Chapter 2

Review of Literature
Cancer may be defined as an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host. In other words, cancer begins when a cell breaks free from the normal restraints on cell division and begins to follow its own agenda for proliferation. From a clinical point of view, cancer is a large group of diseases, perhaps up to a hundred or more, that vary in their age of onset, rate of growth, state of cellular differentiation, diagnostic detectability, invasiveness, metastatic potential, response to treatment, and prognosis (Ruddon, 2007). According to Hanahan and Weinberg (Hanahan and Weinberg, 2000), the malignant transformation of cells is collectively determined by six essential alterations (commonly shared by all types of human tumors) to cell physiology: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues.

Cancer is clearly a worldwide problem. The incidence and mortality rates for various cancers are similar, though not identical, among developed countries. In the developing world, as countries become more westernized and their populations achieve longer life expectancy, cancer rates are increasing. Although there are differences among developing and developed countries in the incidence rates of certain cancers, lung cancer is the most common cancer among men in both regions of the world and breast cancer is the most common cancer in women. Research across last three decades in the area of cancer biology and therapy has revolutionized our understanding of cancer and yielded better ways of preventing, diagnosing and treating cancer. Millions of cancer patients have survived much longer than would have been possible even a few decades ago. The war, however, is far from over, although tremendous progress has been made and a number of battles have been won. In this never-ending fight, older therapies are being refined and practically reinvented. In addition, outstanding scientists all over the world are exploring a number of
exciting possibilities for improved diagnosis, treatment, and prevention of this dreaded disease.

The goal of cancer therapy is to completely eradicate the neoplastic cells without causing any appreciable damage to the normal tissue of the host. This could be achieved either by reversing the neoplastic cell state and/or removing the neoplastic cells completely from the host system. In terms of curing cancer, no unified treatment concept has emerged so far. However, the most commonly employed methods of treating malignant diseases are surgery, radiotherapy, and chemotherapy. Being the oldest and most frequently used method to treat cancer, surgery involves careful and precise removal of the cancerous tissue from the body. It is often the only form of treatment for most benign and some malignant cancer types. Surgery may also be used to confirm a diagnosis (biopsy), determine how far a person's cancer has advanced (staging), relieve side effects (such as an obstruction), or ease pain (palliative surgery). Recent advances in surgical and anesthetic techniques, resuscitation and patient care have made major surgeries relatively safe. However, surgery is effective only in cases, where cancer has been diagnosed at early stages. Surgical procedures also suffer from other disadvantages like, microscopic extensions may be missed; may lead to functional and cosmetic disabilities and is traumatic to the patient; cancer around vital structures can’t be removed and its inability to treat the metastatic forms of the disease. Therefore combination approach involving surgery and other treatment modalities like chemotherapy and/or radiotherapy is often used to improve the therapeutic outcome.

The clinical effectiveness of radiotherapy was established about the year 1900 following the work of Dr. Curie in France and Dr. Roentgen in Germany and since then, radiotherapy is known to be the among the most effective forms of cancer treatment (Connell and Hellman, 2009). Over the last century, the curative potential of radiation therapy for solid tumors has drastically increased. The aim of radiotherapy is to deliver as high a dose as possible to the malignant tissue without causing excessive injury to surrounding healthy tissue (Martin and Harbison, 1986). The increased therapeutic use of radiation and its obvious worth soon brought 2 major problems into focus (Berry, 1987; Orton, 1995):
1. How to reduce the unwanted side effects of radiotherapy such as radiation-sickness and erythema: In clinical practice normal tissue toxicity remains the most important limitation to adequate tumor dose delivery. Doses resulting in high tumor control probability will often cause unacceptable toxicity, a problem related to the width of its therapeutic window. Major avenues of progress in overcoming this limitation in the last decades have included the introduction of altered fractionation regimens, and technical advances resulting in better target visualization and more conformal dose distribution with steeper dose gradients.

2. How to increase the radiosensitivity of the diseased tissue without affecting the normal tissue: Despite improvements in the ability to shape and target radiation beams to deliver higher doses to tumor tissue and lower doses to the surrounding normal tissues, the failure of radiation therapy to control tumor growth locally is still a major clinical problem (Lindegaard et al., 1996; Suit, 1996). Some causes of local tumor recurrence include: (1) the exclusion of part of the gross tumor mass from the radiation field (referred to as a geographic miss); (2) regrowth from tumor cells at the edge of the radiation field that have received less than the full therapeutic dose (a marginal miss); and (3) colonization of irradiated tissues by tumor cells migrating in from regional or distant sites (repopulation). However, it is likely that the major cause of local tumor recurrence is the failure of radiation to eradicate all of the tumor cells within the treatment fields (Rosen et al., 2000) which lead to the quest for alternate approaches for improving the therapeutic outcome.

The concept of administering drugs concomitantly with radiation to enhance the effect of radiation was first established in the preclinical studies of Heidelberger’s and co-workers in the year 1958 (Heidelberger et al., 1958). Subsequently, in the 1964, a pilot study carried out by the scientists from the Mayo Clinic reported improved survival of patients with stomach and pancreatic cancer when the two modalities were combined (Moertel et al., 1964). Although these initial reports showed only modest improvement, with a disease that had such a dismal prognosis, any improvement was welcome. A decade later, in 1974, Nigro’s trial of 5-fluorouracil (FU) in combination with mitomycin C as concurrent therapy with radiation for cancer of the anal canal demanded the attention of both the radiation and
medical oncology communities (Nigro et al., 1993). In 1979, Steel and Peckham introduced a theoretical framework to describe the interaction of cytotoxic chemotherapy and radiotherapy (Steel and Peckham, 1979). They coined a term “spatial cooperation” to describe the scenario whereby radiotherapy acts locoregionally, and chemotherapy acts against distant micro metastases, without interaction between the agents (Figure 2.1). This co operative effect requires the agents to have independent or non-overlapping toxicity profiles (toxicity independence) in order that both modalities can be used at effective doses without increasing normal tissue effects, even if the anticancer effects of the two modalities were simply additive. An alternate interactive scenario was ‘radiation sensitization’, describing a state in which chemotherapy cooperates with radiation within the radiation field, resulting in increased killing of cancer cells. Such an interaction may either be equal to (additive) or greater than (synergistic or supraadditive) the expected sum of killing from each sequentially administered modality (Seiwert et al., 2007).

