4. ATRA LEVEL ASSAY AND LIPID PROFILE ASSAY IN LUNG CANCER MICE MODEL

4.1 Introduction

Retinoic acid modulates the cell proliferation, differentiation and apoptosis of normal and abnormal cells of several cancers in vivo and in vitro including the colon, prostate, lung, and leukemia (Zheng et al., 1999; Liang et al., 1999; Weber et al., 1999; Mologni et al., 1999; Francois et al., 2000). The pharmacokinetic studies of chronic daily dosing schedule have shown a dramatic decrease in systemic exposure to ATRA over time (time-dependent elimination), as measured by the area under the plasma concentration-time curve (AUC) (Adamson et al., 1995, 1997; Regazzi et al., 1997). The observed progressive decrease in plasma AUC values with continued oral therapy has been related to the induction of drug catabolism by cytochrome P450 (CYP) enzymes (Anas et al., 2004). The poor aqueous solubility of ATRA can be a major drawback for its parenteral administration. Incorporation of drugs in lipid-based carriers such as liposome and lipid emulsions is known as an attractive means to overcome the solubility limitations of drugs (Chen et al., 2001). Gradual decrease in ATRA level in blood circulation observed after long term oral treatment however, has been shown to, probably due to the induced cytochrome P-450-dependent metabolism of ATRA (Muindi et al., 1992). Attempts have been made by several researchers to develop i.v. injectable formulation of ATRA by loading in carriers such as solid nano particles, cyclodextrins and liposome in order to improve its aqueous solubility (Ozpolat et al., 2003; Choi et al., 2003). Several studies have shown that the liposome can be a novel injectable formulation of ATRA (Lim and Kim, 2002). In addition to the poor solubility, ATRA has shorter half life and is also known to be chemically unstable (Brisaert et al., 2000).
ATRA encapsulated into liposomes gives a new intravenous ATRA formulation (Drach et al., 1993; Estey et al., 1996; Mehta et al., 1994) that may significantly improve the potency and half life of ATRA in cases of leukemia and potentially other malignancies. Previous studies have shown that the liposomal ATRA effectively induces differentiation in human myeloid leukemia cell lines (HL-60, KG-1, and THP-1) and is as effective as free ATRA in inducing differentiation of cells from patients with acute promyelocytic leukemia (Drach et al., 1993). Diphosphatidyl palmitoylcholine and stearylamine (9:1 [wt/wt]) made liposome encapsulated ATRA showed optimal drug incorporation, high stability, and minimal toxicity toward erythrocytes, while delivering a sufficiently high concentration of ATRA to inhibit the growth of a squamous-cell carcinoma cell line, MDA 886Ln (Parthasarathy et al., 1994). Mehta and coworkers reported that when liposome encapsulated ATRA was administered to rats over a prolonged period, the levels of the drug in the blood did not change over time (Mehta et al., 1994). In vitro studies of isolated liver microsomes in rats that were repeatedly given with the liposome encapsulated ATRA formulation revealed that the catabolism of the drug was not altered whereas microsomes isolated from animals that were orally administered with free ATRA showed a significant increase in the metabolism of the drug (Mehta et al., 1994). For most hydrophilic compounds, intravenous administration results in an instantaneous distribution of the compound within the blood. This is a consequence of homogeneous mixing of the compound with the aqueous components of the bloodstream (Rowland, 1980). However, when compounds are hydrophobic in chemical nature or are incorporated into lipophilic carriers (liposomes), their distribution within the bloodstream may not be instantaneous (Kishor et al., 1998).

This study was therefore designed to enhance the chemo preventive/therapeutic effect of ATRA on lung metastasis by encapsulating it with liposome which might help the ATRA for its persistence in circulation and to reach the lung tissue. In this study,
the level of ATRA in the serum and lung tissue was analyzed using HPLC after treatment with ATRA incorporated in DSPC/cholesterol liposome in B16F10 cells implanted C57BL/6 lung cancer bearing mice model. The results were compared with the ATRA level of free ATRA treated lung cancer model. The reason for using cancer model for this study was to analyze the bioavailability of ATRA in cancer condition and its reachability to target site (lung tissue). The same experimental mice model was further used for the analysis of cancer parameters.

Lipids are major cell membrane components essential for various biological functions including cell growth and division of normal and malignant tissues. Evidence has accumulated that specific retinoids impact on developmental and biochemical processes influencing fatty acid oxidation in tissues. Nevertheless, controversial data have been reported, particularly regarding retinoids' effects on hepatic lipid and lipoprotein metabolism and blood lipid profile (Bonet et al., 2012). An earlier research study has reported that the ATRA has down-regulated the apolipoprotein A-I mRNA expression in rat hepatocytes, which is a major component of HDL-cholesterol and as a result HDL-cholesterol was shown to be decreased. Which suggests that retinoids treatment can induce other long term metabolic regulatory pathways by which lipid metabolism may alter. Moreover, the molecular mechanisms underlying retinoid effects on lipid metabolism are complex and remain incompletely understood (Corbetta et al., 2006).

