene-derivatized nanoparticles (NPs). Using metal, fluorophore, or radioisotope-tagged fullerene derivatives, several reports have documented cellular uptake and subcellular localization via endocytotic processes. For instance, separate studies have demonstrated that malonic acid derivatized $C_{60}C(COOH)_2$ and $C_{63}(COOH)_6$ localize predominantly to the mitochondria [16,17] and the bis-adduct, $C_{60}(C(COOH)_2)_2$ localized to lysosomes [18]. In another report, an amine-functionalized C_{70}-Texas Red conjugate was found to co-localize mostly with the endoplasmic reticulum and to a lesser extent with the lysosomes and mitochondria of mast cells [19]. A cumulative assessment of these reports suggests that subcellular localization of derivatized-fullerenes is dependent on the nature of functionalization and possibly the type of cells investigated. Both observations are attractive avenues for further study, as there is the

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potential to target cancer cells and specific subcellular locations of interest by fullerene cage modification.

The synthesis and characterization of highly water-soluble, non-ionic and functionalized derivatives of C_{60} have been previously reported [20]. Our current investigation pertains to the cellular transport of a malonodiserinolamide-derivatized fullerene C_{60} (C_{60}-ser) in liver cancer cells. In order to study the processing of C_{60}-ser by liver cancer cells, C_{60}-ser has been covalently attached to the fluorophore, PromoFluor-633 (to produce C_{60}-serPF) with an emission spectrum at >600 nm to produce minimal interference with cell auto-fluorescence and absorption by the C_{60} core. The conjugate was synthesized using an amide-linkage to protect against hydrolases within the cell (Fig. 1, Figs. S1 and S2). Using laser-scanning confocal microscopy and flow cytometry, we investigate the cellular localization and mechanism of uptake of C_{60}-serPF in liver cancer cells. Furthermore, we evaluate the biodistribution of these nanoparticles in a mouse model of liver cancer.

2. Materials & methods

2.1. Synthesis and characterization of C_{60}-serPF conjugate

C_{60}-ser has been prepared as described previously [15]. C_{60}-serPF has been synthesized and purified according to the procedure described in the Supporting information. A scheme is represented in Fig. 1.

2.2. Materials, cell culture and cell lines

We obtained the Hep3B cell line from American type culture collection (ATCC) and the Huh7 cell line was a kind gift from Dr. Felipe Samaniego (M.D. Anderson Cancer Center). Cells were maintained in MEM cell culture media and 10% fetal bovine serum, supplemented with sodium pyruvate, non-essential amino acids and penicillin G/streptomycin (GIBCO). The cells were grown in 150 cm^2 flasks placed in a humidified incubator at 37 °C with 5% CO_2 before experimental use. The following inhibitors were used (all from Sigma–Aldrich, Milwaukee, WI); Chlorpromazine (50 μM), di-methyl amiloride (DMA, 100 μM), Methyl β-cyclodextrin (MBCD, 10 mM), Nystatin (50 μM), Dynasore (80 μM), Thapsigargin (0.5 μM).

2.3. Cytotoxicity studies

Cytotoxicity was assessed using propidium iodide staining. Promidium iodide is extruded from viable cells. After varying duration and concentrations of exposure to C_{60}-ser, C_{60}-serPF, or PF-633, cells were trypsinized and washed with PBS. Approximately 0.3 ml of PI/RNase solution was added to cells (BD Pharmingen, San Diego, CA). Cells were incubated on ice for 15 min and analyzed using Flow cytometry (BD LSR II, BD Biosciences, San Jose, CA).

2.4. Laser-scanning confocal microscopy analysis

For confocal microscopy experiments, cells were grown on #1.5 round cover slips in 12-well plates at approximately 50,000 cells per well. After 24 h sub-confluent monolayers were observed. Cells were washed with phosphate-buffered saline (PBS) once and then incubated at the desired concentration of C_{60}-serPF for varying duration. At the end of incubation period, cells were washed with PBS and fixed in 1% Paraformaldehyde for 30 min, then washed again and stained with 4′, 6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA). The cover slips were placed on microscopy slides with a drop of Gold anti-fade reagent (Invitrogen) and sealed. Imaging was performed with Olympus Laser-scanning FV500 microscope using a 60× oil objective and a Z-stack resolution set at 100 nm. Fixed cells were excited with a 405 nm laser and a red 633 nm HeNe laser and emission recorded at 470 nm and 660 nm respectively in sequential steps to prevent bleed-through and photo-bleaching. Images were analyzed in Slidebook (v5.0).