**Figure 2.1.** Rationale for adding chemotherapy to radiation. Spatial and in-field cooperation are the two idealized types of cooperation between radiation and chemotherapy. Both mechanisms can contribute synergistically to clinical benefit (Seiwert et al., 2007).
It is now increasingly believed that combining ionizing radiation with cytotoxic chemotherapy results in better tumor control rates, both within the target volume and potentially also at distant microscopic sites. The introduction of combined modality approaches was a highly significant step in the evolution of curative radiation treatment. Three clinical rationales support the use of combined modality approach involving chemotherapy and radiation. First, concomitant chemoradiotherapy can be used with organ-preserving intent, resulting in improved cosmesis and function compared with surgical resection with or without adjuvant treatment. Second, chemotherapy can act as a radiosensitizer, improving the probability of local control and, in some cases, survival, by aiding the destruction of radioresistant clones. Third, chemotherapy given as part of concurrent chemoradiation may act systemically and potentially eradicate distant micro metastases (Seiwert et al., 2007). A number of factors including drug type, concentration, drug target, metabolism, timing of administration, micro-environmental and genetic factors, etc. are known to influence the efficacy of the combined modality treatment. In several common solid tumor types, landmark clinical studies have clearly demonstrated the benefit of combined modality treatment and the number of patients undergoing such treatment has been increasing steadily for the last two decades.

This possibility of improving the outcome of cancer therapy lead scientists from around the world to look for novel compounds that could preferentially sensitize the cancer cells to the effects of radiation. An ideal radiosensitizer should have the following characteristics

- Should be non-toxic in nature
- Should have potent radiosensitizing potential
- Independent activity against the tumor
- Amenable to dose-intense or prolonged infusion schedules
- Should be effective in all phases of the cell cycle particularly G1 phase (hypoxic cells are known to be arrested in G1 phase)
- Capable of diffusing through non-vascular cell mass to reach hypoxic areas (200 µm from nearest blood vessel)
In that direction, numerous compounds were screened for their radiosensitizing potential with several compounds showing promising potential. However, none of the tested compounds had the ideal properties of a radiosensitizer. Also the literature search reveals that there are no US-FDA approved radiosensitizers available in the market which necessitates the search for novel chemotherapeutic compounds with radiosensitizing potentials.

Chemotherapy, in most simple sense, is the treatment of cancer with chemical agents or cytotoxic drugs. The systemic treatment of cancer has its roots in the initial work of Paul Ehrlich, who coined the term chemotherapy. Alkylating agents represent the first class of chemotherapeutic drugs to be used in the clinical setting. Based on the shrewd observation during World War II that exposure to mustard gas caused bone marrow and lymphoid hypoplasia, first clinical evaluation of nitrogen mustard was conducted at Yale Cancer Center in 1943 to treat patients with hematologic malignancies, including Hodgkin's disease and lymphocytic lymphomas. Treatment with this alkylating agent resulted in dramatic regressions in advanced lymphomas and thereby generated significant excitement in the field of cancer pharmacology. At about this same time, Sidney Farber reported that folic acid had a significant proliferative effect on leukemic cell growth in children with lymphoblastic leukemia (Farber and Diamond, 1948). These observations led to the development of folic acid analogs as cancer drugs to inhibit cellular folate metabolism and thus initiated the era of cancer chemotherapy. In fact, the entire class of antimetabolites, including the fluoropyrimidines, cytarabine, and gemcitabine, and the purine analogs were all designed with the expectation that they would inhibit the normal pathways involved in pyrimidine and purine metabolism, respectively, and thereby inhibit cancer cell proliferation and growth. Since then, extensive efforts to find chemical means of controlling neoplastic disease ensued, resulting in the discovery of a large array of useful chemotherapeutic agents (DeVita et al., 2005).

Although, chemotherapy is one of the frontline treatment modalities in cancer, it comes at a cost of dose-limiting toxicity that may range from mild to lethal. Conventional cytotoxic therapies are known to primarily damage the proliferating cells such as those in the bone marrow, gastrointestinal tract, gonads, and hair
follicles. Therefore, it is imperative that myelosuppression, nausea, vomiting, mucositis, infertility, and alopecia etc. would be the most prevalent side effects of such therapies. Apart from these, many chemotherapeutic agents are also known to cause severe toxicities to vital organs such as heart (Schimmel et al., 2004) and kidney (Ries and Klasterky, 1986; Narins et al., 1990; Perazella and Moeckel, 2010), which may prove to be fatal (Table 2.1). Another major obstacle to the ultimate success of cancer chemotherapy is the ability of tumor cells to develop resistance to cytotoxic compounds. A range of mechanisms, including the mutation or over-expression of the drug target, inactivation of the drug, or elimination of the drug from the cell etc., may be responsible for such an acquired resistance (Harbottle, 2004).

