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Targeted nano-delivery of novel omega-3 conjugate against hepatocellular carcinoma: Regulating COX-2/bcl-2 expression in an animal model

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ABSTRACT

The present approach enumerates the effectiveness of tuftsin tagged nano-liposome for the cytosolic transport of 2,6-di-isopropylphenol-linolenic acid conjugate against liver cancer in mice. Initially, the conjugate in its free form was examined for anticancer potential on HepG2 liver cancer cells. Induction of apoptosis and suppression of migration and adhesion of HepG2 cells confirmed the effectiveness of conjugate as an anticancer agent. After this, role of the conjugate entrapped in a nano-carrier was evaluated in animal model. The nano-formulation comprising of conjugate bearing tuftsin tagged liposome was firsly characterized and then its therapeutic effect was determined. The nano-formulation had 100–130 nm size nanoparticles and showed sustained release of the conjugate in the surrounding milieu. The nano-formulation distinctly reduced the expression of COX-2, an important molecule that is vastly expressed in hepatocellular carcinoma. The utilization of in-house engineered nano-formulation was also successful in significantly up-regulating Bax and down-regulating bcl-2 gene expression eventually helping in better survival of treated mice. Histopathological analysis also revealed positive recovery of the general architecture and the violent death of cancer cells by apoptosis at tumor specific site. The site specific delivery of conjugate entrapped in tuftsin tagged liposomes was highly safe as well as efficaceous. Nano-formulation based approach showed a visible chemotherapeutic effect on liver cancer progression in experimental mice thereby making it a potential candidate for treatment of liver cancer in clinical settings.

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1. Introduction

The treatment with anticancer drugs mostly leads to toxic effects because of lack of specificity towards cancer cells [1,2]. Due to poor solubility their long term use also cause non-targeted accumulation, that further complicates the issue [3]. To circumvent these problems, drug conjugates from natural sources are being in use nowadays. A great deal of attention has been devoted to the use of dietary omega fatty acids because of the fact that they inhibit tumor cell proliferation and induce apoptosis [4–7]. Certain fatty acids synergistically help other chemotherapeutic drugs in inhibiting tumorigenesis [8–10]. Among various fatty acids, omega-3 type has been reported to suppress various neoplastic diseases [4–6,11]. Its use in diet decrease the threat of cancer and also increase the bioavailability of the existing drug in a targeted manner.

Considering the anticancer potential of omega (unsaturated) fatty acids, we have previously synthesized series of ester conjugates where various mono- and polyunsaturated fatty acids were coupled with an anesthetic agent, di-isopropylphenol [5–7,12]. Our preliminary trials on these conjugates have shown effective killing of cancer cells *in-vitro* through the induction of various apoptotic factors [6,7]. Linolenic acid just like other dietary omega 3 s has been shown to possess anticancer activities [13,14]. It also has anti-oxidative and anti-apoptotic properties [15]. Therefore, the present study was envisaged to establish the

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anticancer effect of novel linolenic acid conjugate on liver cancer cells in-vitro and through conjugate entrapped in the delivery vehicle in animal models.

Liposomes are clinically approved biocompatible material that encapsulate both hydrophobic and hydrophilic drug and demonstrate safe delivery with low toxicity. They are able to control the bio-distribution by altering the size, surface charge, composition and other physical characteristics [18,19]. Because of these advantages, use of liposomes promote anti-tumor potential in comparison with the free drug and other therapeutic procedures. Various conjugate bearing liposomal formulations have been used for the cytosolic delivery of antibiotics and anticancer drugs [18,20,21]. Likewise, tuftsin, a naturally occurring tetrapeptide, have been reported as an anticancer agent in animal model [16]. Co-administration of tuftsin enhance the anticancer efficacy of commercially available drugs and are also able to activate the tumoricidal macrophages that can engulf and suppress the various kind of malignant cells [17].

In the current study, the chemotherapeutic action of the conjugate (2,6-di-isopropylphenol-linolenic acid) entrapped in tuftsin tagged liposomes against the treatment of diethyl nitrosoamine (DEN) induced liver cancer in murine experimental model was assessed. The efficacy of the nano-formulation was ascertained in the management of liver cancer in Swiss albino mice on the basis of regression, survival, histopathology as well as the expression of COX-2, bcl-2 and Bax in the treated animals.

2. Materials and methods

2.1. Materials

All the reagents used were of analytical grade with highest purity. Polyvenylidene difuoride [PVDF] membranes and 0.22 μm size sterile filters were purchased from Millipore (Germany). Diethylnitrosoamine (DEN), propofol, linolenic acid and tuftsin were obtained from Sigma Aldrich (St. Louis, MO, USA). Tuftsin was modified by coupling a hydrocarbon fatty acyl residue to the C-terminus through an ethylenediamine spacer arm (Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-NH-CO-$C_{15}H_{31}$), according to the published protocol that permits almost quantitative incorporation into liposomes [22]. Cell migration and adhesion kits were obtained from Cell Biolabs, Inc. Vybrant Apoptosis Assay Kit #2 was procured from Molecular Probe (Eugene, Oregon).

