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Pharmaceutical Nanotechnology

All-*trans*-retinoic acid nanodisks

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Abstract

Nanodisks are nanoscale, disk-shaped phospholipid bilayers whose edge is stabilized by association of apolipoprotein molecules. Self-assembled ND particles enriched with all-*trans*-retinoic acid (ATRA) (phospholipid:ATRA molar ratio = 5.5:1) were generated wherein all reaction components were solubilized. ATRA-ND migrated as a single band (Stokes' diameter ~20 nm) on native gradient polyacrylamide gel electrophoresis. ATRA, phospholipid and apolipoprotein co-eluted from a Sepharose 6B gel filtration column, consistent with stable integration of ATRA into the ND particle milieu. Spectroscopic analysis of ATRA-ND in buffer yielded an absorbance spectrum characteristic of ATRA. ATRA-ND mediated time-dependent inhibition of cultured HepG2 cell growth more effectively than free ATRA. The nanoscale size of the formulation particles and the stable integration of biologically active ATRA suggest ND represent a potentially useful vehicle for solubilization and *in vivo* delivery of ATRA.

Keywords: All-*trans*-retinoic acid; Apolipoprotein; Nanodisk; Phospholipid; Cell culture; Drug delivery

1. Introduction

All-*trans*-retinoic acid (ATRA) is a naturally occurring vitamin A derivative that functions as a regulator of gene transcription (Umesono et al., 1988; Gianni et al., 2000; Duprez et al., 2003). ATRA interacts with members of the hormone receptor superfamily including the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) (Sun and Lotan, 2002). ATRA binding to these transcription factors modulates their interaction with "retinoid response elements" on a wide range of genes that function in cell proliferation, differentiation and apoptosis. When ligand-activated, RAR–RXR heterodimers dissociate from co-repressor proteins they recruit co-activators that lead to chromatin decondensation and activation of gene transcription (Freedman, 1999; Sun and Lotan, 2002). Subsequently, pathways controlling growth, differentiation and cell death are activated.

Recognized as a potent pharmacological agent, ATRA has been used to treat various forms of cancer including leukemia, breast cancer and liver cancer (Freemantle et al., 2003; Arce et al., 2005). In the case of hepatomas, retinoid therapy has successfully reduced both primary and secondary malignancies. This effect may be attributed to a defect in retinoic acid metabolism in patients with hepatocarcinoma, many of whom have significantly lower levels of circulating retinol (Arce et al., 2005). At the same time, other cases may be related to RAR activation following integration of hepatitis B virus (Benbrook et al., 1988).

While beneficial as an anti-cancer agent, the use of ATRA is not without complications. ATRA is a water insoluble, toxic agent with limited bioavailability (Freemantle et al., 2003). Pharmacological levels can cause retinoic acid syndrome and neurotoxicity, particularly in children (Takitani et al., 2006). In addition, drug resistance has been reported in cases of sustained ATRA treatment requiring the use of additional cytotoxic chemotherapy (Freemantle et al., 2003). Although liposomal formulations were developed a number of years ago in an effort to address these issues, they have not progressed past the clinical trial stage (Estey et al., 2005). Despite this, the potential benefits of associating ATRA with a lipid-based carrier are many. Not only do lipid-drug formulations address solubility issues, they also decrease toxicity and potentially avoid triggering ATRA resistance, thereby minimizing the need for additional chemotherapy (Freemantle et al., 2003).

In the present study we show that significant quantities of ATRA can be stably incorporated into novel, nanometer

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scale, apolipoprotein stabilized phospholipid bilayer disk complexes, termed nanodisks (ND). ND associated ATRA is stably associated, fully soluble and retains potent biological activity, suggesting its potential for use as a delivery vehicle for this compound.

2. Materials and methods

2.1. Materials

ATRA was obtained from Sigma Chemical Co. Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids Inc. Recombinant apolipoprotein E3 N-terminal domain (apoE3-NT) was expressed in *E. coli* and isolated as described previously (Fisher et al., 1997). HepG2 cells were purchased from ATTC.

