LITERATURE REVIEW
2. LITERATURE REVIEW

2.1 Cancer

Cancer, a disease characterized by uncontrolled growth and spread of abnormal cells, is one of the major causes of death in humans. The uncontrolled growth of cells is illustrated in Figure 2.1 (www.patient.co.in). Carcinogenesis is generally divided into three stages: initiation, promotion and progression (Yang et al., 2002). Cells divide and grow uncontrollably in cancer, forming malignant tumors and invade nearby cells of the body. The cancer may also travel through the whole body and spread to other distant parts of the body through the lymphatic system or bloodstream. There are more than 200 different known cancers that afflict humans (Jemal et al., 2011).

Besides the rising trend in cancer cases, some of the cancers, like lung cancer, showed remarkably high incidence rates. It is well known that lung cancer rates are high in developed countries; nonetheless, the developing countries do not lag behind and the rates are getting closer to the rates that are seen in developed countries. There is a great variation in the prevalence of lung cancer in different geographical areas. Nearly 70% of all the new cases of lung cancer in the World occur in the developed countries. Worldwide, lung cancer is the most common cancer in terms of both incidence and mortality (1.61 million new cases per year and 1.38 million deaths), with the highest rates in Europe and North America (Ferlay et al., 2010).

2.2 Lung Cancer

Lung cancer is a form of disease wherein the cells of the lungs begin growing invariably and continuously (Figure 2.2) (http://usana.family.my). Mostly, the growth of cell and tissues is uncontrollable. Lung cancer is one of the most commonly found types of cancer often resulting into death. One of the major causes of lung cancer stated
Figure 2.1: Illustration for developing cancer

- Normal cells which line the surface of the airways / gut / milk duct of breast / pancreatic duct / uterus / cervix / skin / mouth / bladder / etc
- Primary tumour
- Cancer cells (look abnormal)
- Nucleus inside cells contains genes made from DNA
- Cancer cells invade into local tissue
- Some cells break off from the primary tumour
- Deeper tissues such as muscle layer in the gut or bladder / fat beneath the skin / etc
- Tiny blood vessel (capillary)
- New blood vessels stimulated to grow to supply cancer cells with a blood supply
- Cancer cells spread to other areas of the body via the blood vessels or lymph channels
Figure 2.2: Lung cancer formation
is smoking and the chances of developing lung cancer are 87% (Hanspeter, 2001).
Early detection of cancer leaves us some hope of positive therapy. In order to detect lung cancer at the earliest possible stage, one has to be conscious of the symptoms of lung cancer.

2.2.1 Lung Cancer Scenario in India (Kirmani et al., 2010; Notani and Sanghavi, 1974; Kenfield et al., 2008)

- **Sex distribution of smokers**
  - Males: 33.4%
  - Females: 1.4%
- **Smoking prevalence in varied areas**
  - Rural areas: 31.3%
  - Urban areas: 21.5%
- **Relative risk of developing lung cancer**
  - *Beedi* smokers: 2.64
  - Cigarette smokers: 2.23
  - Overall: 2.45
- **Incidence rates (age-standardized) per 100,000 population (2008)**
  - Males: 10.9
  - Females: 2.5
- **Increase in new cancer cases per 100,000 population each year over 24 years**
  - Chennai: 160%
  - Bangalore: 100%
  - Delhi: 40%
  - Mumbai: (-) 60%
- **Disability Adjusted Life Years (DALYs) due to lung cancer (2004)**
  - Males: 0.55 million
  - Females: 0.13 million
• Most prevalent is NSCLC amongst lung cancers
  • Squamous cell carcinoma: 44.73%
  • Adenocarcinoma: 30.26%

2.2.2 Evolution and the Major Cause of Lung Cancer

Historically, in India, tobacco was introduced in Karnataka by the Portuguese during A.D 1600 (Thankappan and Thresia, 2007; Reddy and Gupta, 2004). A couple of centuries later, the British people produced cigarettes commercially and established tobacco production in the country. Beedi (0.2-0.3gm of tobacco wrapped in a temburni leaf and tied with a small string) smoking was reported as early as 1711 in India (Thankappan and Thresia, 2007; Shimkhada and Peabody, 2003). Lung cancer was not recognized as a disease until 1761; the first link between lung cancer and smoking was reported way back in 1929 by physician Fritz Lickint from Germany (Hanspeter, 2001).

Smoking is responsible for upwards of 80% of all lung cancers worldwide (Hanspeter, 2001). In India, smoking is prevalent in (Thankappan and Thresia, 2007):
  • 29% of adult males
  • 2.5% of adult females
  • 11.7% of male collegians
  • 8.1% among school children and adolescents
A quarter of the cigarette or beedi smokers in India would be killed by tobacco at the ages of 25-69 years, losing 20 years of life expectancy (Thankappan and Thresia, 2007; Gajalakshmi et al., 2003).

2.2.2.1 Other Causes

Non-smokers account for 15% of lung cancer cases and these cases are often attributed to a combination of genetic factors, radon gas, asbestos, pesticides (Kirmani et al., 2010) and air pollution including passive and static smoking (Hanspeter, 2001).
Farmers mostly end up with lung cancer, which may be because of the heavy use of chemical pesticides to get rid of their farm’s pest problems. Farmers use 85% of the 2.6 million metric tons of active ingredient of pesticides produced annually for crop production in developing countries (Kirmani et al., 2010).

2.2.3 Types of Lung Cancer

90% of lung content is air and only 10% is solid tissue; the latter’s significant components include the bronchi, bronchioles (over a million) and alveoli (300 million) (http://www.ummm.edu/patiented/articles/what_lung_cancer_000072_1.htm). Over 95% of lung cancer is bronchogenic carcinoma. There are 40 types of cells in the lung of which 27 varieties make up the lung tissue itself. Four amongst these are unique for the lung and include (Sorokin, 1970):

- Non ciliated bronchiolar cells (Clara cells)
- Squamous cells (Type I)
- Great alveolar cells (Type 2)
- Alveolar macrophages

Depending upon its origin from a certain cell type, the cancer of lung is classified as (http://www.cancer.gov/):

2.2.3.1 Non-Small Cell Lung Cancer (NSCLC)

- Squamous cell carcinoma: arises from cells lining the bronchi.
- Adenocarcinoma: arises from glands and cells lining the alveoli. In those who have never smoked, and in women, adenocarcinoma is the commonest amongst lung cancers.
- Large cell carcinoma: a form of adenocarcinoma but the cells appear much larger on microscope.
2.2.3.2 Small Cell Lung Cancer (SCLC): also called ‘oat cell’ carcinoma, it arises from neuroendocrine cells variety called “Kulchitsky” cells. Enterochromaffin cells (synonym for Kulchitsky cells) are present primarily in the linings of digestive and respiratory tracts; they store 90% of the body’s serotonin content. Kulchitsky cells could release biologic amines and peptides which could regulate bronchial muscle tone and also control the pulmonary circulation.

Approximately 80% of cancers are NSCLC whilst 20% are SCLC. In India, however, SCLC is predominant over NSCLC having an occurrence ratio of 2.5:1 and 2.7:1 (Bhutani et al., 2006).

2.2.4 Manifestations of Lung Cancer

As is expected, most Indian patients with lung cancer complain of cough with expectoration, breathlessness, fever and anorexia consistently (Jindal and Behera, 1990). The study data is given in Table 2.1.

2.2.5 Treatment Options and Prognosis

The three standard treatments for lung cancer are surgery, radiotherapy and chemotherapy. One or more of these therapies may be used depending on the type of cancer, the stage of the disease and the age of the patient. Localized cancers (that is, cancer that has not spread to any surrounding tissue) detected at an early stage may be successfully treated using surgery and radiation. Up to 70 % of patients survive for at least five years after diagnosis if treated at this stage, with a proportion of these patients being cured. However, the majority of NSCLC cases are diagnosed at an advanced stage, when the cancer has already spread to other parts of the body. In spite of the use of chemotherapy as the first-line treatment option, less than five percent of advanced NSCLC patients survive for five years and most die within six months (Wilking and Jonsson, 2005).
Table 2.1: Presentations of lung cancer patients in India

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>As per large Indian studies</th>
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</thead>
<tbody>
<tr>
<td>Cough with Expectoration</td>
<td>40.0-94.3%</td>
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<tr>
<td>Chest Pain</td>
<td>16.0-66.7%</td>
</tr>
<tr>
<td>Loss of Weight</td>
<td>11.4-90.0%</td>
</tr>
<tr>
<td>Breathlessness</td>
<td>24.0-59.0%</td>
</tr>
<tr>
<td>Weakness</td>
<td>4.0-90.0%</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>8.0-69.2%</td>
</tr>
<tr>
<td>Fever</td>
<td>19.6-68.6%</td>
</tr>
<tr>
<td>Anorexia</td>
<td>20.5-90.0%</td>
</tr>
<tr>
<td>Hoarseness of Voice</td>
<td>9.0-33.0%</td>
</tr>
<tr>
<td>Nausea and Vomiting</td>
<td>6.0-25.0%</td>
</tr>
<tr>
<td>Puffiness of Face</td>
<td>2.9-19.8%</td>
</tr>
<tr>
<td>Dysphagia</td>
<td>2.9-20.8%</td>
</tr>
</tbody>
</table>
2.2.6 Lung Cancer Development Pathways