Table 2.1. Cardiotoxicity of select chemotherapeutic agents (Schimmel et al., 2004).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Toxicity dose</th>
<th>Toxicity</th>
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<tbody>
<tr>
<td>Doxorubicin</td>
<td>400 – 550 mg/m² (emerging data implicate lower doses, mostly in setting of radiation)</td>
<td>Arrhythmia, pericarditis-myocarditis syndrome, myocardial infarction, sudden cardiac death, cardiomyopathy, congestive heart failure</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>&gt;100 – 140 mg/m²</td>
<td>Congestive heart failure, decreased left ventricular ejection fraction, myocardial infarction, ECG changes, arrhythmia</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>&gt;100 – 120 mg/kg (over 2 days)</td>
<td>Hemorrhagic cardiac necrosis, reversible systolic dysfunction, ECG changes, congestive heart failure</td>
</tr>
<tr>
<td>Ifosfamide</td>
<td></td>
<td>ECG changes, congestive heart failure, arrhythmias</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Standard dose</td>
<td>Myocardial ischemia, Raynaud’s phenomenon, ECG changes</td>
</tr>
<tr>
<td>Fluorouracil</td>
<td>Standard dose</td>
<td>Myocardial infarction, angina, cardiogenic shock, sudden death, dilated cardiomyopathy</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td></td>
<td>Ventricular dysfunction, congestive heart failure, Cardiomyopathy</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Standard dose</td>
<td>Sudden death, bradyarrhythmia, myocardial dysfunction, myocardial infarction</td>
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These drawbacks of conventional chemotherapeutic agents have inspired scientists from around the world to search for improved treatment approaches to enhance outcome of cancer therapy. The two most commonly used approaches to address these problems include

- Discovery and development of novel compounds that possess better anticancer and radiosensitizing potential with acceptable toxicity profiles
- Making use of the targeted delivery platforms including liposomal/nanoparticulate carriers for better targeting of these anticancer compounds to the tumor tissue leading to improved anticancer properties and reduced toxicities.

**Discovery and development of novel compounds from natural sources**

Natural products, especially plants, with their diverse spectrum of pharmacologically active principles, have been used for the treatment of various diseases for thousands of years. Terrestrial plants have been used as medicines in Egypt, China, India and Greece from ancient time and an impressive number of modern drugs have been developed from them. Large-scale anticancer drug discovery and screening programs such as those promoted by the National Cancer Institute (NCI) have led to the discovery of several potent chemotherapeutic agents from natural sources (either as naturally occurring or synthetically modified forms) and have played a pivotal role in the management of cancer for over 40 years. The most important classes of plant derived compounds that are used as antitumor agents include the bis-indole alkaloids from vinca, the camptothecins, the epipodophyllotoxins, and the taxanes (Kinghorn, 2008). In addition, there are several examples of promising natural product-derived antineoplastic agents currently in advanced stages of clinical trials or recently approved, not only from plants (e.g., the combretastatin and homoharringtonine analogs) and microbes (e.g., the epothilones and the enediynes), but also of marine origin (e.g., the bryostatins, ecdysteroids and the kahalalide F) (Gullo et al., 2006). Most of these compounds are known to be of relatively small molecular weight (< 3000 daltons) and in the correct chiral form to
exhibit biological activities (Williams et al., 1989). They are reported to exhibit their action through a variety of mechanisms including interaction with microtubules, inhibition of topoisomerase I or II, alkylation of DNA, and interference with tumor signal transduction etc. (Table 2.2). Of a total of 155 anticancer agents approved for use in Western medicine and Japan since the 1940, 47% were classified as either natural products per se (14%), semi-synthetic derivatives of natural products (28%), or otherwise derived from natural products (5%) (Newman and Cragg, 2007). Keeping in mind the contributions of the natural compounds to the existing arsenal of potent anticancer compounds, it is only logical and imperative to search for more novel anticancer compounds from these natural sources.

Table 2.2. Diverse natural products forming bases of cancer drugs (Brower, 2008).

<table>
<thead>
<tr>
<th>Natural product</th>
<th>Source</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halichondrin B (Eisai)</td>
<td>South Pacific sea sponge</td>
<td>Blocks tubulin formation</td>
</tr>
<tr>
<td>Yondelis</td>
<td>Sea squirt</td>
<td>Interferes with cell division, blocks transcription</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Pacific yew tree</td>
<td>Stabilizes microtubule formation</td>
</tr>
<tr>
<td>Combretastatin</td>
<td>South African bush willow</td>
<td>Targets tumor vasculature</td>
</tr>
<tr>
<td>Vinca alkaloids</td>
<td>Madagascar periwinkle plant</td>
<td>Inhibits tubulin formation</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>Chinese Camptotheca acuminata tree</td>
<td>Inhibits DNA topoisomerase I</td>
</tr>
<tr>
<td>Homoharringtonine</td>
<td>Cephalotaxus harringtonia, an evergreen tree</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Podophyllum peltatum plant (mayapple)</td>
<td>Inhibits topoisomerase II</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>Iban Thapsia garganica plant</td>
<td>Induces apoptosis</td>
</tr>
<tr>
<td>Bryostatin 1</td>
<td>Marine moss protozoa</td>
<td>Activates protein kinase C</td>
</tr>
<tr>
<td>Dolastatins</td>
<td>Indian Ocean sea hare</td>
<td>Inhibits mitosis</td>
</tr>
</tbody>
</table>
Quinones represent one such class of naturally occurring compounds that are widely distributed in nature and are most commonly found in plants, animals, fungi and bacteria. They are one of the oldest recognized classes of organic compounds and have fascinated chemists since the early days of modern chemistry. The name “quinone” (“chinon” in German) originates from the name given in the 1830s by Woskresensky, to a bright yellow colored compound obtained by oxidation of quinic acid (from cinchona bark) who referred to the product as “chinoyl” and is now known as 1,4-benzoquinone (Eliel and Ramirez, 1997).