2.2. ATRA-ND

Ten mg of a DMPC/DMPG mixture (7:3 weight ratio) were dissolved in chloroform/methanol (3:1, v/v) and dried under a stream of N_2 gas, forming a thin film on the vessel wall. Residual organic solvent was removed under vacuum. The prepared lipids were then dispersed in 1 ml phosphate buffered saline (PBS; 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.0) by vortexing and intermittent heat (37 °C). Subsequently, 0.8 mg ATRA was added from a 30 mg/ml stock solution in dimethylsulfoxide (DMSO). Following this, 4 mg apoE3-NT (3-4 mg/ml in PBS) was added and the solution (2.0 ml final volume) subjected to bath sonication under a N_2 atmosphere, with the sample temperature maintained between 22 and 25 °C. After 1–4 h the turbid mixture became clear, indicating apolipoprotein/phospholipid complexes (i.e. ND) had formed. The solution was then dialyzed overnight to remove DMSO, followed by 0.22 μm filter sterilization.

2.3. Analytical procedures

Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical Co.) with bovine serum albumin as standard. Choline containing phospholipids were quantified by enzyme based colorimetric assay (Nie et al., 1993). Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed on 4–20% acrylamide slab gels. Samples were electrophoresed at a constant 150 V for 20 h and stained with Coomassie Blue.

2.4. UV/vis absorbance spectroscopy

Absorbance spectroscopy was performed on a Perkin-Elmer Lambda 20 spectrometer at 20 °C. ATRA levels were determined using an extinction coefficient at 341 nm = 45,300 M^{-1} cm^{-1} in ethanol (Barua and Furr, 1998). Samples were scanned from 250 to 450 nm. Spectra of ND samples in aqueous media were obtained in PBS. Control spectra of "empty" ND that lack ATRA confirmed that other ND components do not interfere with or contribute to the spectral absorbance of ATRA between 300 and 450 nm.

2.5. Gel filtration

A sterile filtered sample (2 ml) of ATRA-ND was applied to a 75 ml bed volume Sepharose 6B (GE Biosciences) gel filtration column equilibrated in PBS. Following application of the sample, the column was eluted with collection of two ml fractions. Subsequently, each fraction was analyzed for protein, phospholipid and ATRA content as described above. In control experiments, empty ND gave rise to the same elution profile as ATRA-ND.

2.6. Cell growth inhibition assays

HepG2 human hepatoma cells were maintained in minimal essential media supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS). Prior to experiments, 4×10^4 cells per well were seeded into 24 well culture plates with 2 ml complete media. After 24 h, the media was replaced with 4% FBS complete media. At 48 h, cells were washed, and provided fresh 4% FBS complete media in addition to specified concentrations of free ATRA (in ethanol) or ATRA-ND. 72 and 120 h after administering the ATRA, cell viability was determined using the MTT assay (Mosmann, 1983). Values expressed are the mean ± S.D. ($n = 3$) percent cell viability relative to control.

3. Results and discussion

ND are nanoscale (8–20 nm diameter) noncovalent assemblies organized as a disk-shaped phospholipid bilayer that is circumscribed by two or more amphipathic apolipoprotein molecules (Narayanaswami and Ryan, 2000). The bilayer portion of ND provides an environment capable of solubilizing and sequestering hydrophobic molecules and, as such, they can serve as bioactive agent delivery vehicles. Previous studies have shown that ND enriched with the water insoluble polyene antiobiotic, amphotericin B (Hargreaves et al., 2006) possess potent biological activity *in vitro* and *in vivo* (Oda et al., 2006; Nelson et al., 2006). To further explore the utility of ND as a drug delivery vehicle, experiments were designed to incorporate the bioactive lipid ATRA into ND. The formulation strategy takes advantage of the unique ability of amphipathic apolipoproteins to transform specific phospholipid vesicle substrates into ND (Weers et al., 2001). By introducing ATRA into the reaction mix, it was hypothesized that a ternary ND particle, comprised of phospholipid, apoE3-NT and ATRA, would be generated. A general scheme describing ATRA-ND formation and particle structure is depicted in Fig. 1.

3.1. Formulation and characterization of ATRA-ND

When DMPC, DMPG, and ATRA (phospholipids:ATRA molar ratio = 5.5:1) were incubated with recombinant apolipoprotein at 24 °C with bath sonication, the sample changed from an opaque, turbid suspension to a clarified solution. No precipitate appeared upon centrifugation of the product solution indicating all of the reaction components had