There is an enormous complexity of Lung cancer associated signaling events; however it is nonetheless possible to dissect some discrete biochemical cascades. Autocrine and paracrine loops are characteristic for all lung tumors. Activation of receptor tyrosine kinases in non-small cell lung cancer (NSCLC) often involves members of epidermal growth factor receptor (EGFR) family, whereas up-regulation of tyrosine kinase-protein kinase gene (c-KIT) is more characteristic for small cell lung cancer (SCLC). RAS/RAF/MEK/MAPK mitogenic pathway responds to a variety of receptors and its pathological activation is often attributed to the mutation of K-ras oncogene in lung cancer. Nearly all lung tumors demonstrate loss of function of two major suppressor pathways, p16INK4a/CyclinD1/CDK4/RB and p53/MDM2/p14ARF. Other lung cancer related events include up-regulation of MYC family oncogenes, angiogenic factors, molecules responsible for invasion and metastases (e.g. matrix metalloproteinases (MMPs) found to degrade syndecans), telomerase, COX2, etc., as well as inactivation of a spectrum of tumor suppressor genes. These alterations eventually determine the appearance of “The Hallmarks of Cancer”, such as self-sufficiency in growth signals, insensitivity to antigrowth signaling, evasion from apoptosis, limitless replicative potential, genomic instability, tissue invasion and metastasis, sustained angiogenesis and contribution of surrounding stroma (Figure 2.3) (Imyanitov et al., 2005).

2.2.7 Chemotherapy

Chemotherapy medications work by killing rapidly dividing cells. Since cancer cells divide more frequently than most normal cells, they are particularly susceptible to these drugs. Some normal cells also divide continuously, such as hair follicles, the stomach lining, and the bone marrow that makes red and white blood cells. This accounts for many of the side effects experienced during chemotherapy, such as hair loss, nausea and low blood cell counts. Different chemotherapy medications work at
Figure 2.3: Lung cancer (LC) pathways
different stages of cell division. For this reason, often two or more medications are
given at the same time to kill as many cancer cells as possible.

2.3 All Trans Retinoic Acid (ATRA), a Derivative of Vitamin A

All trans retinoic acid (ATRA) is a nutrient that the body needs in small
amounts to function and stay healthy. ATRA is made in the body from vitamin A and
helps cells to grow and develop, especially in the embryo. A form of ATRA made in
the laboratory is used to treat the skin, especially acne. ATRA is being studied in the
prevention and treatment of other types of cancers and is also called as retinoic acid,
tretinoin, and vitamin A acid. Retinol is ingested in a precursor form; animal sources
(liver and eggs) contain retinyl esters, whereas plants (carrots, spinach) contain pro-
vitamin A carotenoids. Hydrolysis of retinyl esters results in retinol, while pro-vitamin
A carotenoids can be cleaved to produce retinal. Retinal, also known as retinaldehyde,
can be reversibly reduced to produce retinol or it can be irreversibly oxidized to
produce retinoic acid. Retinol is the most usable form of vitamin A, and the other
usable forms include Retinal (aldehyde form), Retinoic acid (acid form) and retinyl
ester (ester form). These chemical compounds are collectively known as Retinoids, and
all possess the biological activity of all trans retinol as a common feature in their
structure. Structurally, retinoids possess a β-ionone ring and a polyunsaturated side
chain, with either an alcohol, aldehyde, a carboxylic acid group or an ester group
respectively. The side chain is composed of four isoprenoid units, with a series of
conjugated double bonds which may exist in trans or cis configuration (Gropper et al.,
2009). The major metabolic forms of vitamin A in vivo are retinol, retinal and ATRA.
The 9-cis retinoic acid and 13-cis RA are natural ATRA isomers, whereas 4-
hydroxyphenyl retinamide (4-HPR) is a conformationally restricted synthetic retinoid
(McBurney et al., 1993). It has been established that the ATRA inhibits the
proliferation and induces the markers of apoptosis and squamous differentiation in non-
small cell lung carcinoma (NSCLC) cell lines (Lokshin et al., 1999). ATRA has been
shown to exert anti-cancer activities in a number of types of cancer cells (Kim et al., 1996). The activity of ATRA is mediated by regulation of a variety of forms of gene expression through ATRA-dependent activation of retinoic acid receptors (RAR) which then bind to retinoic acid response elements (RAREs) in the regulatory regions of direct target genes (including Hox genes), thereby activating gene transcription, leading to the growth inhibition, differentiation, and apoptosis of cancer cells (Freemantle et al., 2003).

Retinoic acid receptors mediate transcription of different sets of genes of a cell differentiation, thus it also depends on the target cells. The retinoic acid level is controlled and maintained by a suite of proteins that control synthesis and degradation of retinoic acid (Holland, 2007). Response of non-small cell lung carcinoma (NSCLC) to ATRA is modulated by additional factors. However, a gradual decrease in the ATRA concentration in the blood circulation after prolonged treatment and highly variable bioavailability after oral administration were observed (Ozpolat et al., 2003). Therefore, unique formulations needed which can maintain the ATRA concentration in the blood and this could enhance its pharmacological effects. General structure and properties of ATRA is given in Figure 2.4 (www.hfchem.cn) and Table 2.2 respectively.

The Dietary Reference Intake (DRI) or Recommended Daily Amount (RDA) for Vitamin A for a 25-year old male is 900 micrograms/day, or 3000 IU. An early estimate on the rate at which β-carotene converted to vitamin A in the human body of 6:1 was revised to 12:1. From recent studies and experimental trials carried out in developing Nations, it is revised again to 21:1 (Sommer, 2008).
Table 2.2: Properties of ATRA

<table>
<thead>
<tr>
<th>Properties of ATRA</th>
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<tbody>
<tr>
<td>FORMULA</td>
</tr>
<tr>
<td>MOLECULAR MASS</td>
</tr>
<tr>
<td>MELTING POINT</td>
</tr>
<tr>
<td>PROTIEN BINDING</td>
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</tbody>
</table>
Figure 2.4: Structure of ATRA
2.3.1 Retinoid Overdose and Toxicity

The Tolerable Upper Intake Level (UL) for vitamin A, for a 25-year old male, is 3,000 micrograms/day, or about 10,000 IU. Too much vitamin A in retinoid form can be harmful or fatal, resulting in what is known as hypervitaminosis A. The body converts the dimerized form, carotene, into vitamin A as it is needed, therefore high levels of carotene are not toxic compared to the ester (animal) forms. The livers of certain animals, especially those adapted to polar environments, often contain amounts of vitamin A that would be toxic to humans. Thus, vitamin A toxicity is typically reported in Arctic explorers and people taking large doses of synthetic vitamin A. Toxicity of vitamin A is believed to be associated with the intervention methods used to upgrade vitamin A levels in the body such as food modification, fortification and supplementation, all of which are employed to combat vitamin A deficiency (Gropper et al., 2009). Toxicity is classified into two categories: acute and chronic toxicities. The former occurs few hours or days after ingestion of large amounts of vitamin A accidentally or via inappropriate therapy. The later toxicity (Chronic) takes place when about 25,000 IU/Kg or more of vitamin A is consumed for a prolonged period of time. Symptoms associated with both toxicities include, but not limited to nausea, blurred vision, fatigue, weight-loss and menstrual abnormalities (Mohsen et al., 2008).

2.3.2 Biotransformation of 13-cis- and 9-cis Retinoic Acid to ATRA

13-cis-Retinoic Acid (13-cRA) is a potent human teratogen, but it binds to retinoid X receptors (RARs) very weakly (Levin, 1995). Experimental results showed that the biotransformation of 13-cRA to ATRA (a high-affinity ligand for RARs) is probably a prerequisite process for the teratogenic effects of 13-cRA (Levin, 1995; Kim et al., 1994; Repa et al., 1993). Studies also demonstrated that 9-cRA is a high-affinity ligand for retinoid X receptors (Levin et al., 1992; Heyman et al., 1992) and is a potent, directly acting dysmorphogen (Creech and Juchau, 1993; Creech et al., 1994). Although 9-cRA can be generated from its carotenoid precursors, isomerization of
ATRA to 9-cRA has been suggested as an alternative pathway for the biogeneration of 9-cRA (Napoli, 1996). Therefore, interconversions between 9-cRA and ATRA may contribute significantly to the regulation of endogenous levels of both ligands in embryonic tissues and steric interconversions of retinoic acids have been observed, both in vitro and in vivo (Napoli, 1996; Zile et al., 1982; Kalin et al., 1981; Sundaresan and Bhat, 1982; Kojima et al., 1994). At equilibrium, ATRA is the dominant isomer, accounting for approximately 60–70% of total retinoic acids, depending on the conditions used (Shih et al., 1986; Urbach and Rando, 1994a). The reactions can be catalyzed by a variety of low-molecular weight sulfhydryl compounds in vitro and thus are often referred to as thiol-dependent, nonenzymatic, isomerization reactions (Shih et al., 1986, 1997; Urbach and Rando, 1994a, 1994b).