Chemically, they are diketones derived from monocyclic or polycyclic aromatic hydrocarbon compounds that contain two carbonyl groups which may either be on the same or on different rings. The naturally occurring quinones may be broadly classified, based on the parent aromatic hydrocarbon compound, as benzoquinone (benzene as the parent), naphthoquinone (naphthalene as parent) or anthraquinone (anthracene as parent) as shown in figure 2.2.

![Figure 2.2. Showing basic types of quinones viz., benzoquinones, naphthoquinones and anthraquinones](image)

**Figure 2.2.** Showing basic types of quinones viz., benzoquinones, naphthoquinones and anthraquinones

**Distribution of naturally occurring quinones and their physiological roles**

Benzoquinones are the simplest class of quinones and found most often in insects, arthropods, millipedes, beetles, arachnids and termites where they serve as important toxic chemical defense systems (Monro et al., 1962; Eisner et al., 1964). On the other hand, naphthoquinones and anthraquinones are the most widely occurring quinones and often found to co-exist in plants and marine organisms (including sea urchins and other echinoderms) (Thomson, 1987).
In general, most naturally occurring quinone pigments are yellow, red or brown in color (but when present as salts of hydroxyquinones, the colors are purple, blue or green), because of which many of these compounds are used as hair dyes and skin coloring agents. Some quinones have key roles in the biochemistry of energy production and serve as vital links in electron transport and biosynthetic processes. For example coenzyme Q (ubiquinone) functions as an electron carrier in the respiratory chain. Vitamin K, a naphthoquinone derivative, is required for blood coagulation and participates in the carboxylation of glutamate to \( \gamma \)-carboxyglutamate.

**General mechanisms of quinone toxicity**

From a toxicological perspective, two major mechanisms have been proposed for the cytotoxic action of quinones, such as menadione (2-methyl-1,4-naphthoquinone: vitamin K3) and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) in a variety of biological systems. First, quinones (Q) undergo one electron reduction by enzymes such as microsomal NADPH-cytochrome P-450 reductase or mitochondrial NADH ubiquinone oxidoreductase, yielding the corresponding semiquinone radicals (Figure 2.3, reactions 1 and 2). Under aerobic conditions, most semiquinones are readily re-oxidized and can enter a redox cycle with molecular oxygen, forming superoxide anion radicals (Figure 2.3, reaction 3). The reaction of superoxide anion radicals with hydrogen peroxide, formed by the enzymatic or spontaneous dismutation of superoxide anion radical, in the presence of trace amounts of iron salts gives hydroxyl radicals, powerful oxidizing species that may be responsible for most of the damage to essential macromolecules involved in oxidative cytotoxicity.

Second, quinones are potent electrophiles, known to alkylate the intracellular GSH leading to its depletion in the cell. The GSH conjugates are transported out of the cell and further metabolized by the kidney to form urinary mercapturic acids. Following GSH depletion, cellular macromolecules, e.g. membrane proteins bind the electrophiles covalently thereby causing irreversible changes and resulting in cell death (Gant et al., 1988; Monks and Jones, 2002). In fact, generation of GSH-conjugates catalyzed by glutathione tranferase isoforms (GST) with depletion of GSH
has been associated with menadione-induced cytotoxicity and oxidative stress (Kim et al., 1996).

Although the mechanistic aspects of quinone induced cytotoxicity have been investigated extensively, no single causative mechanism has been identified up till now. However, it is well documented that the rate of production of oxygen radicals and the importance of these radical reactions in producing cytotoxic damage vary for different quinones and for different environments. Also, the nature and amount of enzymatic reducing systems in cells are known to have an influence on the cytotoxic effect of the quinone (Rockwell et al., 1993). Further, the structure-activity relationship (SAR) studies within a range of quinones suggest that for simple unsubstituted compounds, the Michael addition to glutathione is a major cause of toxicity. SAR studies also revealed that mono- or di-hydroxy substitution (at the 5-position or the 5- and 8-positions in the aromatic ring) of 1,4-naphthoquinone results in higher toxicity as compared to 1,4-naphthoquinone itself due to increased efficiency of redox cycling (Ollinger and Brunmark, 1991).

**Figure 2.3.** GSQH$_2$ or ((GS)$_2$QH$_2$) represent mono- or diglutathione conjugated hydroquinone respectively; GPx represents glutathione peroxidase (Inbaraj and Chignell, 2004).
Pharmacological properties of quinones with particular reference to clinically approved chemotherapeutic agents

From a pharmacological standpoint, quinones have also received considerable attention of the scientific community due to their broad range of activities including antibacterial, antiviral, antifungal, antiparasitic, insecticidal as well as anticancer (Babula et al., 2007). In particular, quinones constitute one of the largest classes of antitumor agents known and several naturally occurring quinones, as well as a number of synthetic derivatives, have been developed as important anticancer therapies (Asche, 2005). To name a few, Mitomycin C, Doxorubicin and Daunorubicin are among the most important quinone containing chemotherapeutic agents currently used to treat solid tumors of breast, lung, ovary, head and neck, bladder, endometrium and prostate. However, these anticancer drugs also caused significant dose-limiting toxicities such as cumulative and often irreversible cardiomyopathy, which may proceed to clinical congestive heart failure. Secondly, it has to be mentioned that many tumor cells also show primary or acquired resistances to anthracyclines.