In addition to the effect of ATRA on cell differentiation, it also can have dramatic effects on serum lipid metabolism. Administration of retinoids to both experimental animals (Gerber and Erdman, 1981b; McMaster et al., 1989; Oliver and Rogers, 1993; Standeven et al., 1996) and humans (Lyons et al., 1982; Bershad et al., 1985) often results in increases in serum triglyceride levels. The hypertriglyceridemia induced by retinoic acid isomers (either ATRA or 13-cis-RA) is due to alterations in
several aspects of serum triglyceride metabolism. In rats, retinoic acid-induced hypertriglyceridemia is due to both increased hepatic production of very-low-density lipoproteins (VLDL) (Gerber and Erdman, 1981a) and a suppression of lipoprotein lipase (LPL) activity in peripheral tissues (Gerber and Erdman, 1981a; Oliver and Rogers, 1993). In humans there are no such data on the effects of retinoids on hepatic VLDL production but 13-cis-RA increases the levels of apolipoprotein (apo)-CIII a hepatic protein that binds to VLDL and interferes with their lipolytic processing (Vu-ac et al., 1998). ATRA induced hypertriglyceridemia was initially encountered in patients receiving retinoic acid isomers that either directly or indirectly activate RARs (Peter et al., 2001). RAR specific retinoids induce hypertriglyceridemia in both experimental animals (Standeven et al., 1996) and humans (Takeuchi et al., 1998).

Furthermore, researchers have reported an association of plasma/serum lipids and lipoproteins with different cancers (Schatzkin et al., 1988; Halton et al., 1998; Simo et al., 1998; Allampallam et al., 2000). The alterations in the circulatory cholesterol levels have been found to be associated with etiology of breast cancer and colorectal cancer (Gerber et al., 1989, 1988; Forones et al., 1998) and few reports are available on plasma lipid profile in head and neck cancer and lung cancer (Schatzkin et al., 1988; Chyou et al., 1992; Patel et al., 2004). Cancer and lipid profile are closely related and it is under study now (Ravi et al., 2009).

Hence attempt is made in this study to analyze the lipid profile also in mice treated with free ATRA and encapsulated ATRA in cancerous and non cancerous mice model.
4.2 Rationale

The highly unstable ATRA has limited half life in the blood and it reaches the target tissues in very less amount. Hence it is essential to study the level of ATRA in serum and lung tissue to find out the efficiency of encapsulation to obtain beneficial clinical outcomes. Retinoid therapy is known to be associated with hypertriglyceridemia and often with low HDL-cholesterol. Lipid constituents such as cholesterol and triglycerides are essential to carry out several vital physiological functions including cell growth and division of normal and malignant tissues. The lipid profile is therefore needed to be analyzed in the same experimental mice model while treating with ATRA.

4.3 Objectives

To evaluate the ATRA level in the serum and lung tissue as well as serum lipid profile upon treatment with free ATRA and encapsulated ATRA in lung cancer mice model.

4.4 Hypothesis to be analyzed

When we encapsulate the ATRA with high percent entrapment, it may extend circulation time, increase stability, reach target site and improve efficacy of ATRA action. Our hypothesis is that the encapsulation of therapeutic ATRA with a promising delivery system liposome may increase the persistence of ATRA in blood, reach the target site of lung where the metastatic cancer cell line proliferates. The encapsulation may prevent the changes in lipid profile which is being reported in ATRA treatment.
4.5 Materials and Methods

4.5.1 Animals

C57BL/6 male mice (4-6 weeks old) were purchased from National Institute of Nutrition (Hyderabad, Andhra Pradesh, India). The animals were kept in well-ventilated cages in a facility maintained at 22 (± 1)ºC, with a 40 (±10)% relative humidity, and a 12-hr light-dark during the experimental period. Mice were provided access to normal mouse chow (Sai Feeds, Bangalore, India) and filtered water ad libitum. All the animal experiments were carried out according to the rules and regulation of the Animal Ethics Committee of the Government of India.

4.5.2 Chemicals

ATRA was purchased from Sigma Chem. Co. (St Louis, MO). Lipid profile diagnostic kit was purchased from Span diagnostic kit, Gujarat, India. All other chemicals used were of analytical grade.