The physiological significance of such nonenzymatic catalysis can be questionable due to following reasons; I) not all thiol compounds are capable of effectively catalyzing the reactions. For example, L-cysteine shows virtually no catalytic activity for conversions of 13-cRA/9-cRA to ATRA, although it contains a free thiol group (Shih et al., 1986, 1997). II) Some sulfhydryl compounds that have high catalytic activities, such as mercaptoethanol, apparently do not exist endogenously in biological tissues (Shih et al., 1986, 1997). III) GSH, a very important endogenous sulfhydryl compound, is effective in catalyzing isomerizations of retinoic acids but only at very low (nonphysiological) concentration (0.05–0.15 mM) (Shih et al., 1986, 1997). Above this concentration range, rates of GSH-catalyzed retinoid isomerizations are very low (Shih et al., 1986, 1997) and IV) it is difficult to imagine that interconversions of important retinoic acid ligands would be completely regulated by nonenzymatic processes (Napoli, 1996). The oxidative conversions of 13-cis- and 9-cis-retinal produced predominant product ATRA, catalyzed by conceptual cytosol and microsomes (Chen and Juchau, 1997). One possible explanation for those observations is that 13-cRA and 9-cRA were rapidly converted to the thermodynamically more
stable ATRA. When 13-cRA was incubated with conceptual cytosol under the same conditions used for the oxidative conversions of retinals to retinoic acids, 13-cRA was found to quickly convert to ATRA and this rapid reaction presumably was catalyzed, at least in part, by an embryonic isomerase (Chen and Juchau, 1997).

2.3.3 Metabolism of ATRA

ATRA metabolism is complex and only partially understood at the present time. However, it is known that ATRA can be oxidized by cytochromes P450 (P450s) to various metabolites, including 4-hydroxy-RA (4-OH-RA), 18-OH-RA, 5, 6-epoxy-RA, and 4-oxo-RA, which may be further bio-transformed into glucuronides via UDP-glucuronosyl transferase-mediated conjugation (Napoli, 1999; Samokyszyn et al., 2000). The most prominent pathway begins with a rate-limiting hydroxylation at the C-4 position of the cyclohexenyl ring leads to the formation of 4-OH-RA. Major involvement of cytochromes P450s CYP3A4/5/7, CYP2C8/9, CYP1A1, and CYP4A11 in the oxidation of ATRA, and the CYP3A subfamily as the most active P450s in the formation of 4-OH-RA, 4-oxo-RA, and 18-OH-RA were reported (Marill et al., 2000). A unique ATRA inducible P450, CYP26 that specifically metabolizes ATRA was also identified, and this enzyme is considered to be an important regulator of endogenous ATRA homeostasis (Ray et al., 1997; White et al., 1997). Due to its main role in a feedback loop where ATRA levels are controlled in an auto regulatory manner, CYP26 was supposed to be one of the mechanisms causing resistance to continuous ATRA therapy.

A series of experiments were conducted that investigated the metabolism and dysmorphogenesis of ATRA and precursor retinoids in Xenopus laevis during early stages of development (Creech et al., 1995, 1995a, 1995b). Results from these studies have shown that the embryos can metabolize ATRA to 13-cis RA, all-trans retinoyl β-glucuronide (all-trans RAG), 4-oxo-all-trans RA, and 4-oxo-13-cis RA through a
variety of isomerization, glucuronidation, and oxidation pathways. These retinoids and 9-cis RA, 5,6-epoxy RA, 4-hydroxy RA, and 3,4-didehydro RA have been identified as metabolites of ATRA in a number of mammalian and rodent studies (Barua et al., 1991; Blaner and Olson, 1994; Howard et al., 1989). These metabolites of ATRA identified (Barua et al., 1991) were further evaluated for their dysmorphogenic effects in X. laevis embryos. All-trans RAG were marginally teratogenic and likely represented a deactivation pathway for ATRA. Similarly, both 4-oxo-13-cis RA and 13-cis RA were less potent teratogens than their trans isomers and were also thought to be formed to inactivate ATRA. Both 4-oxo-ATRA and ATRA have been shown to specifically bind and activate RARs in X. laevis embryos (Pijnappel et al., 1993).

However, ATRA resistance still occurs in patients who relapsed from regimens combining chemotherapy with ATRA, despite limited and intermittent ATRA exposure. Most importantly, treatment of cancer is usually not done with a single anticancer agent, but with a variety of combined chemotherapy regimens, including several anticancer drugs, and other concomitantly administered supportive drugs. Therefore, the possibility exists that another pathway is induced by concomitantly administered drugs that regulates ATRA metabolism and that may be responsible for some form of ATRA resistance.

2.3.4 Receptors, Binding Proteins and Mechanisms of Action of ATRA

The actions of retinoids are mediated through the nuclear retinoid receptors, which are members of the steroid/thyroid/retinoid hormone receptor family (Gudas, 1994). Retinoid receptors act as ligand-inducible transcription factors that enhance the transcription of target genes by binding to retinoic acid response elements (RAREs) in the promoter region of retinoid-responsive genes. The retinoid receptors can be divided into six regions designated A through E (Tate et al., 1994; Lavau and Dejean, 1994). The C domain is a cysteine-rich DNA-binding domain which contains two zinc finger
structures. The E domain contains the ligand (retinoid)-binding site and also has a region required for dimerization with other receptors. The amino acid sequences of C and E domains are highly conserved across the classes of retinoid receptors, whereas the A, B, and F domains are less conserved across receptors but remain highly conserved for the same receptors across different species. Two families of retinoid nuclear receptors have been described, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Giguere et al., 1987; Mangelsdorf et al., 1990, 1991). The RARs (α, β, and γ) bind the naturally occurring retinoid ATRA with high affinity, whereas the RXRs (α, β, and γ) do not bind ATRA (Mangelsdorf et al., 1992). 9-cis-retinoic acid (9cRA) is a naturally occurring, biologically active isomer of ATRA (Heyman et al., 1992) that is capable of binding and trans-activating both the RXRs as well as the RARs. RAR-β expression is prevalent in neural tissues but hardly detectable in skin; and RAR-γ is expressed predominantly in the skin (Chambon, 1996; Mangelsdorf et al., 1995). Many of the therapeutic effects of Retinoic acid, including cancer chemoprevention and treatment of dermatologic disorders, are mediated through RAR-β (Lee et al., 2009). RAR-β has lung tumor–suppressor activity (Berard et al., 1996). Present in normal tissue, RAR-β expression is progressively lost in early stages of both head and neck and non–small-cell lung carcinogenesis (Xu et al., 1999). Although the mechanism of RAR-β mRNA-expression loss during carcinogenesis is not well understood, data indicate that this loss in NSCLC rarely is a result of loss of the RAR-β gene, despite its location on chromosome 3p24, which frequently experiences loss of heterozygosity (Gebert et al., 1991). In normal tissue, RAR-β is transcriptionally regulated by retinoic acid via a retinoic acid response element in the RAR-β gene promoter (Hoffmann et al., 1990). Phase III trials in oral and lung premalignant lesions and analyses of bronchial brushings found that RAR-β can be upregulated by 13-cis-retinoic acid. The seminal study suggesting tumor-suppressor activity of RAR-β in vivo was conducted by Houle et al. in NSCLC (Houle et al.,
1993). This result was supported by more recent in vitro transfection of NSCLC cells and in vivo antisense studies in transgenic mice (Khuri et al., 2000).

Under normal physiologic conditions, the concentration of ATRA and other naturally occurring retinoids is under tight metabolic control. The physiologic plasma concentration of ATRA is approximately 5.0 nM (Napoli et al., 1991). Although circulating ATRA enters cells via passive diffusion, its contribution to intracellular ATRA levels is likely to be inconsequential under normal conditions because cells derive retinoic acid from intracellular oxidation of retinaldehyde, a metabolite of retinol (Ross, 1993). Intracellular ATRA is bound to specific binding proteins, the cellular retinoic acid binding proteins (CRABPs) (Donovan et al., 1995). CRABP I and CRABP II are highly conserved throughout evolution and appear to regulate the amount of retinoic acid capable of binding to their nuclear receptors (Boylan and Gudas, 1992). Binding of ATRA to CRABP appears to facilitate intracellular oxidative catabolism of ATRA to the inactive metabolite, 4-hydroxy-retinoic acid (Napoli et al., 1995).