Because of their fundamental role in cancer chemotherapy, a number of derivatives of these two naturally occurring anthracyclines have been developed in order to increase their efficacy and to decrease their toxicity. For example, Idarubicin, the 4-demethoxy derivative of daunorubicin, has acceptable bioavailability via the oral route of administration. In epirubicin, the orientation of the 4’-hydroxyl group is reversed as compared with doxorubicin. In zorubicin, the side chain at C-9 of daunorubicin is replaced by a benzoylehydrazone substituent and in pirarubicin an additional tetrahydropyran is attached to the O-4’ of doxorubicin forming an acetal. Aclarubicin has several modifications in the aglycone and bears a trisaccharide moiety attached to the C-7 (Asche, 2005). However, only a few of them have been approved for clinical use, which necessitates the search for novel compounds with acceptable toxicities.

Given the often potent biological activity associated with many naturally occurring quinones, it is not surprising that a considerable effort has been expended in isolating, evaluating and synthesizing novel quinones in the search for compounds with potentially useful and selective pharmacological properties. To that effect, more than 1500 quinones have been tested for their antitumor activity and several others
still under investigation. Naphthoquinones represent one such class of compounds that have been extensively studied for their potential as anticancer compounds.

Naturally occurring naphthoquinones with anticancer and/or radiosensitizing properties

Structurally simple naphthoquinones (in particular derivatives of 1,4-naphthoquinone), are found in abundance in a series of unrelated families of plants - the most significant ones being families like Droseraceae, Plumbaginaceae, Juglandaceae, Acanthaceae, Avicenniaceae, Bignoniaceae, Boraginaceae, Dioncophyllaceae, Ebenaceae, Lythraceae and Nepenthaceae (Babula et al., 2006; Babula et al., 2009). 1,4 naphthoquinone based compounds isolated from these plant families like plumbagin, lapachol, juglone, lawsone, menadione and shikonin have been extensively studied for their potential as anticancer agents (Figure 2.4).

![Naphthoquinones](image)

**Figure 2.4.** Some naturally occurring 1, 4 naphthoquinones with anticancer potential

Plumbagin (2-methyl, 5-hydroxy,1,4-naphthoquinone), a naphthoquinone isolated from Plumbago zeylanica L., (Family - Plumbaginaceae) that has shown promising anticancer activity *in vitro* against a broad spectrum of human tumor cells including cervical cancer (ME-180, HeLa), chronic myeloid leukemia (KBM-5), promyelocytic leukemia (HL-60), histiocytic lymphoma (U937), multiple myeloma (U266), lung adenoma (H1299), breast adenocarcinoma (MCF7), non small cell lung cancer (A549), ovarian adenocarcinoma (BG1), prostate cancer (PC3), melanoma
(Bowes) (Srinivas et al., 2004; Hsu et al., 2006; Sandur et al., 2006; Kawiak et al., 2007; Powolny and Singh, 2008; Thasni et al., 2008; Wang et al., 2008). Further, several groups including ours have also shown the ability of plumbagin to inhibit the growth of tumors in vivo (Uma Devi et al., 1994; Sugie et al., 1998). The cytotoxic effect of plumbagin is closely related to its ability to induce oxidative stress by the generation reactive oxygen species. It is known to induce apoptosis in variety of cells types not only through activation of caspase-dependent (by the release of mitochondrial cytochrome C) but also through caspase–independent pathways (involving apoptosis inducing factor (AIF)) (Srinivas et al., 2004; Sandur et al., 2006). Plumbagin down-regulates the expression of NF-kappa B regulated anti-apoptotic (IAP1, IAP2, Bcl-2, Bcl-xL, cFLIP, Bfl-1/A1, and survivin), proliferative (cyclin D1 and COX-2), and angiogenic (matrix metalloproteinase-9 and vascular endothelial growth factor) gene products (Sandur et al., 2006; Thasni et al., 2008). Plumbagin was also shown to cause G2/M cell cycle arrest in H460 cells, which was correlated with the increased levels of p21 protein and reduced amounts of cyclinB1, Cdc2, and Cdc25C (Gomathinayagam et al., 2008). Synergic antitumor effect of plumbagin may be also connected with inhibition of efflux of cytostatic drug mitoxanthrone by multidrug resistance-linked ATP binding cassette drug transporter (ABCG2) (Shukla et al., 2007). Besides, several groups including ours have shown plumbagin to possess potent radiosensitizing potential against various tumor models (Krishnaswamy and Purushothaman, 1980; Uma Devi et al., 1994; Prasad et al., 1996; Uma Devi et al., 1999; Nair et al., 2008) where ROS mediated activation of the apoptotic was proposed to be the key mechanism of action.

Shikonin, another naphthoquinone found abundantly in the roots of L. erythrorrhizon (Family - Boraginaceae), has also been reported to possess anticancer properties against various tumor models in vitro including hepatoma cells (SK-Hep-1), premyelocytic leukemia cells (HL-60), malignant melanoma cells (A375-2), cervical cancer cells (HeLa), prostate cancer (PC3), bladder cells (T24) etc. Previous reports indicate that shikonin induced ROS mediated cell death was mediated through apoptosis (Wu et al., 2004a; Wu et al., 2004b; Yeh et al., 2007; Mao et al., 2008). However, recent studies have also shown a shikonin-induced necroptotic mode of cell
death (necroptosis - refers to a regulated form of necrosis, which is biochemically defined as a form of cell death that is dependent on the serine–threonine kinase receptor-interacting protein 1) in caspase-3 deficient MCF 7 cells, that contributed to overcome Bcl-2- and Bcl-XL-mediated apoptotic resistance (Han et al., 2007). In addition, both plumbagin as well as shikonin have been shown to inhibit the activity of topoisomerase II (Topo II) through the stabilization of the Topo II–DNA cleavable complex (Fujii et al., 1992) and thereby inhibiting the DNA replication process. In another study, Yang and co-workers (2009) observed apoptotic cell death in prostate cancer cells (PC3) treated with shikonin both in vitro as well as in vivo and attributed it to the suppression the proteosomal activity in tumor cells.