4.5.3 Tumor Cell Line

The B16F10 melanoma cell line was purchased from National Centre for Cell Science (NCCS, Pune, India). The cells were maintained in RPMI 1640 medium buffered with 2 g/L of HEPES and sodium bicarbonate, and supplemented with dextrose, penicillin, streptomycin and 10% of fetal bovine serum. The cells were maintained in a humidifified atmosphere containing 5% CO₂ at 37ºC. When needed for experiments the cells were harvested with trypsin:EDTA (0.05:0.03 [w/v]) solution, and then washed in phosphate-buffered saline (PBS, pH 7.4). For the animal experiments, the recovered cells were adjusted to 1x10⁶ cells/ml in PBS and then 100 µl of the suspension was injected into the tail-vein.
4.5.4 ATRA Dose Selection for Treatment and Mode of Administration

The treatment dose of ATRA was selected based on the available research reports as 0.60 mg/kg-body weight (Kawakami et al., 2006; Suzuki et al., 2006). We have given the drug by intra-peritoneal (IP) injection because it is the preferred one over intravenous (i.v) injection in mice for 21 days of treatment. Then the in vivo drug toxicity study was carried out for a range of ATRA dose including the selected dose to confirm the toxicity of ATRA.

4.5.5 In-vivo Drug Toxicity Studies in C57BL/6 Mice

Six groups of six C57BL/6 mice each were given graded doses of the ATRA (i.e., 0.5-1.0 mg/kg-body weight) via intra-peritoneal (IP) and continuously observed for 5 hours for any changes in various autonomic and behavioral responses. Thereafter, the mice were examined daily for 50 days for an extended observation of any ATRA-induced mortality.

4.5.6 Study of the Level of ATRA in Serum and Lung Tissue

4.5.6.1 Study Design and Experimental Groups

C57BL/6 mice were divided into 5 groups of 15 mice each to carry out the level of ATRA present in lung tissue and serum in the lung tumor bearing mice model. B16F10 cell line was injected (1 x 10^6 cells in 0.1ml) by tail vein to group I, IV and V. Free ATRA and encapsulated ATRA were given for respective group at a dose of 0.60mg/kg/body weight/day for 21 days after cell line implantation. Extra 3 mice were analyzed for normal level of ATRA in serum and lung tissue (sacrificed) to get pretreatment level.
Group I = Cell line alone injected (n=15)  
Group II = Free ATRA alone (n=15)  
Group III = Liposome encapsulated ATRA alone (n=15)  
Group IV = Cell line injected + Free ATRA (0.60 mg/kg body weight/day and n=15)  
Group V = Cell line injected + Encapsulated ATRA (0.60 mg/kg body weight/day and n=15)  

4.5.6.2 Sample Collection for ATRA Level Analysis

After 21st dose, three mice from each group were sacrificed by cervical dislocation at different time points such as 1, 3, 6, 12 and 24 hours respectively and on same day the extra 3 normal healthy mice were also sacrificed by cervical dislocation. Blood was collected by heart puncture and lung tissues were dissected out for ATRA level analysis.

Serum was prepared by centrifugation of the blood for 10 min at 4 ℃ and 1500xg and stored at -80 ℃ until analysis. Lung tissues were cut into pieces with scissor (200 mg) and washed with cold saline solution. Lung tissues were homogenized and supernatant was subjected for analysis of ATRA level in lung tissue.

4.5.6.3 Sample Processing for HPLC Analysis

Concentration of ATRA was measured by HPLC (Wyss, 1990). Prior to injection into the chromatograph, serum samples were subjected to the following extraction method which was modified from relevant literature to deproteinate at dark room (Pikkarainen and Parviainen, 1992): 100 μl of acetonitrile was added to 100 μl of serum sample and the mixture was shaken by hand (for 10 seconds), placed in the refrigerator at -4 ℃ for 10 minutes, and then centrifuged (2000 g for 5 minutes). The supernatant was transferred to a clean tube and stored at -20 ℃ until injection (200 μl) into the chromatograph.
Tissues were homogenized with 250 μl of 10% ascorbic acid and then 250 μl of ethanol was added to each tube and vortexed for 1 min. later 2ml of n-hexane was added and vortexed. The sample was then centrifuged at 2000 rpm for 10 mins. The hexane containing upper layer was removed, evaporated and dried under nitrogen stream. The residue was dissolved in 200μl of methanol for the HPLC analysis.

4.5.6.4 HPLC Analysis and the Pharmacokinetics Study of ATRA in Serum and Lung Tissue

All samples were analyzed for ATRA concentration by reversed-phase HPLC in a Shimadzu Prominence HPLC system with LC 10 AT pumps, SPD M20 A detector, with Rheodyne injector loop volume of 20µl. The column was a Phenomenex Luna (250mm×4.6mm) column, 5μm particle size, and the mobile phase was a mixture of acetonitrile and water (45:65, v/v) delivered at a flow rate of 1 ml/min. Wavelength used for the study was 310 nm.

