Journal of Pharmaceutical Sciences xxx (2016) 1-11

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Nanotechnology

# Cholesterol-Targeted Anticancer and Apoptotic Effects of Anionic and Polycationic Amphiphilic Cyclodextrin Nanoparticles

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ARTICLE INFO

*Article history:*
Received 31 January 2016
Revised 7 June 2016
Accepted 24 June 2016

*Keywords:*
apoptosis
cancer
cell culture
cyclodextrin
nanoparticles

ABSTRACT

Amphiphilic cyclodextrins (CDs) are biocompatible derivatives of natural CDs and are able to form nanoparticles or polyplexes spontaneously. In this study, nanoparticles prepared from nonionic (6OCaproβCD) or cationic amphiphilic CD (PC βCDC6) were used comparatively to develop nanoparticles intended for breast cancer therapy. The characterization of these nanoparticles was performed both by *in vitro* and cell culture studies. Furthermore, the apoptotic and cytotoxic effects of blank amphiphilic CDs were demonstrated by various mechanistic methods including Caspase-8 activity, lipid peroxidation assay, TUNEL assay, Tali®-based image analysis, cholesterol assay, and gene expression studies. Blank nanoparticles exerted cytotoxicity against a variety of cancer cells (MCF-7, HeLa, HepG2, and MB49) but none to healthy cells (L929, G/G). Interestingly, blank 6OCaproβCD and blank PC βCDC6 derivatives were found to be intrinsically effective on cell number and membrane integrity of MCF-7 cells in apoptosis studies. Further in-depth studies were performed to elucidate the selective mechanism of anticancer action in MCF-7 cells caused by these amphiphilic CDs. In conclusion, blank amphiphilic CD nanoparticles induced apoptosis through mitochondrial pathway targeted to cholesterol microdomains in cancer cell membrane.



## Introduction

Breast cancer is the most prevalent cancer in women, characterized by uncontrolled proliferation of lining cells in the mammary gland and ducts which may also spread into other parts of the body eventually. Parallel to its high incidence, breast cancer was reported to be the third leading cause of cancer death in 2014.[1-3] According to the National Cancer Institute, by the year 2030 breast cancer incidences are expected to rise up to 50%, meaning that 1 in 8 women will be estimated to develop breast cancer. Success in breast cancer treatment depends on selective, efficient, and safe delivery of chemotherapeutic drugs to tumor site. However, when it comes to conventional chemotherapy, there exist certain limitations. Pharmacokinetic variability of the drug, cellular or non−cellular-based resistance mechanisms, drug solubility, or stability problems may also be crucial parameters adding up to toxicity and side effects or ineffective chemotherapy.[2,3] To overcome the side effects of chemotherapy and provide effective cancer therapy, it is necessary to recognize cellular and molecular basis of cancer.

Amphiphilic cyclodextrins (CDs) are cyclic oligosaccharide derivatives designed by grafting hydrocarbon chains on the hydroxyl groups of either the primary or the secondary face of natural CDs.[4] Their unique structure gives them the ability to form nanoparticles spontaneously and to include active molecules in their hydrophobic inner cavity as well as within their long aliphatic chains.[5] Major advantages of amphiphilic CDs are the improved interaction with biological membranes, enhancement of inclusion complex capacity, prevention of CD-induced hemolysis, and spontaneous formation of nanospheres or nanocapsules avoiding the use of surfactants or other surface active agents.[6] Moreover, the properties of amphiphilic CDs can be tailored by implementing selective chemical manipulation schemes[7] to facilitate, for instance, cell membrane receptor targeting.[8,9]

Cholesterol is one of the main components in the structure of cell membranes and plays an important role in cell viability.

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http://dx.doi.org/10.1016/j.xphs.2016.06.021
0022-3549/

Cholesterol is responsible for (1) the integrity of cell membrane, (2) regulation of membrane fluidity, and (3) facilitating the transmission of cell signals. The cholesterol content of cell membranes varies according to the type of the cell.[10] This difference is more evident between healthy cells and cancer cells. Cancer cells require more nutrients and various receptors due to the high growth rate.[11] For this reason, new membrane is synthesized rapidly in growing cancer cells. Cholesterol-enriched microdomains are necessary for tumor cells to provide growth factors having an essential role on cell proliferation and survival.[12] Moreover, the cholesterol content of sensitive and resistant cells is also different.[13] For these reasons, cholesterol targeting or cholesterol-based conjugates attract attention in recent years for cancer therapy. Cholesterol or cholesterol conjugation approach is tumor selective. CDs are reported to have affinity to cholesterol due to their lipophilic inner cavity. It is well known that CDs, especially methylated CDs, extract cholesterol from cell membrane.[14] Thus CDs are used in the biomedical field to control the level of cholesterol in the membrane as well as to achieve targeting to cholesterol-rich domains in the cell membranes.

The aim of this study was to evaluate the potential of cationic amphiphilic CDs as nanocarriers for anticancer drugs in comparison to their nonionic analogs. Effects of surface charge on interaction with cancer cell membrane were evaluated thoroughly focusing on the intrinsic apoptotic effect of amphiphilic CDs through mechanistic and molecular studies. Quality, safety, and efficacy of both blank negatively and positively charged amphiphilic CD nanoparticles were evaluated for cancer therapy.