Lawsonone, another 1, 4 naphthoquinone derivative from Henna plant (Lawsonia inermis, Family - Lythraceae) has also been shown to possess anticancer properties (Kamei et al., 1998). The growth inhibitory effect of lawsonone has been attributed to its ability to block the synthetic phase of the cell cycle. Lawsonone has also been shown to interfere with the process of de novo pyrimidine synthesis by inhibiting the recombinant human and rat dihydro orotate dehydrogenase activity (an enzyme that catalyses oxidation dihydro-orotate to orotate in the fourth level of de novo pyrimidine biosynthesis) by upto 50 %. However, further studies are required to advocate on its therapeutic potential as anticancer agent.

The anticancer activity of menadione (vitamin K3) has been shown in various cancer cells in vitro and in vivo (Nutter et al., 1991). In rats, menadione was shown to be active against adriamycin-resistant leukemia cells (Parekh et al., 1992). Su and co-workers (1991) demonstrated increased survival rate of 60 days compared to 17 days for controls when hepatoma-bearing rats were treated with intraperitoneal injections of menadione (10 mg/2mL weekly for four weeks). Laux and Nel (Laux and Nel, 2001) demonstrated that the cytotoxic potential of menadione on human T cell lymphoma line jurkat (subclone BMS2) was mediated through oxidative stress induced apoptosis involving both Fas dependent and Fas independent pathways with mitochondria being the major target of menadione induced cytotoxicity. In cancer cells, menadione specifically inhibits DNA polymerase (polγ), causing impairment of mitochondrial DNA replication, and promoted ROS generation leading to apoptosis.
A recent study also found that menadione could bind at the colchicines binding site of tubulin and inhibit microtubule polymerization (Acharya et al., 2009). In another recent study, menadione was shown to modulate the sensitivity of MCF 7 breast cancer cells to the effects of calcitriol (negative growth regulator of MCF7 breast cancer cells) (Marchionatti et al., 2009). This compound has also been evaluated in clinical trials in patients with advanced malignancies. A phase I clinical trial showed that intravenous infusion of menadione starting at 40 mg/m^2 every 3 weeks with escalation to 1360 mg/m^2 produced no objective partial or complete responses (Lim et al., 2005). High dose vitamin K3 daily infusion has been evaluated in patients with advanced liver cancer, and produced objective response in 17.4% patients with improved survival in the responsive patients but did not affect the overall survival (Sarin et al., 2006). In addition, menadione is also reported to possess radiosensitizing properties based on the studies where, pretreatment of mice with transplanted mouse liver tumors by oral or intraperitoneal injection of vitamins K3 and C greatly potentiated the action of radiation (20 - 40 Gy dosages) compared to controls (Taper et al., 1996). However more large scale trials may be required to establish its clinical usefulness.

Lapachol is a naphthoquinone that was first isolated by E. Paterno from *Tabebuia avellaneeae* (Family - Bignoniaceae) in 1882. Way back in 1968, lapachol was shown to possess significant activity against cancerous tumors in rats. β-Lapachone is known to selectively induce cell death in several human cancer cell lines and that its antitumor activity may be due to its inhibitory effects in topoisomerase 1 (Li et al., 1993). Woo and co-workers (2006) studied the effects of β-lapachone on the growth of the human hepatoma cell line (HepG2) and concluded that treatment of cells with β-lapachone resulted in down-regulation of anti-apoptotic Bcl-2 and Bcl-XL and upregulation of pro-apoptotic Bax expression. However, β-lapachone treatment did not affect the inhibitor of apoptosis proteins family and the Fas/FasL system. Besides, β-lapachone is also shown to cause selective necrotic cell death in cancer cells through activation of DNA damage response pathway (Sun et al., 2006). Further, Kung and co-workers (2007) recently investigated the *in vitro* effect of β-lapachone on endothelial cells, including the human vascular endothelial cell...
line, EAh926, and human umbilical vascular endothelial cells (HUVEC) and concluded that β-lapachone may have promising antiangiogenic activity.

Based on the mechanisms of action of these important naturally occurring naphthoquinones, the following illustration (Figure 2.5) may explain the major modes of cell death induced by these naphthoquinones (Babula et al., 2009).

![Figure 2.5](image)

**Figure 2.5.** Different mechanisms of 1,4-naphthoquinone derivates cytotoxicity – inhibition of orotate synthesis (1), inhibition of thymidine incorporation to DNA (2), inhibition of DNA replication by topoisomerase I and/or topoisomerase II cleavage (3), reactive oxygen species (ROS) generation and mitochondrion damage with down-regulation of antiapoptic genes and releasing of apoptosis inducing factor (Babula et al., 2009).

