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Pharmacokinetics and therapeutic efficiency of a novel cationic liposome nano-formulated all *trans* retinoic acid in lung cancer mice model

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ABSTRACT

Purpose: The externally given all *trans* retinoic acid (ATRA) for the treatment of certain solid cancers faces challenges such as stability, persistence, and effective targeted delivery into cell which necessitate the development of a suitable liposomal formulation for ATRA treatment.
Methods: Lipo-ATRA was developed using 1, 2- Dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol and ATRA (70:20:10) by dry thin film method and investigated for its *in vitro* characteristics, *in vivo* ATRA bioavailability and pharmacokinetic properties in normal and cancer mice using HPLC. The *in vivo* therapeutic efficiency of lipo-ATRA was also studied.
Results: DOTAP lipo-ATRA (91.676 ± 1.29%) of 262.776 ± 1.045 d nm size with smooth spherical surface was developed which was stable at up to 60 days with sustained ATRA release through dialysis membrane. The serum pharmacokinetics for lipo-ATRA in cancer bearing mice has shown a higher half-life-(14.8200h), C_{max} (0.66 μg/ml) and a lower CL (46.6061 μg/ml/h) *in vivo* when compared with free ATRA group ($t_{1/2}$-13.2205h, C_{max}-0.29 μg/ml, CL-136.2725 μg/ml/h). A significantly higher bioavailability in blood and lung even after 24 h with a promising therapeutic efficiency was observed in lipo-ATRA group.
Conclusions: The results showed that the formulation of lipo-ATRA in DOTAP and cholesterol in the ratio of 70:20 was the suitable carrier for ATRA in treating lung cancer.

1. Introduction

All *trans* retinoic acid (ATRA) has been reported to treat various types of cancers such as acute promyelocytic leukemia (APL), lung cancer, head and neck cancer, breast cancer [1–4]. Due to its pleotropic regulator function in cell, ATRA participates in many physiological pathways such as cell proliferation, differentiation, embryogenesis, morphogenesis, apoptosis, and inflammation [5]. ATRA being a lipid – acid, is highly lipophilic with low water solubility which is sensitive to light and heat [6]. During oral treatment with ATRA, the main disadvantage is its poor bioavailability and also its persistence (stability) in the blood [7]. Hence, ATRA as a single agent could not achieve a long duration of complete remission due to its poor pharmacokinetics profile [8]. To resolve these kinds of problems it is necessary to develop a suitable delivery system for efficient and safer delivery of ATRA to the target site for better therapeutic outcomes. Various types of vehicles in the form of liposomes, lipid nanoparticles, microspheres and polymeric micelles have been formulated for ATRA in the past years [9,10].

Among the solid cancers, lung cancer is the one which needs topmost attention for such targeted molecular therapy as it is the top most cause of cancer death in the globe [11] and the currently available chemotherapeutic drugs are not effective. Furthermore, intense research is going on ATRA therapy for lung cancer in recent years [12]. Liposomes which helps to incorporate the hydrophilic (in the aqueous cavity), hydrophobic (within lipid membrane) and amphipathic substances [13] are the promising vehicles to carry the drugs to target site and deliver the drugs into the cells. It is also reported to reduce the exposure toxicity of the drugs and also providing sustained drug release [14]. These liposomes are relatively biocompatible, biodegradable, and non-immunogenic lipid complexes that are used to carry both the hydrophilic and hydrophobic drugs. Specific types of lipid and ratio need to be used to develop lipo-drug based on the characteristics of the drugs for proper entrapment, stability and controlled delivery.

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Abbreviations

ATRA	All *Trans* Retinoic Acid
DOTAP	1, 2- Dioleoyl-3-trimethylammonium-propane
APL	Acute Promyelocytic Leukemia
PBS	Phosphate Buffered Saline
DLS	Dynamic Light Scattering
SEM	Scanning Electron Microscope
HPLC	High Performance Liquid Chromatography
CL	Clearance
Cmax	Maximal Concentration
T_{max}	Maximal Time
$t_{1/2}$	Terminal half-life
AUC_{0-t}	Area Under the concentration – time curve up to last time
$AUC_{0-\infty}$	Area Under the concentration – time curve up to infinite time
B(a)P	Benzo(a)Pyrene
DPX	Distyrene Plasticizer Xylene
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
PAH	Poly Aromatic Hydrocarbon