2.5. Nuclear extraction and flow cytometry analysis

After incubation with C_{60}-serPF for varying duration cells were washed with PBS and re-suspended in ice-cold nuclei extraction buffer (sucrose (320 mM), $MgCl_2$ (5 mM), Hepes (10 mM), Triton (1% v: v) was mixed and pH adjusted to 7.4), vortexed for 10 s, and incubated on ice for 10 min. Nuclei were then pelleted at 2000 g for 5 min and re-suspended in wash buffer (same as nuclei extraction buffer but without triton). This was repeated twice and nuclear extraction was confirmed by bright-field microscopy and trypan blue staining. The extracted nuclei were then stained with propidium iodide for 15 min before flow cytometry analysis. Single-parameter (nuclear localization studies) and multi-parameter (cell cycle studies) flow cytometry analysis was performed using a BD LSR II FACS (Beckton Dickinson, Franklin Lakes, NJ). For C_{60}-serPF detection, nuclei or cells were excited with 640 nm SORP Red Laser and emission was recorded at 635–684 nm. For DNA content measurements, propidium iodide fluorescence was recorded at 570–597 nm after excitation with a 561 nm laser. To measure internalization of C_{60}-serPF, approximately 1 million cells were incubated with or without pharmacological inhibitors for 45 min on ice in cell culture media as described above. C_{60}-serPF was then added and cells were restored to 37 °C. Aliquots (50 μL) were removed at various time points and fluorescence was measured as described above. At $t = 0$ min, cells exposed to the conjugate were immediately assessed to calculate the background signal due to passive adsorption of C_{60}-serPF.

2.6. Animal experiments

A mouse model of liver cancer was generated using 4 wk old CB17 SCID mice. Animals were anesthetized using isoflurane and a transverse incision was made in the upper abdomen under sterile conditions. Approximately 1.5 million Hep3B cells were injected in the liver in a 10 μL volume (Hamilton Syringes). Hemostasis was restored using gentle pressure and silver nitrate cautery. Biodistribution experiments were conducted 4 weeks after injection. C_{60}-serPF was injected in the dorsal tail vein. Animals were sacrificed at various time points starting at 16 h and tissues were harvested for fluorescence imaging and histology. Fluorescence imaging was performed on harvested tissues immediately, with Xenogen optical *in vivo* imaging system (IVIS 200, Caliper Life Sciences, Hopkinton, MA) and using Cy5.5 excitation (615–665 nm) and emission (695–770 nm) filter set.

3. Results

3.1. C_{60}-serPF localization in liver cancer cells

Laser-scanning confocal microscopy was used to study fluorescently labeled C_{60}-serPF uptake in human liver cancer cells. We exposed Hep3B and Huh7 cells to varying concentrations of C_{60}-serPF for 2 h and the cells were fixed for imaging. As shown in Fig. 2 and Fig. S3, a dose-dependent uptake was observed in a linear fashion. We observed that at lower concentrations, 78 ± 8% of the total fluorescence intensity from each cell localized to the cytosol. However, at higher concentrations this proportion decreased to approximately 50%. Conversely, nuclear uptake increased from 22 ± 8% at 1 μg/ml to 47 ± 5% at 100 μg/ml. Similar observations were obtained with Huh7 cells. Since the relative physical dimensions of the nucleus and cytoplasm vary in each cell, we calculated the sum fluorescence intensity per μm^3 from the nucleus and the cytoplasm of each cell. As shown in Fig. 2A (bottom, left), C_{60}-serPF preferentially accumulated in the nucleus, reaching concentrations up to three times that in the cytoplasm at higher incubation concentrations. In order to evaluate the integrity of the conjugate within the nucleus, we measured fluorescence-decay of free PromoFluor-633 dye and C_{60}-serPF conjugate in separate experiments. The half-life of the free PromoFluor was 54 s and that of C_{60}-serPF was 146 s in the nucleus. The fluorescence decay observed is comparable to the decay observed when the compounds are loaded in Sephadex microbeads and subjected to photo-bleaching (Fig. S4). These findings imply that the C_{60}-serPF conjugate remains intact in the cellular environment.

In order to quantify the proportion of cells with uptake in the nucleus, nuclei were extracted from unfixed cells after a 2-h exposure to C_{60}-serPF. The nuclear extraction procedure resulted in a yield of >97% as determined through microscopic analysis after trypan blue staining and a decrease in forward scatter on flow cytometry. As shown in Fig. 2B, 88% and 87% of the nuclei extracted from Hep3B and Huh7 cells were positive for C_{60}-serPF, respectively. This data is consistent with confocal microscopy observations that the majority of nuclei were observed to emit varying intensity of fluorescence signal above background.

We investigated whether nuclear localization could occur because of membrane permeabilization caused by cytotoxicity of the conjugate. Propidium iodide is a fluorescent cell impermeant dye that fluoresces after intercalating with nucleic acids in membrane-damaged cells. Our data demonstrated that C_{60}-ser, PF