Colloids and Surfaces B: Biointerfaces 147 (2016) 65–72

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Cellular uptake and anticancer activity of salvianolic acid B phospholipid complex loaded nanoparticles in head and neck cancer and precancer cells

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ARTICLE INFO

Article history:
Received 5 March 2016
Received in revised form 25 July 2016
Accepted 28 July 2016
Available online 28 July 2016

Keywords:
Salvianolic acid B
Head and neck cancer
Cellular uptake
Nanochemoprevention
Lipid nanocarrier

ABSTRACT

Salvianolic acid B (SalB) was demonstrated to be a promising chemopreventive agent for head and neck squamous cell carcinoma (HNSCC) in the previous studies by our and other research institution, but the properties like low efficacy, poor systemic delivery, and low bioavailability has hampered its clinical applications. To continue our research program focused on the use of natural compounds on cancer chemoprevention, we propose a first example of phospholipid complex loaded nanoparticles (PLC-NPs) encapsulating SalB as a potential carrier for intervention of HNSCC (HN13, HN30) cells and precancer Leuk1 cells in this study. Qualitative and quantitive studies of cellular uptake showed that intracellular accumulation of SalB was significantly higher when HN13, HN30 and Leuk1 cells were incubated with SalB-PLC-NPs complex (nano-SalB) as against free-SalB. Cell viability assay revealed that the cell growth of HN13 and HN30 cells was significantly inhibited of 56.1% and 29.3%, respectively, for nano-SalB compared to an equivalent amount of free-SalB ($P < 0.001$). Moreover, cell cycle and apoptosis assay showed that a clear trend of cell cycle arrest and induction of apoptosis was also observed within the HNSCC cells treated with nano-SalB. Collectively, this study demonstrated that nano-SalB was significantly more potent had an anticancer effect against HNSCC cells, which serves as the first step toward establishing SalB nano-formulations as promising cancer chemopreventive agents. The current study could pave a new way for the development of drugs that target HNSCC in the future.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies and a leading cause of cancer death worldwide, and leukoplakia is the best-known potentially malignant disorder and precursor lesion of HNSCC [1–3]. The early prevention and effective intervention options of HNSCC are of utmost importance to reduce both the morbidity and mortality. HNSCC especially in oral mucosa have been considered to be a typical multistep and multifocal carcinogenesis processes with stepwise accumulations of genetic alterations resulting in aberrant cellular appearance, deregulated cell growth and carcinoma. The processes can take many years to undergo the multiple cellular and genetic alterations which lead to malignant changes [4]. Therefore, it remains an appealing strategy to explore effiective, nontoxic and affordable novel pharmacological agents for preventing the carcinogenesis processes and development of HNSCC. Notablely, chemoprevention by phytochemicals is emerging as one such promising strategy to delay or block the early carcinogenic processes and the development of HNSCC [5].

Salvianolic acid B (SalB) is the most abundant and bioactive component of *Salvia miltiorrhiza* Bge (Danshen or Tanshen) and used as a quality control ingredient and active marker for *Salvia miltiorrhiza* Bge products by the National Pharmacopoeia Council of China [6]. Increasing evidence indicates that SalB exerts its effects by inhibiting cancer initiation and development and its anticarcinogenic activities have been clearly demonstrated in both cell

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http://dx.doi.org/10.1016/j.colsurfb.2016.07.053
0927-7765/

cultures and animal models of HNSCC [7–13], which serves as a promising chemopreventive agent for HNSCC. Gu and colleagues demonstrated the anticancer effectiveness of SalB by evaluating cell viability, proliferation, and tumor xenograft growth in cultured HNSCC cells and HNSCC tumor xenograft mice model [8,9]. Consistently, our group also demonstrated anti-proliferative effect of SalB in cultured HNSCC cells [10,11]. Furthermore, our group demonstrated the anticarcinogenic effect of SalB in a classical model of 7,12-dimethylbenz(a) anthracene (DMBA)-induced oral carcinogenesis in hamsters [12,13].

Regardless of these encouraging findings, the extensive use of SalB has met only limited success, largely due to its low efficacy, poor systemic delivery, and low bioavailability [14–17]. To overcome these limitations, nanochemoprevention, encapsulation of natural components capable of interfering with a carcinogenesis process using nanoparticle-mediated delivery systems, has been approved as a powerful strategy for cancer management [18–21]. Interestingly, earlier studies demonstrated that the bioactivity of SalB was improved using nanotechnology-based approach through targeted drug delivery [14–17]. Notablely, Peng et al. demonstrated that the oral bioavailability of SalB was significantly enhanced using SalB phospholipid complex loaded nanoparticles (SalB-PLC-NPs) by in vitro study and in vivo rats model study [14,15].

In the current study, we hypothesized that the anticancer activity of SalB-PLC-NPs (nano-SalB) would be superior to SalB as a chemopreventive agent for HNSCC. Thus, we attempted for the first time to examine the anticancer effects of SalB-PLC-NPs that provided by Peng and colleagues [14–17], compared with free-SalB by evaluating cell viability, cell cycle, cell apoptosis and cellular uptake in cultured HNSCC cells and precancer Leuk1 cell line.