In this study, we have developed the formulation of liposome ATRA (Lipo-ATRA) using a cationic lipid 1, 2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and Cholesterol. DOTAP is a synthetic lipid which consists of one positive charge at the head group so called cationic which can bind with negatively charged compounds of cell membrane due to its high affinity. This enhances the internalization and delivery of the drugs to the specific target cells [15]. The previous *in vitro* and *in vivo* studies using different cationic liposomes have shown their potency in enhancing the anti-tumour immune response against cervical cancer and an immunologic adjuvant effect on dendritic cells [16,17] which also might be helping for enhancing anti-cancer effect. Furthermore the cationic liposome DOTAP formulated ATRA has been demonstrated to have an enhanced anti-lung cancer activity on A549 human lung cancer cell lines and also an anti-lung metastatic activity on *in vivo* metastatic mice model [18,19]. Cholesterol affects the mechanical properties of lipid bilayer by increasing their mechanical strength, membrane elasticity, and increases the packaging density, by its ordering and condensing effects [20,21]. However, the drug delivery or release is also equally important as packaging. Previous studies on the optimization of cholesterol level in liposomes have reported that the increasing amount of cholesterol decreased the percentage of drug release from the liposome due to its planar steroid ring and a rigid structure [22]. In this study we have formulated the liposomal encapsulated ATRA in a suitable ratio by using high level of lipid and minimal concentration of cholesterol to enhance the drug release percentage without compromising the percentage entrapment efficiency or integrity. We have evaluated the characteristics, pharmacokinetic property, *in vivo* bioavailability and the anti-cancer efficacy of the developed formulation.

2. Methods

2.1. Chemicals

DOTAP was purchased from Santacruz Biotechnology, Inc. (Santacruz, CA, USA) and cholesterol was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). All *trans* retinoic acid was purchased from Sigma Chem. Co. (St. Louis, MO, USA). All other chemicals used for encapsulation studies were highly pure and of analytical grade.

2.2. Preparation of liposome encapsulated ATRA

The liposome formulation was prepared as per the method described by Bhowmick et al. [23]. The mixture of liposome DOTAP: Cholesterol: ATRA at the molar ratio of 70:20:10 was formulated with or without ATRA (in ethanol). Briefly, the mixture with or without ATRA, was first dissolved in chloroform in a round bottom flask and run in the rotary evaporator at 55 °C in 150 rpm for developing the thin film of liposome layer. After vacuum drying, phosphate buffered saline (PBS) (pH 7.4) was added for hydration. The preparation was then subjected to sonication to get unilamellar liposomes and ultra-centrifugation at 100,000×g to remove the unentrapped ATRA if any. This liposome encapsulated ATRA was then mixed with PBS for further study.

2.3. Characterization of ATRA encapsulated liposome

2.3.1. Percent entrapment analysis

The efficiency of ATRA loaded into the liposome was estimated by reading absorbance at 340 nm after dissolving ATRA incorporated in liposomes in ethanol since the ATRA alone is soluble in ethanol. A standard graph for free ATRA (1–10 μg/ml) was developed. The percent entrapment was then calculated [24,25].

Entrapment percent = [Concentration of entrapped ATRA/Total Concentration]×100.

2.3.2. Stability studies

The stability of liposomal formulation to retain the drug was studied by storing the drug at different temperatures and durations [26]. The liposomal formulation with ATRA was stored up to 3 months in 4 °C (Refrigerated Temperature), 25 ± 2 °C (Room Temperature), and at physiological temperature 37 ± 2 °C (Incubator). The samples were kept in aluminum foil covered glass tubes due to the light sensitivity of ATRA. The drug content (ATRA) present in the liposomal formulation was assessed periodically at 1st, 15th, 30th, and 60th day as the method of percent entrapment analysis after subjecting to ultracentrifugation.

2.3.3. Particle size analysis

The size of the prepared ATRA entrapped liposome and free liposome was measured by Dynamic Light Scattering (DLS) method after diluting the liposomes (1:5000) with PBS. These diluted samples were subjected to the Zeta sizer Nano ZS instrument (Malvern instruments Ltd., Worcestershire, UK).

2.3.4. Morphological analysis

The ultra-centrifuged liposomal drug and free liposomal pellets were dried, and the solid powdered sample was processed by gold coating for the morphological analysis by Scanning Electron Microscope (SEM). The morphological appearance of the surface as well as the vesicle size of the dried gold coated samples of ATRA entrapped liposome and free liposomes were observed under the SEM (JEOL JSM 6390) at 20 kV.

2.3.5. In vitro drug release study

The *In vitro* drug release profile of ATRA was performed using dialysis bag as per the method reported earlier [27]. Briefly, 2 ml of ATRA entrapped liposome in PBS was poured in to the pre-soaked (12 h in distilled water) dialysis bags (Hi-media, 12,000–14,000 MW cutoff) having the ability to retain the