Chemico-Biological Interactions 252 (2016) 1–8

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

A novel biologically active acid stable liposomal formulation of docosahexaenoic acid in human breast cancer cell lines

Christine G. Skibinski [a], Arunangshu Das [a], Kun-Ming Chen [a], Jason Liao [c], Andrea Manni [b], Mark Kester [d], Karam El-Bayoumy [a, *]

[a] Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA, 17033, USA
[b] Department of Medicine, Pennsylvania State University College of Medicine, Hershey, PA, 17033, USA
[c] Department of Public Health Sciences, Pennsylvania State University College of Medicine, Hershey, PA, 17033, USA
[d] NanoSTAR Institute, Department of Pharmacology, The University of Virginia, Charlottesville, VA, 22908, USA

ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form
23 March 2016
Accepted 30 March 2016
Available online 1 April 2016

Keywords:
Liposomal DHA
Human breast cancer cell lines
Cancer prevention

ABSTRACT

Purpose: The risk of breast cancer can be influenced by certain dietary components, such as the amount and type of dietary fatty acids ingested. Docosahexaenoic acid (DHA), a component of fish oil, is known to suppress rat mammary carcinogenesis, reduce cell growth and induce apoptosis in human breast cancer cell lines. The purpose of this study was to develop a novel nanoliposomal formulation that would encapsulate a concentrated amount of DHA and utilize lipids that could protect DHA from pH fluctuations and oxidation.

Methods: We developed an acid stable liposome formulation of DHA by utilizing ether and phytanyl lipids similar in structure to those found in Archaea, known to endure high acidity and temperature; we compared its biological activities with free DHA in human breast cancer cells.

Results: The mean size of the liposomal DHA was 137 ± 12 nm with a slightly negative charge; the encapsulation efficiency of DHA in the liposomes as determined by LC-MS/MS ranged from 60 to 80%; our formulation is resistant to oxidation and stable over a range of pH (1.0–7.4) at 37 °C for a duration of two hours. In MCF-7 cells, liposomal DHA (IC_{50} 38.8 μM) significantly reduced cell viability more effectively than free DHA (IC_{50} 72.5 μM, $p = 0.0017$). In MDA-MB-231 cells, liposomal DHA was also marginally more effective. Liposomal DHA was more effective than free DHA in inducing apoptosis in both cell lines. It altered proteins involved in cell growth, cell cycle, and apoptosis more effectively than free DHA in both cell lines; it up-regulated p21 and cleaved PARP, while P-AKT and P-S6 were down-regulated.

Conclusions: We developed a novel biologically active acid stable liposomal DHA as a potentially useful formulation for breast cancer prevention.

1. Introduction

Breast cancer is the second-most diagnosed cancer in women with 231,840 cases expected as of 2015, resulting in 40,290 deaths [1]. While therapies [2–4] are available for the treatment of breast cancer, prevention is the ideal approach to decrease breast cancer morbidity and mortality. The risk of breast cancer can be influenced by certain dietary components such as the amount and type of dietary fats ingested [5,6]. Specifically, omega-3 fatty acids (n-3 FAs), a component of fish oil, and omega-6 fatty acids (n-6 FAs) have been suggested to decrease and increase breast cancer incidence, respectively [7–9]. We have demonstrated that dietary administration of a high ratio of n-3:n-6, 25:1, reduced tumor burden induced by 1-methyl-1-nitrosourea (MNU) by 79% [10]. An extensive analysis of the molecular signature underlying inhibition of mammary carcinoma by dietary n-3 FAs revealed a dominant effect on suppression of proliferation over induction of apoptosis, although both processes were significantly affected [8].

The high n-3 FA: n-6 FA ratio needed to induce an antitumor effect suggests that the tumor protective effect of fish oil may be due to one of its components which may be present in small

* Corresponding author. Penn State Hershey Cancer Institute, 500 University Drive, PO Box 850, Penn State University College of Medicine, Hershey, PA, 17033, USA.
E-mail address: kee2@psu.edu (K. El-Bayoumy).

http://dx.doi.org/10.1016/j.cbi.2016.03.035
0009-2797/

amounts [7,9]. Since, administration of such high ratios of n-3 FA: n-6 FAs in the diet is not feasible, it is necessary to develop a practical formulation of one of its active ingredients, such as docosahexaenoic acid (DHA) which can easily be administered to high risk women.

Presently, the oral availability of DHA is limited, which can be attributed to its high unsaturation being vulnerable to oxidation and degradation under acidic conditions, as posed by the digestive track [11–14]. Therefore, an ideal oral delivery system would be a nanoliposomal formulation that would encapsulate a concentrated amount of DHA and utilize lipids that could protect DHA from pH fluctuations and oxidation. This protection from physiological stress would allow for the nanodelivery system to cross the enterocytes, enter the bloodstream, and increase the bioavailability of DHA at the breast tissue. Hussain et al., has demonstrated that particles under 200 nm are ideal to cross the enterocytes of the intestine [15].

One source of lipids (highly saturated diphytanyl ether lipids) for the development of an acid stable liposome belong to the unique species, Archaea, which inhabit harsh environments including acidic conditions, high pressure, and heat [15–18]. Therefore, in the present study we utilized the commercially available ether lipids: 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphatidylcholine, and 1,2- di-*O*-phytanyl-*sn*-glycero-3-phosphatidylethanolamine to construct an acid stable liposome formulation of docosahexaenoic acid and compared its biological activities with free DHA in human breast cancer cell lines.