However, the clinical testing of many of these quinones poses considerable problems due to their toxicity profiles. For example, plumbagin (being structurally
related to vitamin K) on chronic administration is known to prolong the bleeding times by altering the platelet adhesiveness and coagulation process (Vijayakumar et al., 2006). Similarly, some other quinones are also known to cause hemolytic anemia, hepatotoxicity, cardiotoxicity as well as nephrotoxicity (Gant et al., 1988; Munday et al., 1991; Munday et al., 1994). Apart from that, many naphthoquinones are also known to have short biological half lives, rapid clearance from the circulation which may result in poor anticancer efficacy in vivo (Chandrasekaran and Nagarajan, 1981; Loadman et al., 2002; Phillips et al., 2004). These above mentioned problems associated with naphthoquinones anticancer agents, have in the past been overcome by the use of novel drug delivery platforms that help not only in reducing the toxic side effects and improving the anticancer efficacy but also in enhancing the elimination half life of the naphthoquinone compounds. With that perspective, plumbagin has been successfully formulated as poly (lactic-co-glycolic) acid (PLGA) based microparticles, non ionic surfactant vesicles (niosomes), as well as thermo-sensitive liposomes which have resulted in significant improvement in its anticancer efficacy against in vivo mouse tumor models (Singh et al., 1997; Singh and Udupa, 1997; Tiwari et al., 2002). Very recently, Blanco and co-workers (2010) reported improved anticancer activity of β-lapachone when formulated as micelles using biocompatible polymers and concluded that nano-carriers provided an attractive and clinically viable platform for the delivery of β-lapachone with enhanced safety, pharmacokinetics, and antitumor efficacy for the specific treatment of non small cell lung carcinoma (NSCLC).

**Delivery of anticancer compounds using nano-carriers for better tumor targeting**

One of the main goals of any treatment employing xenobiotics is to increase the therapeutic index of the drug while minimizing its side-effects. Different approaches have been attempted in order to provide “selective” and “sufficient” delivery of cytotoxic agent only to those organs, tissues or cells affected by the disease, while restricting its access to non-target normal cells. The use of drug delivery systems (DDS), such as molecular conjugates and colloidal particulates, is one such approach that can improve the pharmacological properties of chemotherapeutics by altering the pharmacokinetics and biodistribution patterns of
the encapsulated drug. Colloidal particulates result from the physical incorporation of the drug into a particulate colloidal system. These colloidal carrier systems are known to offer numerous advantages over the conventional dosage forms (Vyas and Khar, 2006) viz., improved efficacy, reduced toxicity, prolonged release and improved patient compliance and convenience of administration. Among the various particulate drug carriers (such as liposomes, niosomes, micro- and nano-spheres, erythrocytes, and polymeric and reverse micelles) used for chemotherapeutic drug targeting, liposomes have gained most attention.

**Liposomes in drug delivery**

The origins of liposome research can be traced to the contributions of Alec Bangham who described lecithin dispersions as containing “spherulites composed of concentric lamellae” (Bangham and Horne, 1964). A year later in 1965, another startling observation made by Bangham and co-workers, that “the diffusion of univalent cations and anions out of spontaneously formed liquid crystals of lecithin is remarkably similar to the diffusion of such ions across biological membranes” (Bangham et al., 1965) inspired investigators to evaluate the utility of liposomes as models to understand the structure and function of biological membranes. Later in 1976, two landmark papers of Gregory Gregoriadis, published in New England Journal of Medicine, outlined the huge potential of liposomes as carriers in biology and medicine (Gregoriadis, 1976a; Gregoriadis, 1976b). The following 2 decades saw immense efforts in academia and in soon-to-be-founded start-up companies to turn Gregoriadis’ vision into clinical reality. These 20 years of intense work in liposome laboratories around the world finally culminated with the FDA (USA) approval of the first injectable liposomal drug, Doxil, in February of 1995. Today, liposome science and technology is one of the fastest growing scientific fields contributing to areas such as drug delivery, cosmetics, structure and function of biological membranes and investigations of the origin of life to name a few.

As a definition, liposomes are the small vesicle of spherical shape in which an aqueous volume is entirely enclosed by a membranous lipid bilayer and can be
produced from cholesterols, non toxic surfactants, sphingolipids, glycolipids, long chain fatty acids and even membrane proteins (Bangham and Horne, 1964).

**Attractive biological properties of liposomes** (Torchilin, 2005)

- Liposomes are biocompatible/biodegradable and cause very little or no antigenic, pyrogenic, allergic and toxic reactions.
- Liposomes can entrap both hydrophilic and hydrophobic pharmaceutical agents.
- Liposome-incorporated pharmaceuticals are protected from the inactivating effect of external conditions; yet do not cause undesirable side reactions.
- Liposomes provide a unique opportunity to deliver pharmaceuticals into cells or even inside individual cellular compartments.
- Liposomes are regarded as flexible in that their size and charge can easily be varied. Also, their surfaces can be easily modified with a variety of functional moieties such as polyethylene glycol (PEG) and other targeting ligands (Figure 2.6).

**Figure 2.6.** Diagram of a drug-loaded liposome both with (sterically stabilized liposomes) and without (conventional liposomes) a PEG coating (Adapted from Cukierman and Khan, 2010).
Classification of liposomes (Sharma and Sharma, 1997)

Liposomes may be classified based either on its composition or on its size and lamellarity.

A. On the basis of size and lamellarity: The vesicle size is a critical parameter in determining circulation half life of liposomes, and both size and number of bilayers (lamellarity) influence the extent of drug encapsulation in the liposomes. Therefore, based on their size and number of bilayers, liposomes can also be classified into one of following three categories (Table 2.3).

Table 2.3. Classification of liposomes based on the size and lamellarity.