The pharmacokinetic parameters were assessed from the analysis of the ATRA level present in the serum and lung tissue at a particular time point. Elimination rate constant (ERC), C\text{max}, T\text{max}, Terminal half-life (t\text{1/2}), area under the concentration-time curve up to last time (AUC\text{0–t}) or infinite time (AUC\text{0–∞}) were analyzed.

4.5.6.5 Standard Curve Calibration for ATRA Level Analysis

Calibration graphs were constructed by linear least squares regression analysis of the plot of the peak height against the concentration of ATRA for serum and lung tissue respectively. For serum sample, 5 standards covering the range of 200 to 1000 ng/ml and for lung tissue sample, 5 standards covering the range of 400 to 2000 ng/ml were taken. The concentration of ATRA in serum and lung tissue samples were
determined by interpolation in the calibration graph of the corresponding peak height values obtained in the chromatograms.

4.5.7 Biochemical Assays for Lipid Profile

Serum was prepared from eight groups mentioned below and analyzed for total cholesterol (Parekh and Jung, 1970), triglycerides (TG) (Itaya, 1977), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and high density lipoprotein (HDL) (Wilson and Spiger, 1973). For this lipid profile study, cell line was not injected in group I-III and remaining groups from IV-VIII were injected with B16F10 cell line. All the treatment was carried out for 21 days to analyze the lipid profile in mice serum after cell line injection. Six mice were used in each group for this lipid profile study.

**Experimental Groups for Lipid Profile Study**

Group I = Normal
Group II = Free ATRA alone treated
Group III = Encapsulated ATRA alone treated
Group IV = Cell line alone injected
Group V = Cell line injected + olive oil
Group VI = Cell line injected + Free liposome
Group VII = Cell line injected + Free ATRA
Group VIII = Cell line injected + Encapsulated ATRA

4.5.7.1 Serum Total Cholesterol Level Estimation

Briefly, 5 μl of serum sample was mixed with 500 μl of working reagent that contained cholesterol oxidase, cholesterol esterase, peroxidase, 4-amino phenanzone, surfactant, phenol, buffer, preservatives and stabilizer. The mixture was incubated at 37°C for 10 minutes and absorbance was read at 505 nm.
4.5.7.2 Serum HDL Cholesterol Level Estimation

Briefly, 0.3 ml serum sample was mixed with 0.3 ml precipitating reagent (PEG 6000, stabilizer and preservative), followed by 10 minutes incubation at room temperature. The mixture was centrifuged at 2000 rpm for 15 minutes. The supernatant obtained was mixed with working cholesterol reagent. After incubation for 10 minutes at 37°C, absorbance was read at 505 nm.

4.5.7.3 Serum Triglyceride Level Estimation

Briefly, 10 μl serum sample was mixed with 1000 μl of triglycerides assay reagent containing Pipes buffer, lipase, 4–chlorophenol, Magnesium ion, ATP, lipase, peroxidase, glycerol kinase, sodium azide, 4–amino antipyrene, glycerol-3-phosphate oxidase and detergents. The mixture was then incubated for 10 minutes at 37°C and absorbance was read at 505 nm. VLDLC and LDLC levels were calculated as shown below

VLDLC = Triglyceride/5
LDLC = Total Cholesterol – [(VLDLC) + (HDL)]

4.5.8 Statistical analysis

All data were expressed as mean (± SD). The statistical analysis was done using a one-way analysis of variance (ANOVA) followed by a Dunnett’s test (using Graphpad InStat version 3.00 for Windows XP; GraphPad Software, Inc., La Jolla, CA). P-value ≤ 0.05 was considered significant.
4.6 Results

4.6.1 In-vivo Drug Toxicity Study Result in C57BL/6 Mice

The drug did not show any predictable changes in the autonomic and behavioral responses in C57BL/6 mice. All the mice lived healthy even after 50 days even at a dose of 1.0 mg/kg body weight of C57BL/6 mice.

4.6.2 Levels of ATRA by HPLC Analysis in the Serum and Lung Tissue

The ATRA levels in the serum and lung tissues and the pharmacokinetic parameters that were quite consistent are given in Figure 4.1 and 4.2 respectively. In non cancer mice model, encapsulated ATRA treatment showed the serum ATRA level as 0.601±0.04 µg/ml at 1st hour and 0.273±0.012 µg/ml at 12th hour while ATRA level in free ATRA group was found to be 0.423±0.04 µg/ml at 1st hour and 0.111±0.008 µg/ml at 12th hour (Figure 4.1). The serum ATRA level in cancer bearing mice model was found to be 0.523±0.05 µg/ml at 1st hour and 0.189±0.02 µg/ml at 12th hour for encapsulated ATRA treatment and that of for free ATRA treatment were found to be 0.212±0.01 µg/ml at 1st hour and 0.089±0.01 µg/ml at 12th hour respectively.