2.3.5 Successful ATRA Therapy in Acute Promyelocytic Leukemia (APL)

ATRA has marked a major advance in the treatment of acute promyelocytic leukemia (APL) with differentiation therapy. However, the duration of remission induced and maintained by ATRA therapy alone is generally short-lived, and, when relapse occurs, ATRA alone fails to induce a second remission in a majority of patients (Warrell et al., 1993; Degos et al., 1995). ATRA can selectively induce terminal differentiation of promyelocytic leukemic cells into normal granulocytes without causing bone marrow hypoplasia or exacerbation of the frequently occurring fatal hemorrhagic syndromes associated with chemotherapy. Thus, ATRA-induced differentiation of promyelocytic cells provides an excellent in vitro model for studying myeloid cell differentiation. Although development of quick resistance to the
differentiation therapy is commonly observed, when combined with chemotherapy, this therapy can dramatically increase patient’s survival by enhancing the efficacy of chemotherapy.

2.3.5.1 Mechanism of Action of ATRA at Cell Level in APL

The persistence of chromosomal translocation t (15; 17) in a large number of morphologically matured granulocytes during in vivo remission induction is a strong indicator that ATRA drives the differentiation of immature neoplastic cells into mature granulocytes (Huang et al., 1988; Castaigne et al., 1990; Elliott et al., 1992). It has been suggested that ATRA acts on at least two stages of myeloid cell development: promyelocytes and earlier neoplastic progenitor cells which are capable of self-renewal but are already committed to the myeloid lineage (Warrell et al., 1993). After an irreversible commitment to differentiation induced by ATRA, the maturing cells originating from the leukaemic clone eventually enter into programmed cell death (Martin et al., 1990; Gianni et al., 2000; Altucci et al., 2001). The recent data has been shown that ATRA induces an immediate increase in the intracellular cAMP level and activation of the protein kinase A (PKA) pathway in APL cells after exposure to the drug, and this effect appears to be abrogated by PKA antagonists, suggests a coordinated activation mechanism between RA nuclear signaling and cell-membrane-associated cAMP/PKA signaling (Zhu et al., 2002).

2.3.5.2 Mechanism of Action of ATRA at Gene Level in APL

There is considerable evidence to indicate that remission induction by ATRA in patients with APL is associated with the differentiation of immature promyeloblasts into mature granulocytes, followed by the recovery of normal hematopoiesis (Warrell et al., 1993) i) APL patients treated with ATRA do not experience a period of marrow hypoplasia during induction; ii) immuno-phenotypically intermediate cells which express both immature (CD33) and mature (CD15) cell surface antigens are observed
during induction therapy (Castaigne et al., 1990), and iii) in situ hybridization studies demonstrate the presence of the (15;17) translocation in maturing cells (Warrell et al., 1991). The hallmark cytogenetic alteration found in the leukemic cells of patients with APL is the reciprocal translocation between the long arms of chromosomes 15 and 17 (Golomb et al., 1976; Rowley et al., 1977). In 1987, the gene encoding the retinoic acid receptor-alpha (RARα) was mapped to chromosome 17q21 (Mattei et al., 1988). After that, it was demonstrated that in APL the breakpoint on chromosome 17 involved the gene encoding for the RARα receptor (Chomienne et al., 1990). The breakpoints on chromosome 15 cluster in a region containing a previously undefined gene initially termed myl (de Thé et al., 1990) but subsequently renamed promyelocytic leukemia (PML) (Borrow et al., 1990; Goddard et al., 1991; Kakizuka et al., 1991). The fusion of the RARα and PML genes on chromosomes 15 and 17 results in the generation of two reciprocal transcripts PML/RARα, which is expressed in all patients studied to date, and the RARα/PML transcript, expressed in approximately two-thirds of patients (Warrell et al., 1993). The PML/RARα fusion protein acts as an ATRA-dependent transcription factor and appears to interfere with retinoic acid transcriptional regulation (Early and Dmitrovsky, 1995). In myeloid leukemic cell lines, overexpression of PML/RARα blocks normal maturation and differentiation (Rousselot et al., 1994). ATRA has the ability to overcome promyelocytic leukemia (PML)/retinoic acid receptor (RAR) fusion protein. It thus appears that disruption of RARα in APL is linked to leukemogenesis, and that this disruption can be overcome by super physiologic concentrations of ATRA.

2.3.5.3 Molecular Mechanism of ATRA in APL

ATRA induces differentiation of immature leukemic blasts into terminally differentiated granulocytic cells, which is associated with clinical remissions (Breitman et al., 1981; Warrell et al., 1991). ATRA-induced differentiation of APL blasts requires expression of PML-RARα receptor protein (Slack, 1999). PML-RARα can
heterodimerize with RXR or form homodimers and subsequently binds to RARE, located in the promoters of the ATRA-responsive target genes. ATRA can bind to PML-RARα with an affinity comparable to RARα. In the absence of ligand, RAR-RXR in normal blasts and PML-RARα-RXR heterodimers in APL cells recruit nuclear co-repressor proteins, NCoR or silencing mediator of retinoid and thyroid hormone receptor (SMRT), and Sin3A or Sin3B, which in turn form a complex with histone deacetylase enzymes (HDAC1 or HDAC2), resulting in transcriptional repression or silencing (Grignani et al., 1998; Grunstein, 1997; Heinzel et al., 1997). The transcriptional suppression occurs because deacylation of histone protein creates conformational changes, limiting access and binding of transcription factors and RNA polymerase to related genes (Kouzarides, 1999). At physiologic concentrations of ATRA (10^{-9}-10^{-8} M), the NCoR protein and HDAC complex are dissociated from RARα in normal blasts, which in turn results in recruitment of co-activators with histone acetyltransferase (HAT) activity, such as steroid receptor coactivator-1 (SRC-1), PCAF, p300/ CBP, ACTR, TIF2 or P/CIP (Collingwood et al., 1999; Glass et al., 1997). Acetylation of lysine residues in the N-terminal of histones by HAT activity results in trans-activation of responsive genes leading to differentiation. However, the physiologic concentration of ATRA does not cause dissociation of NCoR protein and HDAC complex from the PML-RARα fusion receptors in APL blasts, leading to differentiation block. The CoR complex is dissociated from PML-RARα at only pharmacological concentrations (10^{-7}-10^{-6} M) of ATRA, resulting in removal of transcriptional repression and transcription of genes related to differentiation (Heinzel et al., 1997; Kouzarides, 1999; Collingwood et al., 1999; Glass et al., 1997).

In addition to release of transcriptional repression, the other possible mechanisms involved in ATRA effectiveness in myeloid cell differentiation include expression of different classes of genes including induction of expression of (p21^{waf1/cip1}) cyclin-dependent kinase inhibitor (Casini and Pelicci, 1999), up-regulation
of C/EBP-γ, β, and ε (Morosetti et al., 1997), interferon regulatory factor-1 (IRF-1) (Pelicano et al., 1997), and regulation of the localization of promyelocytic leukemia protein (PML) oncogenic domains (PODs) (Weis et al., 1994). In APL cells isolated from patients, ATRA up-regulated expressions of RARα at mRNA and protein levels were observed (Agadir et al., 1995; Chomienne et al., 1991), whereas it causes the degradation of PML-RARα (Raelson et al., 1996; Yoshida et al., 1996). Therefore, the ratio of RAR/RXR to PML-RARα would be higher, which helps in overcoming the dominant negative effects of PML-RARα protein.

2.3.6 Chemotherapeutic Role of ATRA and Other Retinoids in Solid Cancers

Retinoids are a family of molecules that are structurally related to retinol, and are known to play a critical role in many physiological functions, such as vision, metabolism, bone development, cell proliferation, differentiation, apoptosis, homeostasis, reproduction, fetal development and inhibiting proliferation in vascular smooth muscle cells (VSMCs) (Mangelsdorf et al., 1994; Lotan, 1980). Intense research is going on the increasing use of ATRA as anti-tumour therapeutical agent for the treatment of various tumoral diseases such as Kaposi’s sarcoma, head and neck squamous cell carcinoma, ovarian carcinoma, bladder cancer and neuroblastoma (Zuccari et al., 2005) and has shown against vascular disorders with endothelial damage and anti-angiogenic effects in several systems (Maiti et al., 2006). Various experimental data of retinoids have been demonstrated to inhibit development of a number of different types of tumors such as epithelial tumors associated with the skin, respiratory tract, stomach and mammary gland (Aylsworth et al., 1986; Munker et al., 1987) and to inhibit the growth of a variety of neoplastically transformed cells like F9 teratocarcinoma cells and malignant melanoma cells by differentiating these cells (Breitman et al., 1980; Edward et al., 1988), suggesting their potential role as a cancer chemotherapeutic agent. RA treatment was also found to reduce DNA synthesis, induce morphological changes, prolong cell doubling time and reduce saturation density and
colony formation in soft agar assays. The retinoid suppression of growth does not involve the death of tumor cells but rather their arrest during the G₁ stage of the cell cycle (Wu et al., 1997).

