International Journal of Pharmaceutics 509 (2016) 244–254

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Dendrosomal curcumin nanoformulation modulate apoptosis-related genes and protein expression in hepatocarcinoma cell lines

Maryam Montazeri[a,b], Majid Sadeghizadeh[c,**], Yones Pilehvar-Soltanahmadi[a,b], Faraz Zarghami[d], Samaneh Khodi[e], Mina Mohaghegh[f], Hadi Sadeghzadeh[a], Nosratollah Zarghami[a,b,*]

[a] Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[b] Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[c] Department of Genetics, School of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[d] Imam Reza Teaching Hospital, Tabriz University of Medical Sciences, Tabriz, Iran
[e] Department of Medical Genetic, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
[f] Department of Molecular Biology and Biotechnology, University of Aix-Marseille, Marseille, France

ARTICLE INFO

Article history:
Received 20 February 2016
Received in revised form 16 May 2016
Accepted 21 May 2016
Available online 24 May 2016

Keywords:
Dendrosomal curcumin
p53 mutant
Apoptosis
Hepatocarcinoma
Real-time PCR

ABSTRACT

The side-effects observed in conventional therapies have made them unpromising in curing Hepatocellular carcinoma; therefore, developing novel treatments can be an overwhelming significance. One of such novel agents is curcumin which can induce apoptosis in various cancerous cells, however, its poor solubility is restricted its application. To overcome this issue, this paper employed dendrosomal curcumin (DNC) was employed to in prevent hepatocarcinoma in both RNA and protein levels. Hepatocarcinoma cells, p53 wild-type HepG2 and p53 mutant Huh7, were treated with DNC and investigated for toxicity study using MTT assay. Cell cycle distribution and apoptosis were analyzed using Flow-cytometry and Annexin-V-FLUOS/PI staining. Real-time PCR and Western blot were employed to analyze p53, BAX, Bcl-2, p21 and Noxa in DNC-treated cells. DNC inhibited the growth in the form of time-dependent manner, while the carrier alone was not toxic to the cell. Flow-cytometry data showed the constant concentration of 20 μM DNC during the time significantly increases cell population in SubG1 phase. Annexin-V-PI test showed curcumin-induced apoptosis was enhanced in Huh7 as well as HepG2, compared to untreated cells. Followed by treatment, mRNA expression of p21, BAX, and Noxa increased, while the expression of Bcl-2 decreased, and unlike HepG2, Huh7 showed down-regulation of p53. In summary, DNC-treated hepatocellular carcinoma cells undergo apoptosis by changing the expression of genes involved in the apoptosis and proliferation processes. These findings suggest that DNC, as a plant-originated therapeutic agent, could be applied in cancer treatment.

1. Introduction

Hepatocellular carcinoma (HCC), which is ranked as the fifth lethal cancer in men and the eighth one in women, is the third leading cause of cancer-related deaths worldwide estimated about 700,000 deaths per year. Since p53 gene is mutated in approximately about 50% of human cancers, and does not accumulate in normal unstressed cells, indicating its essential role in both clinical marker and therapeutic target, therefore it is important to detect its modification in therapeutic responses (Farnebo et al., 2010). 74% of p53 mutations are missense mutations occurring in DNA-binding domains. The ninth most common mutation in hepatocarcinoma cells is Y220C, occurred in exon 6 (Tyr-Cys). Y220C mutant exists in Huh7cell line, in which mutant p53 protein is overexpressed with a prolonged half-life leading to its accumulation in the nuclei (Boeckler et al., 2008). Conventional therapeutic methods, including surgery and liver transplant, have several limiting difficulties. For instance, the scarcity of donor organs makes the liver transplantation applicable in a small number of HCC patients (Darvesh et al., 2012). Therefore, regarding the alarming increase in HCC, new alternative treatment

* Corresponding author at: Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
** Corresponding author at Department of Genetics, School of Biological Sciences, Tarbiat Modares University, Tehran, Iran.
E-mail addresses: Sadeghma@modares.ac.ir (M. Sadeghizadeh), zarghami@tbzmed.ac.ir (N. Zarghami).

http://dx.doi.org/10.1016/j.ijpharm.2016.05.039
0378-5173/

approaches are needed. Curcumin (diferuloyl methane), as a major active ingredient of turmeric, is derived from the dried roots of the plant *Curcuma Longa* and is used as a spice and a traditional medicine for many centuries in India and other Asian countries (Kazemi-Lomedasht et al., 2013). It has chemo-preventive and therapeutic features including anti-proliferative, anti-mutagenic and anti-carcinogenic properties (Esmatabadi et al., 2015; Nasiri et al., 2013). It induces the release of apoptosis inducing factor from mitochondria and eliminates the cancer cells (Rashmi et al., 2003). Although, the underlying mechanisms are not well-known, many molecular targets have been suggested including various transcription factors, inflammatory enzymes, cytokines, adhesion molecules and cell survival proteins (Badrzadeh et al., 2014; Milacic et al., 2008). Curcumin has shown a reduced solubility and poor uptake in both in vitro and in vivo studies, however its application in medicine and more specifically drug delivery is spreading rapidly (Mollazade et al., 2013). A promising method to improve the solubility of curcumin is the application of polymeric nanoparticles, such as dendrosomes which were first applied as gene reporter (Sadeghizadeh et al., 2008).