The t1/2 was found to be 9.03 and 5.42 hours for encapsulated ATRA and free ATRA respectively in non-cancer bearing mice model and that of 9.03 and 7.2 hours respectively in lung cancer bearing mice model. The AUC0-1 was found to be 4.86 and 2.67 µg.h/ml for encapsulated ATRA and free ATRA in non-cancer bearing mice model and that of 4.86 and 2.15 µg.h/ml respectively in lung cancer bearing mice model (Table 4.1). ATRA level varied in serum sample of different groups up to 12th hour and at 24th hour all groups showed the normal level of ATRA.

In lung tissue, in non cancer mice model, encapsulated ATRA group showed the ATRA level as 0.70 ±0.05 µg/ml at 1st hour and 0.21±0.03 µg/ml at 24th hour
respectively. While Free ATRA group showed 0.71±0.08 µg/ml and 0.08±0.011 µg/ml ATRA levels in lung tissue sample at 1st hour and on 24th hour respectively (Figure 4.2). The ATRA level in lung tissue in cancer bearing mice model was found to be 0.86±0.07 µg/ml at 1st hour and 0.18±0.02 µg/ml at 12th hour for encapsulated ATRA treatment and that of for free ATRA treatment were found to be 0.26±0.02 µg/ml at 1st hour and 0.088±0.016 µg/ml at 12th hour respectively.

The t_{1/2} was found to be 14.79 and 2.81 hours for encapsulated ATRA and free ATRA respectively in non-cancer bearing mice model and that of 10.39 and 8.34 hours respectively in lung cancer bearing mice model. The AUC_{0-t} was found to be 7.68 and 3.79 µg.h/ml for encapsulated ATRA and free ATRA respectively in non-cancer bearing mice model and that of 7.68 and 1.63 µg.h/ml respectively in lung cancer bearing mice model (Table 4.2).

### 4.6.3 Results for Lipid Profile

The effect of ATRA on the levels of serum triglycerides and total cholesterol is shown in Figure 4.3. There was also little elevation in the levels of triglycerides and total cholesterol in non cancerous groups (Group II and III) treated with free ATRA and encapsulated ATRA. There was a significant (P ≤ 0.01) increase in the levels of triglycerides and total cholesterol in cancer mice without any drug treatment (Group IV-VI) when compared to normal mice (Group I). Free ATRA and encapsulated ATRA treatment in cancer groups significantly (P ≤ 0.01) lowered these alterations on the lipid profile in Group VII and VIII mice when compared to cancer control mice (Group IV). B16F10 cells induced lung cancer mice (Group IV) showed significant (P ≤ 0.01) elevations in the levels of serum LDL and VLDL with a concomitant decrease in HDL levels when compared to group I normal mice. These alterations were reverted back close to normalcy in free ATRA and encapsulated ATRA treated (Group VII and VIII) mice respectively (Figure 4.3).
Encapsulated ATRA treated showed highest concentration of ATRA in serum both in non-cancer mice and cancer mice models compared with free ATRA treatment. Values are expressed in mean ± SD (n = 3). The significant difference was determined between:

Normal vs. Free ATRA treated in non cancer mice: **p≤0.01 and non-significant=ns
Normal vs. Encapsulated ATRA treated in non cancer mice: **p≤0.01 and non-significant=ns
Normal vs. Free ATRA treated in cancer mice: **p≤0.01 and non-significant=ns
Normal vs. Encapsulated ATRA treated in cancer mice: **p≤0.01 and non-significant=ns
Encapsulated ATRA treated showed highest concentration of ATRA in lung tissue both in non-cancer mice and cancer mice models compared with free ATRA treatment. Values are expressed in mean ± SD (n = 3). The significant difference was determined between:

Normal vs. Free ATRA treated in non cancer mice: **p≤0.01 and non-significant=ns
Normal vs. Encapsulated ATRA treated in non cancer mice: **p≤0.01
Normal vs. Free ATRA treated in cancer mice: **p≤0.01 and non-significant=ns
Normal vs. Encapsulated ATRA treated in cancer mice: **p≤0.01
Table 4.1: Representative pharmacokinetics of ATRA in serum