Squamous cell carcinomas (SCCs) are an ideal therapeutic target for retinoids because these cancers arise from epithelial tissues whose growth and differentiation are modulated by retinoids in vivo (Lippman et al., 1987, 1987a). Retinoids have been successfully used in vivo to prevent the progression of preneoplastic oral, bronchial and head and neck lesions to frank malignant tumors (Hong et al., 1986, 1990; Geisen et al., 1997) and also successfully used in the treatment of oral SCCs.

Vitamin A and retinoids modulate the growth of cervical cells and several studies have shown that various retinoids inhibit cellular proliferation in cervical cancer cells, which suggests their potential as chemopreventive agents for cervical neoplasia (Geisen et al., 1997; Lotan et al., 1995). Moreover, many of studies (Surwit et al., 1982; Meyskens et al., 1983) suggested that ATRA is an effective chemopreventive agent for cervical neoplasia. A reduced level of ATRA in the serum of HPV associated uterine cervix cancer patients was reported already (Berlin et al., 2006). Studies using topically applied vitamin A to the cervix has resulted in up to 50 percent complete reversal of cervical dysplasia in Phase-II and Phase-III clinical trials. Vaginal and vulvar side effects of this treatment were mild and reversible at the end of treatment. 13-cis RA in combination with interferon-α has been successfully used to treat SCCs of the cervix and skin (Lippman et al., 1993).

2.3.7 Anti-Metastatic Gene Expression of ATRA in Breast Cancer Cells

Approximately 15–25% of women with node-negative breast cancer will eventually succumb to the disease due to distant metastases (Heimann and Hellman, 2000). Several metastasis-promoting proteins have been identified in differential
screening of cell cultures and primary invasive tumors which include ERB2/Her2/neu, VEGF and stromelysin (Singletary, 2002). It is very exciting to search for proteins those are responsible for inhibition of the metastatic cascade, "metastasis suppressor" genes, and has revealed a set of molecules that include: NM23 (histidine kinase), KAI1 (a tetraspanin integral membrane protein that responds to NFκB), BRMS1 (gap junction function), and MKK4 (mitogen-activated protein kinase) (Steeg, 2003; Yoshida et al., 2000; Shevde and Welch, 2003). The functions regulated by such metastasis suppressors include transcription, signal transduction, cell adhesion, and inflammation. Studies have shown that the RARβ2, with known tumor suppressor functions, also confers anti-metastatic properties as shown in a xenograft model of human breast cancer (Treuting et al., 2002).

Trans-activation via these heterodimeric partners is generally conferred by physiological or pharmacologic levels of retinoid-derived ligands, including ATRA and 9-cis-retinoic acid, for RARs and RXRs, respectively. Various investigations have shown that the RARβ2 or RARβ4 mRNA is diminished in most breast cancer cell lines (Roman et al., 1992; Swisshelm et al., 1994) and in the primary breast cancers (Widschwendter et al., 1997; Xu et al., 1997). The mechanism(s) for diminished RARβ2 expression include transcriptional repression by epigenetic silencing (Sirchia et al., 2000, 2002; Mehrotra et al., 2004). A truncated, oncogenic RARβ protein (RARβ-prime) is exclusively expressed in breast cancer cell lines, and the presence of this isoform likely obstructs tumor suppressor functions of RARβ2 and RARβ4 protein isoforms (Sommer et al., 1999; Chen et al., 2002). RARβ2 activates both tumor suppressor and anti-metastatic programs. Furthermore, RARβ2 has been introduced via a retroviral vector into cell lines and all the breast cancer cells were found to be inhibited in their proliferative capacity, even in the absence of the natural ligand, ATRA. Very few experiments have been conducted to determine the downstream factors regulated by ectopic RARβ2 expression. One such study with F9
teratocarcinoma cells, employing expression microarrays and subtractive hybridization, found differential expression of transcription factors, signaling molecules and metabolic enzymes (Zhuang et al., 2003). Moreover, these investigators found an altered gene expression patterns for RARβ2 even in the absence of ATRA.

2.3.8 Chemo-therapeutic Role of ATRA in Other Disorders

ATRA has shown beneficial effect for atherosclerotic vascular disorders by inducing differentiation and inhibiting proliferation in vascular smooth muscle cells (VSMCs) (Miano and Berk, 2000, 2001; Johst et al., 2003). Moreover, ATRA increases nitric oxide (NO) production by endothelial NO synthase (eNOS) phosphorylation through RAR-mediated phosphoinositide 3-kinase/Akt pathway activation in vascular endothelial cells (ECs) (Uruno et al., 2005). Because NO is a potent vasodilator and signal modulator molecule, plays important roles in controlling vascular function, ATRA may be a candidate for novel therapeutic agents against vascular disorders with endothelial damage. Several researchers’ investigations have shown that the ATRA and its derivatives modulate angiogenesis (Ingber and Folkman, 1988; Oikawa et al., 1989; Kini et al., 2001; Gaetano et al., 2001; Blebea et al., 2002; Arsenou et al., 2005).

2.3.9 Role and Therapeutic Effects of ATRA in Lung Cancer

Emphysema results from progressive destruction of alveolar septae and was considered irreversible until ATRA was shown to reverse anatomic and physiologic signs of emphysema in a rat model. Retinoids, such as ATRA, are known to activate genes involved in lung development and promote alveolar septation and growth in the pre- and postnatal period (Ong and Chytil, 1976; Massaro and Massaro, 1996). In animal model, systemic administration of ATRA reversed manifestations of elastase-induced emphysema (Massaro and Massaro, 1997). There have been several reports about the effects of ATRA on cytokine production (Maeno et al., 2002; Matikainen et
al., 1994; Pelicano et al., 1997; Tkatch et al., 1995) as well as the induction of growth inhibition of myeloma cells, with the reduction of both IL-6 production and its receptor expression (Levy et al., 1996; Ogata et al., 1994; Sidell et al., 1991). That is irradiation was found to stimulate IL-6 production and accelerated transcription of its receptors, and increased cell proliferation via predictable IL-6/IL-6R autocrine/paracrine systems in human lung fibroblastic cell lines (Tabata et al., 2006a). ATRA thus reduced this irradiation-induced production of both IL-6 and its receptors in the lung fibroblasts along with IL-6–dependent cell growth (Tabata et al., 2006). In previous research study, it was reported that the ATRA have inhibited metastatic NSCLC in patients in a phase II trial (Choi et al., 2003).

2.3.10 Role of RARβ in Lung Cancer

It is likely, however, that unique expression profiles will be found with ectopic RARβ2 expression, depending upon the cell of origin. The decreased level of ATRA was found to decrease the expression of RARβ in NSCLC cells (Lotan, 1999). RARβ2 was transfected into two lung cancer cell lines that lacked endogenous RARβ2 expression (Toulouse et al., 2000). It was also suggested that the loss of RARβ expression may be associated with lung cancer development and it was found that the transfection of the RARβ gene into NSCLC cells suppressed their tumorigenicity in the lung of nude mice (Lotan, 1999).

2.3.11 Lung Metastasis Development by B16F10 Melanoma Cell Line

The B16F10 mouse melanoma cell line having high metastatic potential was found in the lung tissue when injected through the lateral tail vein (Fidler, 1970, 1973). It was also reported that the B16F10 cells reached the lung tissue and it started depositing fibrosis and collagen later which turns into lung tumor. B16F10 implanted lung cancer mice model is well accepted for the preclinical studies (Brunda et al., 1993; Teng et al., 2011). Such lung metastatic cancer growth can be assessed in vivo by
tumor nodules, cytopathologic changes and the life time of the experimental mice (Korangath et al., 2010). ATRA is shown to have effects on cellular proliferation and differentiation in various cancers including melanoma (Zhang et al., 2003). The histological examination of lung cancer bearing animals showed loss of architecture with distorted alveoli with increased number of hyper chromatic nuclei in the cells of alveolar wall with extensive proliferation of alveolar epithelium (Ravichandran et al., 2011).