Dendrosomes are inexpensive, non-toxin, neutral, biodegradable, covalent or self- assembled, hyper branched, and spheroidal polymeric nanoparticles nano-carriers derived from oleic acid which were synthesized by our group for the first time. Previous studies by our group introduced a dendrosome specified Den O400, a nonionic biodegradable denderic glycol ester, to improve the solubility of curcumin, with maximum stability in the optimal ratio of 1:25 (curcumin/dendrosome), and to escalate its anti-tumor (Babaei et al., 2012; Tahmasebi Birgani et al., 2015).

The physical and chemical stability of DNC was studied by Mirgani et al. Transmission electron micrographs has shown DNC as a sphere shaped nanostructure and DLS analysis has determined DNC with mean diameter of 200 nm, polydispersity index (PDI) of 0.4, meaning that its colloidal suspension is polydispersed, ζ-potential of around −7 mV, and very high loading efficiency of 87%. The content of curcumin into the dendrosome was also quantified by HPLC-DAD analysis which was at constant level after preparation (Table 1) (Tahmasebi Mirgani et al., 2014b).

However, the toxicity of curcumin is more in cancer cells compared to normal cells, the apoptotic effects in high concentration of curcumin is reported in some normal cell types such as hepatocytes and human normal fibroblasts (Bisht et al., 2007; Singh, 2007). Here, HepG2, harboring wild-type p53, and Huh7, harboring p53 mutant, hepatoma cell lines were used to study the effect of DNC on cancer cells to investigate whether DNC reduces the expression of p53 mutant and leads cancer cells into cell death and if treated cancer cells make changes in the gene expression involved in apoptosis and cell cycle.

2. Materials and methods

2.1. Cell lines, cell culture and reagents

The human cancer cell linesHepG2/Huh7 (derived from hepatocellular carcinoma) and HFSF-PI3 (normal human fibroblast) were obtained from the Iranian national cell bank (Pasteur institute of Iran, Tehran). The Huh7cell line was cultured in DMEM (low glucose) and HepG2 cell was cultured in DMEM (High glucose) culture medium (pH = 7.2), supplemented with 100u/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS, GIBCO, USA), and incubated at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was changed every other day. Curcumin was obtained from Merck (Indianapolis, IN, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture products were obtained from Gibco (UK), Annexin V FLOUS kit and Propidium iodide (PI) were purchased from Roche (Roche Applied Science, Penzberg, Germany).

Table 1
The physical and chemical stability of DNC.

Size (nm)	PDI	ζ-potential (mV)	EE (%)
142.97 ± 4.27	0.4 ± 0.03	−7.81 ± 1.4	87.65 ± 1.82

Abbreviations: EE, encapsulation efficiency; PDI, polydispersity index.

2.2. OA400 carrier and DNC preparation

Dendrosomes are PEGylated micelles derived from oleic acids, which was prepared by an optimized protocol in our Lab (Babaei et al., 2012). Briefly, OA400 was synthetized by esterification of oleoyl chloride (0.01 mol), polyethylene glycol 400 (0.01 mol) in presence of triethylamine (0.012 mol) and chloroform as solvent at 25 °C for 4 h, followed by filtration to remove triethylamine hydrochloride salt and evaporation in vacuum oven at 40 °C for 4 h to remove the chloroform (Tahmasebi Mirgani et al., 2014a). Curcumin was dissolved in various amounts of prepared polymeric carriers in weight/weight ratios of dendrosome/curcumin ranging from 1:10 to 1:50 and checked for absorbance spectra by ultraviolet spectrophotometry (Infinite® 200 PRO, Tecan, Mannedorf, Switzerland). The mixture was incubated at 37 °C for an overnight in order to dissolve the curcumin completely in the polymeric carrier. Then, the appropriate mixture of dendrosome and curcumin was evaluated at wavelength of 420 nm, in comparison with free curcumin dissolved in PBS and 1% methanol as control samples and 1:25 was chosen as appropriate proportion 1:25 was chosen as appropriate proportion and very stable for weeks at room temperature without precipitation of curcumin. The loading of dendrosome nanocarriers with curcumin molecules was performed using Gou et al's protocol (Gou et al., 2011). In summary, curcumin and dendrosome were co-dissolved in 5 mL acetone; this solution was added into 5 mL PBS while stirring constantly. Then, the acetone was evaporated in a rotary evaporator. The curcumin/dendrosome micelle solution was sterilized using a 0.22 μm syringe filter (EMD Millipore). Prepared DNC was kept away from light in 4 °C for further procedures. Due to the innate fluorescence of curcumin, the cells were treated with 10 μM DNC and studied after 4 h by fluorescence microscopy. Non-treated samples were used as a reference value; auto-fluorescence of the cells was set as "Zero" value.

2.3. Uptake of DNC versus void curcumin

To study the uptake of DNC by different cell lines, the 80×10^3 cells per mL fresh cells were transferred into 12-well plate and treated with 18 μM DNC as well as void curcumin for 0–7 h. To evaluate whether DNC is attached to the cell membrane or absorbed into cells, its cellular localization was evaluated by fluorescence microscopy after treating with DNC and void curcumin in various incubation times. Each experiment was repeated at least three times.

2.4. Cell proliferation assays

MTT viability assay was used to assess cell proliferation as follows: Adherent cells were released from their substrate by trypsinization, centrifuged and resuspend at 1×10^6 per mL followed by preparing serial dilutions of cells in culture medium from 1×10^6 to 1×10^3 cells per mL. 200 μL of the dilutions was