<table>
<thead>
<tr>
<th>Groups</th>
<th>Elimination rate constant (ERC) (hour)</th>
<th>AUC$_{0-t}$ (µg.h/ml)</th>
<th>AUC$_{0-\infty}$ (µg.h/ml)</th>
<th>Terminal half life in hour (t$_{1/2}$)</th>
<th>C$_{max}$ (µg/ml)</th>
<th>T$_{max}$ (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: Cell line alone injected</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II: Free ATRA alone</td>
<td>0.127812</td>
<td>2.6775</td>
<td>3.545966</td>
<td>5.423197</td>
<td>0.423</td>
<td>1</td>
</tr>
<tr>
<td>III: Liposome encapsulated ATRA alone</td>
<td>0.07673</td>
<td>4.8665</td>
<td>8.42442</td>
<td>9.033562</td>
<td>0.601</td>
<td>1</td>
</tr>
<tr>
<td>IV: Cell line injected + Free ATRA</td>
<td>0.096259</td>
<td>2.15</td>
<td>3.074588</td>
<td>7.20085</td>
<td>0.212</td>
<td>3</td>
</tr>
<tr>
<td>V: Cell line injected + Encapsulated ATRA</td>
<td>0.098322</td>
<td>4.8665</td>
<td>6.788754</td>
<td>9.033562</td>
<td>0.523</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4.2: Representative pharmacokinetics of ATRA in lung tissue

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ellimination rate constant (ERC) (hour)</th>
<th>AUC\textsubscript{0-t} (µg.h/ml)</th>
<th>AUC\textsubscript{0-∞} (µg.h/ml)</th>
<th>Terminal half life in hour (t\textsubscript{1/2})</th>
<th>C\textsubscript{max} (µg/ml)</th>
<th>T\textsubscript{max} (hour)</th>
</tr>
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<tbody>
<tr>
<td>I: Cell line alone injected</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II: Free ATRA alone</td>
<td>0.140288</td>
<td>3.79</td>
<td>5.001792</td>
<td>2.813621</td>
<td>7.04</td>
<td>1</td>
</tr>
<tr>
<td>III: Liposome encapsulated ATRA alone</td>
<td>0.04684</td>
<td>7.685</td>
<td>18.14609</td>
<td>14.79812</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>IV: Cell line injected + Free ATRA</td>
<td>0.083078</td>
<td>1.634</td>
<td>4.940883</td>
<td>8.343377</td>
<td>2.6</td>
<td>1</td>
</tr>
<tr>
<td>V: Cell line injected + Encapsulated ATRA</td>
<td>0.06671</td>
<td>7.685</td>
<td>13.68115</td>
<td>10.39053</td>
<td>8.6</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 4.3: Effect of encapsulated ATRA on lipid profile in serum

Values are expressed in mg/dl as the mean ± SD (n = 6). The significant difference was determined between:

Normal (Gr-I) vs. Cancer control (Gr-IV): **p≤0.01
Normal (Gr-I) vs. free ATRA treated in cancer mice (Gr-VII): **p≤0.01
Normal (Gr-I) vs. encapsulated ATRA treated in cancer mice (Gr-VIII):
**p≤0.01 and *p≤0.05
Cancer control (Gr-IV) vs. free ATRA treated in cancer mice (Gr-VII): **p≤0.01 and *p≤0.05
Cancer control (Gr-IV) vs. encapsulated ATRA treated in cancer mice (Gr-VIII): **p≤0.01
4.7 Discussion

In the present study we have performed reverse phase HPLC analysis to determine the ATRA level in the serum and lung tissue in non-cancer and lung cancer mice model respectively treated with free ATRA and encapsulated ATRA.

Several research reports have suggested that a high dose of encapsulated ATRA is required to achieve physiological concentrations of ATRA at the level of target cells. In the free ATRA treatment, the ATRA concentrations rapidly decreased in serum, most probably as a result of its catabolism in the liver and other tissues (Maret et al., 2007). Exogenous retinoic acid (RA) can induce alveolar regeneration in a mouse and a rat model and this may be because RA is required during normal alveolar development (Hind and Maden, 2004). Several reports suggest that lipofibroblasts may be a potential target of exogenous RA in the regenerating lung. It has been reported that lipofibroblasts are present in the lung during alveologenesis (Kaplan et al., 1985; McGowan and Torday, 1997; Hind and Maden, 2004) and these cells are the retinoid storage cells of the lung (Okabe et al., 1984; Hind and Maden, 2004). It was observed that the retinoids are used to repair emphysematous lung damage, the capacity for ATRA to generate new septa and increase alveolar surface area in the adult lung has been confirmed by other several models (Belloni et al., 2000; Ishizawa et al., 2004; Kaza et al., 2001; Ofulue et al., 2002). Endogenous ATRA is thus responsible for promoting lung septation and an increase in alveolar surface area during lung development (Ross et al., 2000; Ong and Chytil, 1976; Massaro et al., 2000; Roth et al., 2006).