2. Material and methods

2.1. Nanoformulation

The SalB and lyophilized SalB-PLC-NPs in this study were provided by Peng and colleagues [14,15]. Preparation, characterization and evaluation of SalB-PLC-NPs were performed according to their previous studies [14,15]. The mean size of free SalB embedding phospholipid complex nanoparticles (SalB-PLC-NPs) was 112.2 nm of diameter [15]. Free SalB and SalB-PLC-NPs (nano-SalB) were filtered through 220 nm of diameter for the subsequent cell experiments.

2.2. Cell line and cell culture

Human HNSCC cell lines, HN13 and HN30, were obtained from the National Institutes of Health (Rockville, MD, USA). Human oral leukeplakia cell line, Leuk1, was a generous gift from Prof. Li Mao at University of Maryland School of Dentistry, Baltimore, MD. HN13 and HN30 were cultured in DMEM with 10% fetal bovine serum, 100 units/ml of penicillin and 100 units/ml of streptomycin and Leuk1 cells were cultured in K-SFM medium (1646135, GIBCO, Grand Island, NY, USA) in a humidified incubator at 37 °C, 5% CO2.

2.3. Cellular uptake assay

To qualitatively determine the cellular uptake of a drug to assess the efficacy of the delivery vehicle, intracellular accumulation assay of free- and nano-SalB was performed. Since SalB is autofluorescent according to Chinese Pharmacopoeia, it is used directly to examine cellular uptake without additional markers. HN13, HN30, and Leuk1 cells were seeded into coverglass-containing 24-well plates at density of 2×10^4 cells/well and incubated overnight. The cells were treated with 200 μg/ml of free-Sal B and equivalent dose of nano-SalB. After incubated for 2 h, the cells were washed one time with PBS and fixed with 4% formaldehyde solution and washed with PBS again. Subsequently, the cells were added 200 μl Fluorescent Phalloidin (Acti-stain™ 488, PHDG1, Cytoskeleton, USA) per well and incubated for 30 min in darkness at room temperature. Then, the cells were washed three times with PBS and sealed pieces with 50% glycerol. Finally, the cells were examined under a laser scanning confocal microscopy by using blue excitation filter for monitoring Sal B fluorescence. The internalized SalB emits a blue fluorescence with the filter of 405 nm excitation wavelength and 400–550 nm emission, and phalloidin emits a green fluorescence with the filter of 488 nm excitation wavelength and 520–670 nm emission.

To quantitively check if the drug-loaded nanoparticles were internalized in cells, the concentration- and time-gradient intracellular uptake of free- and nano-SalB was further studied. HN13, HN30, and Leuk1 cells were seeded at a density of 10×10^4 per well in 96-well plates and incubated overnight. Then, cell medium was replenished containing a concentration gradient of free- and nano-SalB (25, 50, 100, 200 μg/ml). Besides, cell medium was replenished containing an equivalent dose (200 μg/ml) of free- and nano-SalB in a time gradient (5, 10, 20, 30, 60, 120 min). Cellular uptake of these treated cells were measured by fluorescence microscopy using a Thermo Scientific™ Varioskan™ Flash (Thermo Fisher Scientific, USA) kit according to the manufacturer's protocol.

2.4. Cell viability assay

To determine cell growth, HN13, HN30, and Leuk1 cells were seeded at a density of 3×10^3 per well in 96-well plates and incubated for 24 h. Cell medium was replenished containing a concentration gradient (25, 50, 100, 200 μg/ml) of free-SalB or equivalent dose of nano-SalB for a time gradient (24 h, 48 h, 72 h, 96 h). Subsequently, cell viability assay was determined by MTS-formazan reduction using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (G3582, Promega, Madison, WI, USA) according to manufacturer's protocol. The absorbance of the samples was measured at 490 nm using an enzyme-linked immunosorbent assay reader. All the samples were performed in triplicates and each experiment was performed at least thrice.

2.5. Cell cycle analysis

To analyze cell cycle, cells were seeded at a density of 3×10^4/ml in 10 cm Petri dishes and incubated for 24 h. HN13, HN30, and Leuk1 cells were treated with 200 μg/ml, 100 μg/ml and 200 μg/ml of free-SalB or equivalent dose of nano-SalB for 24 h, 48 h, and 48 h, respectively. The treated cells were collected by centrifugation at 1000r/min for 3 min, washed with cold PBS, and then fixed in chilled 70% ethanol at 4 °C for 4 h. Cells were centrifuged again, washed with cold PBS twice, incubated with RNase A (10 mg/ml) for 1 h at 37 °C, and stained with propidium iodide (1 mg/ml) for 30 min in the dark. Cells were measured for DNA content by flow cytometry (Beckman Coulter Cytomics Epics Altra, Brea, CA, USA), and cell cycle distribution was analyzed by ModFit LT software (BD Biosciences, USA).

2.6. Annexin V-PI apoptosis assay

To analyze cell apoptosis, cells were seeded at a density of 3×10^4/ml in 6-well plates and incubated for 24 h. HN13, HN30, and Leuk1 cells were treated with a concentration of 200 μg/ml, 100 μg/ml and 200 μg/ml of free-SalB or nano-SalB for 24 h and 48 h, respectively. The treated cells at 24 h and 48 h were measured by annexin V binding and PI staining using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis kit (Thermo Fisher Scientific, USA, 1351935) according to the manufacturer's protocol. The cells ana-