2. Materials and methods

2.1. Reagents

Reagents for Western blots were obtained from Bio-Rad Laboratories (Hercules, CA) and primary and secondary antibodies and GAPDH (SC-32233) were purchased from Santa Cruz Biotechnology and Cell Signaling. Primary antibodies against p21 (2947s), P-AKT (4060p), AKT (9272), P-S6 (4856s), S6 (2217s), PARP and Cleaved PARP (9542s) were obtained from cell signaling. Chemiluminescent immunodetection reagents and autoradiography film were obtained from GE Healthcare. Ether lipids 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (999985) and 1,2-diphytanyl-*sn*-glycero-3-phosphoethanolamine were purchased from Avanti Polar Lipids, Alabaster, AL. Docosahexaenoic acid sodium salt (D8768) for the liposome formulation and MTT reagent (M5655) for cell viability assays were purchased from Sigma Aldrich, St. Louis, MI. The Cell Death Detection Elisa Kit (11544675001) was purchased from Roche Applied Science, Indianapolis, IN.

2.2. Nanoformulation of liposomal DHA and ghost (no DHA, control) liposomes

Docosahexaenoic acid-sodium salt was dissolved in absolute ethanol to a concentration of 10 mg/mL 1,2-Diphytanyl-*sn*-glycero-3-phosphoethanolamine and 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine were dissolved in chloroform to a concentration of 10 mg/mL. For the liposome formulation, 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine, 1,2-diphytanyl-*sn*-glycero-3-phosphoenthanolamine and docosahexaenoic acid-sodium salt were combined in the following molar ratio, 6:3:1, v/v/v. (US Patent Application: 2014/0271,824). For ghost liposomes 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine and 1,2-diphytanyl-*sn*-glycero-3-phosphoethanolamine were combined in a molar ratio, 6.67:3.33, v/v. Lipids were dried to a film under a stream of nitrogen for 4 h, then hydrated with 1 mL of 0.9% degassed saline and heated (70 °C) for 90 min with occasional vortexing. Next, the liposome solution was sonicated at 65 °C for 2.5 min, allowing the liposome suspension to emulsify. The solution of lipids was extruded 13 times at 70 °C through a 0.1 nm polycarbonate membrane with an Avanti-Mini Extruder (Avanti Polar Lipids, Alabaster, AL). Liposomes were purified on a CL-4B column in 0.9% saline and stored at 4 °C until further use. Liposome size (nanometers) and zeta potential (millivolts) for DHA liposome and ghost were later confirmed, respectively, by dynamic light scattering (DLS) and laser Doppler microelectrophoresis (Zetasizer Nano ZS at 25 °C). Three measurements were taken for each nanoliposome batch. Three different batches of nanoliposomes were tested for each parameter. The level of DHA content in the liposome was determined by liquid chromatography tandem mass spectroscopy (LC-MS/MS, Agilent 1100 Series capillary LC/MSD Ion Trap XCT equipped with an electrospray ion source).

2.3. pH stability studies

Solutions of dPBS were adjusted to pH 1–7.4 by adding an appropriate amount of 1.0 N HCl or 10 N NaOH and verified by a pH meter (Denver Instruments, Bohemia, NY). In a 1 mL cuvette 100 μL of liposomal DHA was added to 900 μL of pH adjusted dPBS. The pH of the mixture was further verified by the pH meter. Two batches of DHA nanoliposomes were run in triplicate for each pH point. This solution was submitted to DLS analysis at 37 °C for a duration of 10 min to 2 h. DLS spectra were collected at 10 min, 1 h, and 2 h time points as an indicator of stability by observing the size of the liposome.

2.4. Oxidation studies

DHA and liposomal DHA, both in dPBS, were aliquoted into amber vials and stored at 37 °C or 4 °C for up to two weeks. Aliquots of each sample with D_5-DHA, an internal standard, were analyzed using an API 3200 LC MS/MS triple quadrupole mass spectrometer interfaced with an Agilent 1200 series HPLC using an Agilent extended C18 column (4.6 × 150 mm, 5 μm). The electrospray was in negative ion mode. The MS parameters were: electrospray source temperature and voltage were 230 °C and −4.5 kV, respectively; the declustering potential (DP) collision energy (CE), entrance potential (EP) and cell exit potential (CXP) were −40, -16, -3, and −12ev, respectively; the collision activated dissociation was set to 6 psi, while the curtain gas was set to 20 psi. The elution solvent program was 200 μL/min gradient using solvent A (methanol) and solvent B (water). The gradient was 10% A to 100% A in 5 min, 100% A was held for another 28 min, and continued to 10% A in 2 min. DHA and the internal standard, D_5-DHA, were detected after 31 min.

2.5. Cell lines

MDA-MB-231 (triple negative), and MCF-7 (estrogen and progesterone receptor positive) cell lines were purchased from ATCC (American Type Culture Collection). MCF-7 cells were cultured in EMEM with10% FBS, 0.01% human insulin and 1% Penicillin/Streptomycin. MDA-MB-231 cells were cultured in DMEM with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. Cell lines were maintained in a humidified incubator at 37 °C, 5% CO_2. For subculture, cells were subjected to 0.25% trypsin/EDTA detachment, followed by centrifugation at 1200 rpm for three minutes at 25 °C and then re-plated in growth media.

2.6. Cell viability assay

MDA-MB-231 cells were plated at 6.0 × 10^3 cells per well in a