ATRA which is hydrophobic in nature need to be incorporated with some kinds of delivery systems, especially liposomes, to obtain beneficial clinical outcomes, including extended circulation time, increased stability and improved efficacy. In the earlier research report, the $C_{\text{max}}$ was found to be 4.5 µg/ml at 0.75 h in mice given with
intragastric ATRA (10 mg/kg) (Kalin et al., 1981). The $t_{1/2}$ was reported as $0.438 \pm 0.124$ h (Chou et al., 1997) or 0.69 h (Shelley et al., 1982) after an oral dose of 2 or 13.9 mg/kg, respectively; the $\text{AUC}^{0-\infty}$ was reported as $13,740 \pm 1600$ ng.h/ml after an oral dose of 13.9 mg/kg (Shelley et al., 1982). Another report stated that the pharmacokinetic behavior of orally administered ATRA shows that the drug is rapidly eliminated by humans, with a $t_{1/2}$ of approximately 45 min (Regazzi et al., 1997). Pharmacokinetic studies in patients demonstrated that oral ATRA is unable to sustain steady plasma drug levels during chronic daily administration. Oral ATRA administration resulted in a significant decrease in the plasma $\text{AUC}^{0-\infty}$ and $C_{\text{max}}$ by day 9 of treatment. However, $\text{AUC}^{0-\infty}$ and $C_{\text{max}}$ for encapsulated ATRA did not decrease during the 15 days of treatment. In the same study, encapsulated ATRA also has produced sustained plasma ATRA concentrations over a prolonged period (7 weeks) when compared to oral ATRA (non-liposomal) which resulted in decreased drug plasma concentrations (Ozpolat et al., 2003).

In these approaches, encapsulated ATRA has been the most successful in terms of enhancing the level of ATRA in serum as well as in target tissues that is commonly seen lower level of ATRA during oral ATRA treatment. One of the rationales for using liposomes as a drug delivery system is to improve the performance of ATRA by its delivery to the specific tissue sites where the disease present. Liposomal formulation allows the use of higher doses without increasing toxicity (Lopez-Berestein et al., 1994; Ozpolat et al., 2003).

In the present study, we have found that the intraperitoneal (i.p.) administration of encapsulated ATRA could maintain high ATRA concentrations in the serum as well as lung tissue than the administration of free ATRA following multiple dosing in healthy mice over a period of 21 days. Encapsulated ATRA and free ATRA treatment in non cancer mice model showed higher level of ATRA present in serum as well as in
the lung tissue whereas cancerous mice model showed a comparatively lower level of ATRA in serum as well as in lung tissue. To our surprise in our study, in lung tissue the ATRA level at 1st hour alone was found to be higher in cancer group than in non cancerous group but later from 3rd hour the cancerous group showed lower level in compared with non cancerous group. The decreased level of ATRA found in cancer model may be due to the use of ATRA in lung cancer development. The relatively higher level of ATRA was observed in serum of encapsulated ATRA treated mice in non cancerous condition suggesting that the encapsulated ATRA may prevent the rapid decrease in drug concentrations and clearance of drug from serum. In lung cancer condition ATRA may be used up for the lung development or lung repair mechanism, so the ATRA level was found less in compared with non cancer mice model. But the encapsulated ATRA treated mice showed elevated level of ATRA which means that the encapsulated formulation helps to maintain the ATRA level in lung tissue even in cancer model. Overall the data suggest that the encapsulated ATRA treated mice showed an increased level of ATRA in serum as well as in lung tissue in compared with free ATRA treatment in both lung cancer and non-cancer mice model. The favorable pharmacokinetic profile of encapsulated ATRA along with the higher consistent serum and lung tissue exposure to ATRA. This safety profile of encapsulated ATRA suggest that the encapsulated ATRA has potential advantages over free ATRA and may be highly effective as a monotherapy for the long-term treatment of lung cancer.