2.3.12 Lung Tumor Marker γ- Glutamyl transpeptidase (γ-GT)

In cancer study, we can identify some tumor markers in early stage by which we can prevent the cancer progression. Serum level of gamma glutamyl transpeptidase (γ-GT) plays as one of such tumor marker in cancer patients (Prezioso et al., 1993) and serves as a specific marker for the prognosis of carcinogenic events. The marker enzyme γ-GT is one of the specific indicators of lung damage (Vinodhkumar et al., 2006; Ferrigno et al., 1994) and cancer (Sattu et al., 2007). γ-GT is not only useful in diagnosis but also has extrapolative value in malignancies such as lung cancer and malignant melanoma and is closely associated with oxidative stress too (West et al., 1985). Retinoic acid may provide useful information concerning the identification of differentiation-associated markers of human rectal cancer cells. γ-GT activity was found decreased level in a human rectal adenocarcinoma (HRT-18) cells grown in retinoic acid treatment (Tsao et al., 1982).

2.3.13 Role of Apoptosis and Angiogenesis in Metastatic Cancer Development

Apoptosis is a programmed cell death defined by characteristic morphological and biochemical changes (Sarih et al., 1993; Smeyne et al., 1993; Corcoran et al., 1994). Apoptosis plays a role opposite to that of mitosis in cell population kinetics in almost all tissues, and apoptosis abnormalities are closely associated with multistep carcinogenesis (Wyllie, 1997). Growth of tumor tissue is determined by the balance
between cell proliferation and apoptosis. The inability of cancer cells to undergo apoptosis may provide a growth advantage, and therefore have a better chance of surviving. Thus, induction of apoptosis was recognized as an ideal strategy for cancer chemotherapy. ATRA has been reported to have the capability to inhibit cell growth by inducing apoptosis. Induction of apoptosis is mediated via specifically binding and activating retinoic acid receptors, such as RAR\(\alpha\), RAR\(\beta\), RAR\(\gamma\). The molecular mechanism says that the vitamin-A in the liver is converted to ATRA, diffuses to the target tissues through cellular membranes and is translocated to the RARs through CRABP and induced the apoptosis pathway shown in Figure 2.5 (www.quigen.com) (Lee et al., 2004).

Angiogenesis or formation of new blood vessel from the existing one has been considered as the most important step during tumor progression (Folkman, 2002). Current advancement in cancer research has shown that targeting the angiogenic pathways could be a more rational and promising anti-cancer therapeutic approach (Chakraborty et al., 2012). These processes are controlled by various stimulatory factors and/or inhibitory factors. Especially, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), hepatocyte growth factor (HGF), and angiopoietin (Ang)-1 and 2 are well known major angiogenic factors. Angiogenesis is physiologically important in normal growth, development, wound healing, and reproduction, whereas pathological angiogenesis contributes to tumor growth, exacerbation of diabetic retinopathy, and various inflammations (Akiko et al., 2007). Angiogenesis has been demonstrated as one of the major event during malignant progression of cancer (Chakraborty et al., 2012).
Figure 2.5: ATRA mediates apoptosis
2.3.14 Oxidative Stress in Induced Experimental cancer Model and ATRA as Antioxidant

Superoxide and hydroxyl radicals along with hydrogen peroxide (H$_2$O$_2$) are collectively called as reactive oxygen species (ROS). The sources of generation of ROS in cells are various metabolic reactions with the incomplete reduction of oxygen in mitochondrial electron transport chain during respiration (Halliwell and Gutteridge, 1999). Thus generated ROS can drive the cell to a pro-oxidant state, referred as oxidative stress, affects biological molecules including membrane lipids (Halliwell and Gutteridge, 1999). The lung is exposed to higher levels of oxygen than most other tissues. The intensity of ROS in the lung is increased by cigarette smoke, inflammation, pollutants, chemicals and carcinogens (Cugell and Kamp, 2004).

Reactive oxygen species (ROS) and organic free radical intermediates formed from many carcinogens are suggested to be involved in the initiation and progression of carcinogenic transformation. The effective carcinogen induce enormous amounts of free radicals, which in turn reacts with lipids causing LPO. The products of LPO include malondialdehyde that has been reported to be involved in the formation of tumors. Accumulating evidence suggests that these free radicals and electrophile mediated oxidative stress plays an important role in all stages of induced carcinogenesis and tumorgenesis (Sun, 1990). Several reports stated that the cell line implantation create oxidative stress for example human bladder cancer (T24 cells) causes oxidative stress (Fabbri et al., 2005) and the B16 cells implantation also causes oxidative stress (Shukla and Gude, 2003).

Oxidative damage to cellular macromolecules can arise through overproduction of ROS and lowering the antioxidants. In addition, ROS can stimulate signal transduction pathways and lead to activation of key transcription factors such as Nrf2 and NF-kB. The resultant altered gene expression patterns evoked by ROS contribute
to the carcinogenesis process (James et al., 2010). Anti-oxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic processes (Bagchi et al., 2000). The antioxidant defense system includes SOD which convert superoxide radical (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$). Accumulation of excess H$_2$O$_2$ causes toxic effects on cellular system. In this regard GPx and CAT convert H$_2$O$_2$ into water. Therefore these two toxic species (O$_2^-$ and H$_2$O$_2$) are converted into water (Li et al., 2000). Several studies have reported a decreased activity of SOD and CAT in various carcinogenic conditions that may be due to the increased compensation of these anti-oxidant enzymes to neutralize the oxidative stress (Ramakrishnan et al., 2006). The anti-oxidant enzymes SOD, CAT and GPx that are activated against cell injury also limit the effects of oxidant molecules on tissues (Lee et al., 2010). The induction of GPx and CAT, which are of central importance in the detoxification of peroxides and hydroperoxides, was measured in the lung tissue where these processes have fundamental importance (Gaetani et al., 1989).

The non-enzymic anti-oxidants such as GSH comprise a protective system in the cells against ROS. GSH is found to be present in high concentration in the cells, and it protects cells from free radical generation (Farombi et al., 2000) and acts directly as a scavenger by donating a hydrogen atom and thereby neutralizing the hydroxyl radical. It also reduces peroxides and maintains protein thiols in the reduced state (Nwanjo and Oze, 2007). GPx uses GSH as a substrate to catalyze the reduction in organic hydroperoxide and H$_2$O$_2$ (Bebe and Panemangalore, 2003).

According to previous studies retinoic acid stabilizes the protein levels in brain cells and the activity of SOD at the mRNA level; thereby retinoic acid helps to reduce oxidative stress in brain cells (Ahlemeyer et al., 2001). Among retinoic acid isomers, ATRA is known to control the activity levels of antioxidant enzymes as demonstrated in rat sertoli cells (Tae-Kyong and Lee-kim, 2009). In another research, it is suggested
that retinoic acid isomers (ATRA, 13-cis-RA, and 9-cis-RA) have influence on enzymatic antioxidant system in breast cancer cell lines by assaying the activity levels of antioxidant enzymes. This study on breast cancer cells suggests that ATRA is the most potential modulator of antioxidant enzymes among RA isomers in MCF-7 human breast cancer cells (Tae-Kyong and Lee-Kim, 2009).

2.3.15 Function of ATRA in Inflammatory responses

Retinoids exert their functions through their binding to the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), which belong to the subfamily B (respectively, NR1B and NR2B) of the nuclear hormone receptors. After binding of retinoids, RAR and RXR form a homodimer or a heterodimer and activate the cellular machinery for an increased transcription rate but RAR and RXR can alternatively induce gene transrepression by sequestering transcription factors such as activator protein-1 (AP-1) or nuclear factor-interleukin-6 (NF-IL-6) without binding to DNA (Lefebvre, 2001). This transcription factors control many inflammatory mediators, and the liganded RAR complexes can repress a broad spectrum of genes, including inflammatory proteins, cytokines, or matrix metalloproteases (MMPs) (DiSepio et al., 1997) which are also associated with cancer progression. Huge amounts of inflammatory cytokines are found in the synovial fluid, tissue and in the sera of rheumatoid arthritic patients (Houssiau et al., 1988), and serum levels of IL-6 have been correlated with the activity of the disease (Nishimoto, 2006). IL-6 is synthesized and then secreted extensively by fibroblast-like synoviocytes from rheumatoid arthritis patients (Tan et al., 1990). Among possible pathogenic roles, IL-6 activates T cells and macrophages, induces osteoclast differentiation, causes systemic inflammatory manifestations, and could promote angiogenesis (Nishimoto, 2006). These clinical results have confirmed the pathological role of IL-6 in rheumatoid arthritic (Smolen et al., 2008). Following the successful use in the treatment of skin diseases or cancer, retinoids were shown to be anti-inflammatory in several animal models of Rheumatoid
arthritis. Treated with 13-cis RA in the paws of adjuvant arthritis (AA) rats showed a decrease of cartilage lesions, associated with a reduction of MMP-1 expression (Brinckerhoff et al., 1983). And in the rodent collagen-induced arthritis (CIA) model, ATRA improved the course of the disease and reduced the production of inflammatory cytokines (Nozaki et al., 2006). A RAR agonist Am-80 decreased the anti-collagen II antibody levels and improved joint swelling and bone destruction (Kuwabara et al., 1996). Apart from differences in the pathogenic mechanisms of animal models of Rheumatoid arthritis (Kannan et al., 2005) or in the binding activity of retinoids to RAR subtypes, these experimental data strongly suggest that the anti-arthritic effect of RAR agonists is supported mainly by their ability to reduce the immune response. The inflamed synovial tissue is a major source of IL-6 production (Sugita et al., 1993), and the blockade of IL-6 (Takagi et al., 1998) or its deficiency (Hirota et al., 2007) reduced the severity of experimental arthritis by impairing the T-cell response.