Anti-COX-2, Anti-bcl-2, Anti-Bax antibody and non fat dry milk were procured from BD Biosciences (San Diego, CA). Rabbit anti-mouse HRP tagged secondary antibody was purchased from Amersham and monoclonal anti-GAPDH was purchased from Sigma Aldrich (St. Louis, MO, USA). Chemiluminescence detection kit was brought from Bio Rad Laboratories, Inc. (CA, USA).

2.2. Assessment of anticancer potential of conjugate on HepG2 cells

2.2.1. Measurement of cancer cell migration and adhesion

Assay for migration and adhesion was performed with cytoselect 24-well cell migration and cell adhesion kits as per Khan et al. [6] protocol. For quantitative analysis, three concentrations viz. 5 μM, 15 μM and 25 μM of the conjugate were tested on HepG2 liver cancer cells. Finally after incubation process, the 100 μl of the sample in 96-well plate was read at 560 nm.

2.2.2. Analysis of annexin-V binding by flow cytometry

Annexin-V staining was performed according to the kit's protocol. After treating with 15 μM conjugate for 48 h the HepG2 cells were harvested and incubated with annexin V-FITC and PI. The fluorescence emission of Annexin-V stained cells was measured at 530–575 nm in a flow cytometer (MACSQuant, Germany).

2.3. Preparation of conjugate entrapped liposomes

Tuftsin tagged liposomes were prepared using published methods with slight modifications [23]. The nano-formulation was made by mixing 10:1 ratio of liposomes with conjugate (2,6-di-isopropylphenol-linolenic acid) [7]. The nano-formulation was then reconstituted with phosphate buffered saline (PBS) and centrifuged at 14,000*g* for 5 min. The pellet was washed three times in PBS and then passed through 100 nm polycarbonate filter for 10–12 cycles to get the desired size of nanoparticles.

2.4. Characterization of conjugate entrapped in liposomes

2.4.1. Transmission electron microscopy (TEM)

A drop of nano-formulation (lyophilized and suspended in 20 mM PBS, pH 7.4) was mounted on clear glass stub. The sample was coated with gold-palladium alloy and imaged at an accelerating voltage of 120 kV on a transmission electron microscope (Model HT7700, Hitachi High Technologies, America Inc.).

2.4.2. Nanophox imaging

The 2 mg of nano-formulation in a lyophilized form was suspended in 1 ml of distilled water and a drop of it was analyzed by the Nanophox particle-size analyzer (Sympatec GmbH, Germany).

2.4.3. Entrapment efficiency and zeta-potential

The entrapment efficiency of the conjugate in nano-liposomes was measured by dissolving an aliquot of formulation in chloroform:methanol [1:9 v/v] solution. Isocratic methanol-water (72:28 v/v) was taken as the solvent system. Entrapped amount was calculated from the respective standard curve plotted at λ_{max} = 272 nm. Percent entrapment efficiency (%EE) was calculated as %EE = (amount of conjugate entrapped)/(total amount of conjugate used in the beginning) × 100.

Zeta [ζ]-potential of conjugate entrapped nano-formulation was measured by Zetasizer Nano ZS (Malvern Instrument Limited, UK). For analysis, the nano-formulation was lyophilized and then suspended in phosphate buffer (pH 7.4).

2.4.4. Release kinetics

The nano-formulation [equivalent to 1 mg of conjugate] was dispersed in 1 ml of 20 mM PBS [pH 7.4]. The suspension was kept in an orbital shaking water bath at 120 rpm at 37 °C. Aliquots of the release medium were saved at different time intervals and were read at 272 nm. Release runs were continued for 144 h. The results obtained were the mean values of three independent experiments.

2.5. Assessment of anticancer potential of nano-formulation in mice

2.5.1. Experimental animals

Healthy male Swiss albino mice (20 ± 2 g body weight) were obtained from the animal house facility and housed in propylene cages under standard atmospheric conditions (22 ± 1 °C temperature; 12 h light/12 h dark photoperiod and 50–60% humidity). The care and handling of mice was done regularly with pellet diet and water *ad libitum*.

2.5.2. Induction of liver cancer by DEN

For induction of liver cancer, 2.4 mg/mouse dose of diethylnitrosoamine (DEN) was injected intraperitoneally [24]. During induction period, the mice were quarantined and examined routinely for gross body morphological changes. After 40 days,

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