One of the purposes of this study was to determine the total cholesterol and lipoprotein distribution in mice serum after receiving the treatment of free ATRA and encapsulated ATRA in normal mice and lung cancer bearing mice model. Cholesterol and triglycerides are important lipid constituents of cell, essential to carry out several vital physiological functions. Cholesterol is essential for maintenance of the structural and functional integrity of all biological membranes. For transport in plasma,
triglycerides and cholesterol are packaged into lipoproteins, which are then taken up and degraded by cells to fulfill demands for cellular functions (Ramakrishnan et al., 2008). Hypertriglyceridemia is a frequent complication observed in the patients with either retinoids or rexinoids treatment. The hypertriglyceridemia induced by retinoic acid isomers either ATRA or 13-cis-RA, is due to alterations in several aspects of serum triglyceride metabolism (Peter et al., 2000). In fasted animals, plasma triglyceride levels showed a balance between the rate of hepatic secretion of very-low-density lipoproteins (VLDL) and their clearance rate. VLDL particles are cleared by lipoprotein lipase (LPL) activity present in the tissue vascular beds and hepatic lipase present in the liver. The multiple effects of retinoids on serum triglyceride metabolism may be due to the distinct activities of the different retinoid receptors and the members of the nuclear receptor (Mangelsdorf et al., 1993). RAR-specific retinoids induce hypertriglyceridemia in both experimental animals (Standeven et al., 1996) and humans (Takeuchi et al., 1998). Previous studies also showed in experimental animals that retinoid-induced hypertriglyceridemia is due to both alterations in hepatic lipoprotein secretion and peripheral lipoprotein catabolism (Oliver and Rogers, 1993; Peter et al., 2000). In fasted animals, plasma triglyceride levels represent the equilibrium between the rate of hepatic secretion of very-low-density lipoproteins (VLDL), and the rate of their clearance. VLDL particles are cleared by lipoprotein lipase (LPL) activity present in the tissue vascular beds and hepatic lipase present in the liver (Peter et al., 2000).

However, the increase in ATRA level in serum in encapsulated ATRA treatment group was found to be little when compared with that of in lung tissue (target tissue), which is discussed in the same chapter. As liposome encapsulation is used for targeted drug delivery and to increase half life of ATRA, it might not have influenced much on the serum triglycerides level in our study. Moreover, the serum triglycerides level is maintained by an intricate metabolic pathway of liver.
Research report has shown that by treatment with ATRA as low as 26 to 105 µg/100 g diet daily for 8 days induced hypertriglyceridemia in male Sprague-Dawley rats (Gerber and Erdman, 1979). In another study, elevated triglyceride was found in the plasma of young male rats intramuscularly administered with 30,000 µg of retinol daily for 13 days. It was also observed an elevation of plasma triglycerides in adrenalectomized rats by feeding the same level of retinol (Gerber and Erdman, 1980).

In our present study the liposome encapsulated ATRA and free ATRA treatment have shown a slightly elevated total cholesterol, triglyceride, VLDL and LDL cholesterol concentrations and a decreased HDL cholesterol in the cancerous and non cancer mice models (free ATRA and encapsulated ATRA alone treated). These findings suggest that the ATRA is having the activity to alter the distribution of lipoproteins in mice serum. However, no difference in serum lipoprotein distribution was observed in liposome alone treated group in our study and these findings suggest that a liposome-lipid acts as a solubilizing agent and does not influence the distribution of lipoprotein in serum. Kishore et al. (1998) has also reported already that the liposome used to deliver ATRA does not alter the lipid profile level (Kishore et al., 1998).

In the present study, the higher level of changes in cholesterol and lipoprotein in cancer model than the non cancer model and normal mice may be due to the association of cancer with changes in lipid profile as reported by several studies. Hypertriglyceridemia was observed in patients and experimental animals during the growth of wide variety of tumors and it showed a stronger association with metastatic conditions (Nalini et al., 2006; Liu and Yang, 2003). Elevations in tissue and serum triglyceride levels observed in cancerous conditions may be due to the decreased activity of the enzyme LPL (Lanza-Jacoby, 1984; Anandakumar et al., 2009). The
increased level of VLDL and triglyceride in lung cancer bearing mice observed in our present study is also matching with the above findings.

Upon treatment with ATRA might have improved LPL activity in the cancer bearing mice, thereby restoring normal triglyceride and VLDL levels in free ATRA treated cancer mice (group VII). The encapsulated ATRA treated cancer mice showed even better restoration than free ATRA treatment in cancer mice (group-VIII). Cholesterol is an important constituent of cell membranes and the availability of cholesterol is known to control the rate of cell multiplication and also cholesterol synthesis is known to be increased in cancerous condition (Ramakrishnan et al., 2008). In cancer mice model ATRA may activate the reverse cholesterol pathway in which excess cholesterol moves toward the liver and deposited.

Above findings sated that the in non-cancerous mice, the encapsulated ATRA slightly alter the lipid profile in compared with free ATRA treated mice. In the cancer mice model ATRA helped to restore the lipid profile level towards the normal level by an unknown mechanism. Further work has to be done on these interesting results as well as in-depth studies on lipoprotein distribution with ATRA treatment in cancer model is required.