2.3.16 ATRA as an Immunomodulant

Most immunomodulants have biphasic effects, i.e., some tend to stimulate immune system at low doses but suppress host defense parameters at higher levels (Raphael and Kuttan, 2003). Vitamin A is an essential dietary constituent that has long been known to influence the immune system. ATRA, a metabolite of Vitamin A, has increasingly received attention for having an unexpected and crucial effect on host immune responses. ATRA has been shown to modulate a broad range of immunologic processes, such as lymphocyte activation and proliferation, T-helper-cell differentiation, tissue-specific lymphocyte homing, and production of specific antibody isotypes (Rodrigo et al., 2008). The immunomodulatory effects of naturally occurring terpenoids in experimental animal models are assessed by bone marrow cellularity, α-esterase activity and DTH assays (Raphael and Kuttan, 2003).
2.3.17 Problems Associated with Free ATRA Treatment

Though ATRA treatment considered being a relatively safe drug, use of ATRA can lead to several side effects such as skin problems (dryness, peeling, itching, and sun sensitivity), reversible elevation in liver enzymes, hypothyroidism, headaches retinoic acid syndrome and pseudotumor cerebri (PC) (Dylan et al., 2012). The water solubility of ATRA is lower than 0.1 µg/ml. The poor aqueous solubility and low persistence in blood circulation of ATRA are the principal obstructions for oral treatment. Moreover promising results are not obtained for the ATRA treatment in solid cancers (Sonal et al., 2012) and the reason may be the reachability to target site. Currently, ATRA treatment is given clinically along with arsenic trioxide or with other chemotherapy as combination therapy. Preclinically it is given by encapsulation in liposome or polymeric micelles (Sanz and Lo-Coco, 2011). Liposome made of lipid emulsions, which possess an oil phase in particulate form and have a high solubilizing capacity for lipophilic drugs like ATRA, which might be an interesting alternative (Suzuki et al., 2006).

2.3.18 Lipid Profile in ATRA Treatment and Cancer

Administration of retinoids to both experimental animals (Gerber and Erdman, 1981; 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. Although the modest hypertriglyceridemia most frequently induced by retinoid therapy may be innocuous, the extreme elevations occasionally encountered in retinoid-treated patients can precipitate pancreatitis (McCarter and Chen, 1992). Uptake of low or less antioxidant containing food may lower the lipoprotein and successively leading to various cancers (Mittal and Mittal, 2004; Ravi et al., 2009). Lower levels of vitamin A in patients with cancers of stomach, oesophagus, colon and pancreas also suggest LDL oxidation resulting in low levels of LDL and high triglyceride levels (Mittal and Mittal, 2004). Retinoid therapy is known to be associated with hypertriglyceridemia and often
with low HDL-cholesterol (Zech et al., 1983; Matel-Teeuwisse et al., 2001). However, changes in lipid profile especially increase in cholesterol synthesis has been reported in cancer which promote invasion and metastasis of tumor cells (Anandakumar et al., 2009). Retinoids and retinoid-activated receptors can modulate lipid metabolism and lipid distribution. Whereas, the treatment with ATRA, showed a mild, transient changes in high density lipoprotein cholesterol levels. Retinoic acid treatment induced the expression of RAR and PPARβ/δ target genes involved in the regulation of lipid homeostasis (Rhee and Plutzky, 2012). Hence the study of lipid profile in ATRA treatments becomes mandatory for both experimental models as well as clinical trials.

2.3.19 Need for ATRA Encapsulation

Encapsulation of ATRA in liposomes provides a parenteral ATRA preparation that has been shown to decrease metabolism of ATRA by hepatic microsomes in rats. The liposomal nature of the product may also resulted in less ATRA going to the skin, a major site for cellular retinoic acid binding proteins. Due to these effects, ATRA concentrations are maintained longer in rats given encapsulated ATRA than in rats given the same amount of oral ATRA. Alterations in biodistribution may also be responsible for the reduced toxicity seen on a milligram-to-milligram basis in rats given with encapsulated ATRA versus oral ATRA (Estey et al., 1996).

ATRA incorporated in cationic liposomes composed of DOTAP/cholesterol was efficiently internalized into A549 cells, producing potent cytotoxic and apoptosis-inducing effects on ATRA-insensitive (resistant) A549 cells. The enhanced expression of TIG3 mRNA tumor suppressor gene by this encapsulation might partly explain the mechanism of enhanced activity. These observations provided valuable information to help in the design of differentiation therapy using ATRA in NSCLC (Kawakami et al., 2002). The delivery of ATRA by emulsions can reduce the elimination of ATRA from the blood circulation and referentially accumulate in the liver after intravenous
injection. The retention of ATRA in the liver could successfully suppress the progression of liver metastasis in the mice injected with colon carcinoma cells. These findings indicated that the effective delivery and retention of ATRA in hepatocytes by emulsion as an efficient approach for the treatment of liver metastasis (Chansri et al., 2006). ATRA incorporated in DOTAP/cholesterol liposomes was particularly found to be effective in suppressing tumor metastasis in the lung when injected with CT-26 cells in mice (Suzuki et al., 2006). Atragen also known as liposome encapsulated ATRA, is used to treat APL with success clinically and is sold by Roche under the brand name Vesanoid (Yang et al., 2003).

2.4 Liposomes as Drug Delivery System and Other Uses

The clinical utility of most conventional chemotherapeutics is limited either by the inability to deliver therapeutic drug concentrations to the target tissues or by severe and harmful toxic effects on normal organs and tissues. Controlled drug delivery systems have been attempted to overcome these problems by providing selective delivery to the affected area. Liposomes are small, spherical and enclosed compartments separating an aqueous medium from another by phospholipid bilayer. Liposomal formulations are one of advanced drug delivery systems in clinical application due to their unique properties. Due to the differences in preparation methods and lipid compositions, liposomes can be classified according to their lamellarity (uni- and multi-lamellar vesicles), size (small, intermediate, or large) and charge (anionic, cationic and neutral) (Jesorka and Orwar, 2008; Makino and Shibata, 2006; Irache et al., 2011). A liposome encapsulates a region of aqueous solution inside a hydrophobic membrane; dissolved hydrophilic solutes cannot readily pass through the lipids. Hydrophobic chemicals can be dissolved into the membrane, and in this way liposome can carry both hydrophobic molecules and hydrophilic molecules. To deliver the molecules to sites of action, the lipid bilayer can fuse with other bilayers such as the cell membrane, thus delivering the liposome contents. By making liposomes in a
solution of DNA or drugs (which would normally be unable to diffuse through the membrane) they can be (indiscriminately) delivered past the lipid bilayer (Makino and Shibata, 2006; Irache et al., 2011).

Liposomes are able to encapsulate lipophilic or hydrophilic drugs with their lipidic layers or in their aqueous core respectively and deliver those to target site for in vivo application. Moreover, liposome delivery system can increase the solubility of hydrophobic drugs and stabilize a variety of therapeutic agents such as peptides, proteins and nucleotides in bloodstream (Irache et al., 2011; Allen and Moase, 1996). In clinical studies, liposomes show improved pharmacokinetics and biodistribution of therapeutic agents and thus minimize toxicity by their accumulation at the target tissue (Immordino et al., 2006). Liposomes were first discovered by Bangham in 1965 and the first liposomal pharmaceutical product, Doxil, received FDA approval in 1997 for the treatment of refractory AIDS-related Kaposi’s sarcoma (Allen and Moase, 1996; Immordino et al., 2006). Most of liposomal drug formulations, including Ambisome, Doxil and Myocet, are approved for intravenous application. Other administration routes such as intramuscular delivery have also been approved for delivery of surface antigens derived from the hepatitis A or influenza virus. Oral delivery has been examined which is however more troublesome due to the potential for liposome breakdown following exposure to bile salts (Shaji and Patole, 2008; Chang et al., 2012). Peter et al. showed that daily i.p. injection of liposome encapsulated ATRA for 21 days resulted in much higher levels of ATRA in mice, and produced long term molecular remissions in 88% of immunocompetent animals (Peter et al., 2002).