## Materials and Methods

### Materials

Nonionic amphiphilic CD heptakis (6-*O* hexanoil) cyclomaltoheptose (6OCaproβCD)[15] and polycationic amphiphilic CD (PC βCDC6)[16] were synthetized as described previously in the University of Sevilla, Sevilla, Spain. Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, MO). TUNEL assay kit (ApopTag® Plus Peroxidase In situ Apoptosis Detection Kit, S7101) was purchased from Millipore (Billerica, MA). Caspase-8 kit (ApoTarget Caspase-3/CPP32 Colorimetric Protease Assay; Invitrogen, Carlsbad, CA) was purchased from Thermo Fisher (Waltham, MA). Lipid peroxidation assay kit (ALDetect™ BML-AK170) was purchased from Enzo Life Sciences (Farmingdale, NY). Cholesterol quantitation kit (MAK043) was purchased from Sigma-Aldrich. All other chemicals used were of analytical grade and obtained from Sigma-Aldrich. Ultrapure water was used as obtained from Millipore Simplicity 185 Ultrapure Water System (Millipore).

### Methods

#### Preparation of Amphiphilic CD Nanoparticles

PC βCDC6 nanoparticles and anionic 6OCaproβCD nanoparticles were prepared according to nanoprecipitation method as described previously.[17] Briefly, 1 mg of PC βCDC6 or nonionic 6OCaproβCD was dissolved in 1 mL of suitable water-miscible organic solvent (ethanol, methanol, or acetone) (0.1% wt/vol). This organic phase was added dropwise into an aqueous phase (2 mL) under magnetic stirring at room temperature. The organic phase was then evaporated under vacuum at 40°C to the desired final volume of 2 mL.

#### Physicochemical Characterization of Amphiphilic CD Nanoparticles

*Particle Size Distribution..* Mean particle size (nm ± SD) and polydispersity index (PDI) of amphiphilic CD nanoparticles were determined by dynamic light scattering technique (Malvern NanoZS; Malvern Instruments, Malvern, UK). All formulations were measured at an angle of 173° at room temperature by triplicate.

*Surface Charge Measurement.* In order to determine the surface charge of nanoparticles, zeta potential values (mV) were measured with Malvern NanoZS at room temperature by triplicate.

#### In Vitro *Cellular Interaction Studies*

*Cell Culture.* Mouse fibroblast cells (L929), mouse urinary bladder cell (G/G), human breast cancer cell line (MCF-7), mouse bladder cell line (MB49), human liver hepatocellular carcinoma cell line (HepG2), and human epithelial adenocarcinoma cell line (HeLa) were purchased from American Type Culture Collection (Manassas, VA). For *in vitro* cell culture and cellular interaction studies, 2 different nanoparticle dilution rates were used. The concentration of CDs is 83 μg/mL for 1:2 vol/vol dilution and 10 μg/mL for 1:16 vol/vol dilution. All amphiphilic CD nanoparticles were diluted with complete DMEM containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). Untreated cells that have been incubated in only complete DMEM were used as the control group in all cell culture studies.

*Cytotoxicity Assay for Blank CD Nanoparticles.* L929 mouse fibroblast cells and G/G mouse urinary bladder cells were cultured as a monolayer in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cultures were maintained at 37°C in a humidified 5% $CO_2$ incubator. L929 and G/G cells were seeded in 96-well tissue culture plates at a density of $1 \times 10^3$ cells/well in DMEM (100 μL). After 24 h, DMEM was removed from the cells and fresh medium containing blank amphiphilic CD nanoparticles (1:2 vol/vol) was replaced and incubated for 48 h. In order to determine cell viability, MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was applied. For this purpose, 20 μL of MTT solution in PBS (5 mg/mL) was added in each well and incubated for 4 h. After incubation, 80 μL of MTT lysis solution containing sodium dodecyl sulfate (23% wt/vol) and dimethylformamide (45% vol/vol) in ultrapure water was added in plates and incubated overnight to dissolve formazan crystals. Optical densities were determined by a microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Control group consisted of cells incubated in DMEM alone ($n = 3$).

*Anticancer Efficacy of Blank Nanoparticles.* In order to investigate anticancer efficacy of blank anionic and cationic amphiphilic CD nanoparticles, MCF-7, MB49, HepG2, and HeLa cancer cells were seeded in 96-well tissue culture plates at a density of $1 \times 10^4$ cells/mL in complete DMEM (100 μL). The cultures were incubated in same conditions as L929 and G/G cells At 80% confluency, DMEM was replaced with a new DMEM containing blank 6OCaproβCD or blank PC βCDC6 (1:2 vol/vol) and incubated for 48 h. Untreated cells used as control groups contain only complete DMEM. At the end of the incubation period, cell viability was determined with MTT assay as described previously ($n = 3$).

*Lipid Peroxidation Assay.* Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Measurement of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) has been used as indicators of lipid peroxidation. To measure both MDA and HAE, lipid peroxidation assay kit was used, according to the manufacturer's manual. For this purpose, MCF-7 cells ($5 \times 10^6$ cells/mL) were incubated with blank anionic 6OCaproβCD or blank PC βCDC6 amphiphilic nanoparticles (1:2 vol/vol and 1:16 vol/vol) in 6-well plates for 48 h. Following incubation, the chromogenic