A similar approach can be exploited in the biodetoxification of drugs by injecting empty liposomes with a transmembrane pH gradient. In this case the vesicles act as sinks to scavenge the drug in the blood circulation and prevent its toxic effect (Bertrand et al., 2010). Another strategy for liposome drug delivery is to target
endocytosis events. Liposomes can be made in a particular size range that makes them viable targets for natural macrophage phagocytosis. These liposomes may be digested while in the macrophage's phagosome, thus releasing its drug. Liposomes can also be decorated with opsonins and ligands to activate endocytosis in other cell types. In addition to gene and drug delivery applications, liposomes can be used as carriers of dyes to textiles (Barani and Montazer, 2008), pesticides to plants, enzymes and nutritional supplements to foods, and cosmetics to the skin (Meure et al., 2009).

2.4.1 Liposomal Encapsulation Technology and Its Characterization

Many hundreds of drugs, including anti-cancer and antimicrobial agents, chelating agents, peptide hormones, enzymes, other proteins, vaccines and genetic materials, have been incorporated into the aqueous or lipid phases of liposomes with various sizes, compositions and other characteristics by different preparation techniques. An ideal method of liposome formulation is preparing liposome with high entrapment efficiency, narrow particle size distribution and long term stability.

There are mainly three types of liposomes - MLV (multilamellar vesicles) SUV (Small Unilamellar Vesicles) and LUV (Large Unilamellar Vesicles) available to deliver different types of drugs. Multilamellar vesicles (MLV) consist of several (up to 14) lipid layers (in an onion-like arrangement) separated from one another by a layer of aqueous solution. These vesicles are over several hundred nanometers in diameter. Small unilamellar vesicles (SUV) are surrounded by a single lipid layer and are 25–50 nm in diameter. Large unilamellar vesicles (LUV) are, in fact, a very heterogenous group of vesicle that, like the SUVs, are surrounded by a single lipid layer. The diameter of these liposomes is very broad, from 100 nm up to cell size. Depending on the need, one can use SUV type or MLV type vesicles for effective entrapment and delivery of the drug to the target tissues or cells. MLV can be used for slow drug release and increased persistance of the drug in the blood circulating system (Bertrand
et al., 2010). The representation of bilayer liposomes and multilayer liposome vesicle is shown in Figure 2.6.A and 2.6.B.

Number of techniques have been reported for preparation of liposomes such as Bangham method, the detergent depletion method, the ether/ethanol injection method, the reverse phase evaporation and the emulsion method (Meure et al., 2008). The majority of liposome preparation methods require organic solvents to dissolve lipids but these organic solvents are harmful to the environment and human body. Recently, some alternative methods including dense gas and supercritical fluid techniques have been introduced for liposome preparation without using any organic solvents (Meure et al., 2008; Lesoin et al., 2011; Castor, 2005). Physicochemical properties of liposomal formulations, including size, membrane lamellarity, surface charge, permeability, and encapsulation volume, are depending on the lipid composition (cationic, anionic, and neutral lipid species). The major function of liposome preparation techniques is to obtain efficient drug entrapment and increased stability of the liposome products (Uhumwangho and Okor, 2005; Chang et al., 2012).

Liposome encapsulated drugs are characterized by determining the size, shape and surface changes on the liposome. A number of techniques including TEM, Surface Tensio meter, viscometer, zeta analyser, DLS and fluorescence micrograph are used to characterize the emulsion (Table 2.3) (Pautot et al., 2002).

2.4.2 List of Drugs

As of 2008, 11 drugs with liposomal delivery systems have been approved and 6 additional liposomal drugs were in clinical trials (Table 2.4) (Zhang et al., 2008).
2.4.3 Liposome Targeting Cancer Sites

Another interesting property of liposomes is their natural ability to target cancer (Lasic, 1998). The endothelial wall of all healthy human blood vessels is encapsulated by endothelial cells that are bound together by tight junctions. These tight junctions stop any large particles in the blood from leaking out of the vessel. Tumor vessels do not contain the same level of seal between cells and are diagnostically leaky. This ability is known as the Enhanced Permeability and Retention effect. Liposomes of certain sizes, typically less than 200 nm, can rapidly enter tumor sites from the blood, but are kept in the bloodstream by the endothelial wall in healthy tissue vasculature. Anti-cancer drugs such as Doxorubicin (Doxil), Camptothecin and Daunorubicin (Daunoxome) are currently being marketed in liposome delivery systems (Lasic, 1998).

New Liposomal drugs targeting cancer like Liposomal Cisplatin (Lipoplatin of Regulon Inc.) has received Orphan Drug designation for Pancreatic Cancer from EMEA.
Figure 2.6: A) Unilamellar Vesicle and B) Multilamellar Vesicle
**Table 2.3: Production and characterization of liposomes**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Topic/Reference</th>
<th>Materials / Methods</th>
<th>Measurements</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>“Production of unilamellar vesicles using an Inverted emulsion” (Pautot <em>et. al.</em>, 2002)</td>
<td>Phospholipids (POPC, POPS), Oil (Dodecane), water / Inverted emulsion</td>
<td>Inverted microscope (Leica), Fluorescene Quenching assay, Dynamic light scattering</td>
<td>Used to transport macromolecules through blood stream or through the skin, leading to the widespread use of vesicles in cosmetics and drug delivery</td>
</tr>
<tr>
<td>2</td>
<td>“Physicochemical characterization of PEG coated liposomes loaded with Doxorubicin” (Polo <em>et. al.</em>, 1997)</td>
<td>Phospholipids (PC, PG), Layer Stabilizer (PEG), Oil (methanol), water / Rotary Evaporation</td>
<td>Surface activity (Langmuir balance), Micro viscosity (Membrane interior probe (DPH), Bilayer Fluidity.)</td>
<td>Drug administration, Targeting cells, “<em>in vivo</em>” half life improvement</td>
</tr>
<tr>
<td>3</td>
<td>“Biophysical aspects of using liposomes as delivery vehicles” (Anne <em>et. al.</em>, 2002)</td>
<td>Phospholipids (DMPC, DOTAP), water, oil (propane) / Homogenization</td>
<td>Fluorescent microscopy, Zeta analyzer, Differential scanning colorimeter (DSL)</td>
<td>Biocompatible carriers of drugs, peptides, proteins, plasmic DNA, antisense oligonucleotides or ribosomes, for pharmaceutical, cosmetic, and biochemical purposes.</td>
</tr>
<tr>
<td>4</td>
<td>“Oil in water liposomal emulsions: characterization and potential use in vaccine delivery,” (Jean <em>et. al.</em>, 1999)</td>
<td>Phospholipids (DMPC, DMPG), Mineral oil, water/Extrusion</td>
<td>Fluorescent microscopy, Rotational Viscometer</td>
<td>As constituents of oil in water emulsion adjuvants for vaccines</td>
</tr>
</tbody>
</table>
### Table 2.4: List of clinically approved liposomal drugs

<table>
<thead>
<tr>
<th>Name</th>
<th>Trade name</th>
<th>Company</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomal amphotericin B</td>
<td>Abelcet</td>
<td>Enzon</td>
<td>Fungal infections</td>
</tr>
<tr>
<td>Liposomal amphotericin B</td>
<td>Ambisome</td>
<td>Gilead Sciences</td>
<td>Fungal and protozoal infections</td>
</tr>
<tr>
<td>Liposomal cytarabine</td>
<td>Depocyt</td>
<td>Pacira (formerly SkyePharma)</td>
<td>Malignant lymphomatous meningitis</td>
</tr>
<tr>
<td>Liposomal daunorubicin</td>
<td>DaunoXome</td>
<td>Gilead Sciences</td>
<td>HIV-related Kaposi’s sarcoma</td>
</tr>
<tr>
<td>Liposomal doxorubicin</td>
<td>Myocet</td>
<td>Zeneus</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>Liposomal IRIV vaccine</td>
<td>Epaxal,</td>
<td>Berna Biotech</td>
<td>Hepatitis A, Influenza</td>
</tr>
<tr>
<td></td>
<td>Inflexal V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomal morphine</td>
<td>DepoDur</td>
<td>SkyePharma, Endo</td>
<td>Postsurgical analgesia</td>
</tr>
<tr>
<td>Liposomal verteporfin</td>
<td>Visudyne</td>
<td>QLT, Novartis</td>
<td>Age-related macular degeneration, pathologic myopia, ocular histoplasmosis</td>
</tr>
<tr>
<td>Liposome-PEG doxorubicin</td>
<td>Doxil/Caelyx</td>
<td>Ortho Biotech, Schering-Plough</td>
<td>HIV-related Kaposi’s sarcoma, metastatic breast cancer, metastatic ovarian cancer</td>
</tr>
<tr>
<td>Micellular estradiol</td>
<td>Estrasorb</td>
<td>Novavax</td>
<td>Menopausal therapy</td>
</tr>
</tbody>
</table>