<table>
<thead>
<tr>
<th>Type</th>
<th>Usual size</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multilamellar vesicles (MLVs)</td>
<td>500 – 5000 nm</td>
<td>More than one bilayer; moderate aqueous volume to lipid ratio; greater encapsulation of lipophilic drugs; greater stability on storage; rapid RES uptake and so better for RES targeting.</td>
</tr>
<tr>
<td>Large unilamellar vesicles (LUVs)</td>
<td>200 – 800 nm</td>
<td>Single bilayer; high aqueous volume to lipid ratio; useful for hydrophilic drugs; higher capture of macromolecules; rapid RES uptake and so better for RES targeting.</td>
</tr>
<tr>
<td>Small unilamellar vesicles (SUVs)</td>
<td>≈ 100 nm</td>
<td>Single bilayer; better size homogeneity; thermodynamically unstable and susceptible to aggregation and fusion; limited capture of macromolecules; lower aqueous volume to lipid ratio; longer circulation half life with slower RES uptake; usually prepared by reducing the size of MLV or LUV.</td>
</tr>
</tbody>
</table>
B. **On the basis of composition:** The net physicochemical properties of the lipids composing the liposomes, such as membrane fluidity, charge density, steric hindrance, and permeability, determine liposomes’ interactions with blood components and other tissues after systemic administration. The nature and extent of liposome-cell interaction in turn determines the mode of intracellular delivery of drugs. Thus, the predominant mechanism behind intracellular delivery of drugs by liposomes may mainly depend on their composition (Table 2.4).

**Table 2.4.** Classification of liposomes based on the composition.

<table>
<thead>
<tr>
<th>Type</th>
<th>Usual size</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional liposome (CL)</td>
<td>Neutral or negatively charged phospholipids plus cholesterol</td>
<td>Subject to coated-pit endocytosis; contents ultimately delivered to lysosomes; useful for RES targeting, short half life with dose-dependent pharmacokinetics</td>
</tr>
<tr>
<td>pH-sensitive liposome</td>
<td>Phospholipids like PE or DOPE with either CHEMS or OA</td>
<td>Subject to coated-pit endocytosis; at low pH, fuse with cell of endosome membranes and release their contents in cytoplasm; suitable for intracellular delivery of weak bases &amp; macromolecules; pharmacokinetics similar to conventional liposomes</td>
</tr>
<tr>
<td>Cationic liposome</td>
<td>Cationic lipids: DDAB, DOGS, DOSPA, DOTMA, DMRIE and DORIE with DOPE</td>
<td>Fusion with cell or endosome membranes; suitable for delivery of negatively charged macromolecules (DNA, RNA, oligos); structurally unstable; mainly restricted to local administration</td>
</tr>
<tr>
<td>Long circulating liposome</td>
<td>Neutral high $T_c$ lipids, Cholesterol, plus mPEG-DSP</td>
<td>Hydrophilic surface coating; low opsonization and thus low rate of RES uptake; longer circulation half life.</td>
</tr>
<tr>
<td>Immuno-liposomes</td>
<td>CL or LCL with attached antibody or recognition sequence</td>
<td>Subject to receptor-mediated endocytosis; cell-specific binding; can release contents extracellularly near the target tissue</td>
</tr>
</tbody>
</table>
**Methods of liposome preparation** (Samad *et al.*, 2007; Malam *et al.*, 2009)

An important parameter to be considered when preparing the liposome is the rigidity of bi-layers. There are several groups of phospholipids that can be used for the liposome preparation which may be of either synthetic or natural origin. The predominant physical and chemical properties of a liposome are based on the net properties of the constituent phospholipids, including permeability, charge density and steric hindrance. The most commonly used phospholipids for the preparation of liposomes include Dilauryl phosphotidyl choline (DLPC), Dimyristoyl phosphotidyl choline (DMPC), Dipalmitoyl phosphotidyl choline (DPPC), Distearoyl phosphotidyl choline (DSPC), Dioleoyl phosphotidyl choline (DOPC), Dilauryl phosphotidyl ethanolamine (DLPE), Dimyristoyl phosphotidyl ethanolamine (DMPE), Distearoyl phosphotidyl ethanolamine (DSPE), Dioleoyl phosphotidyl ethanolamine (DOPE), Dilauryl phosphotidyl glycerol (DLPG), Distearoyl phosphotidyl serine (DSPS). Additionally, cholesterol is often added to the bilayers mixture which serves the following purposes:

- Act as a fluidity buffer
- Act as intercalator with phospholipids molecules
- Restrict the transformation of trans to gauche conformation.

The three different strategies for the preparation of liposomes are as follows:

- **Mechanical method or thin film hydration method**: This is the most simple and widely used method for preparation of MLV in which a thin film of lipids is hydrated with an aqueous buffer at a temperature above the transition temperature of lipids. The drug to be encapsulated is included either in the aqueous hydration buffer (for hydrophilic drugs) or in the lipid film (for lipophilic drugs). The lipids spontaneously swell and hydrate to form liposome. This method yields a heterogeneous sized population of MLVs that are normally over 1 µm in diameter which can either be sonicated or extruded through polycarbonate filters to produce small (up to 0.025 µm) and more uniformly sized population of SUV. This
method however suffers from poor encapsulation efficiencies (5 – 15 % for hydrophilic drugs)

- **Methods based on replacement of organic solvent:** In this method lipids are co-solvated in organic solution, which is then dispersed into aqueous phase containing material to be entrapped within the liposome. This method is of two types:
  
  - **Reverse Phase Evaporation:** In this method, the lipid mixture is added to a round bottom flask and the solvent is removed under reduced pressure. The system is then purged with nitrogen and lipids are re-dissolved in the organic phase which is the phase in which the reverse phase vesicle will form. Diethyl ether and isopropyl ether are the usual solvents of choice. After the lipids are re-dissolved, the emulsion is obtained following which the solvent is removed from an emulsion by evaporation to a semisolid gel under reduced pressure. The resulting liposomes are called reverse phase evaporation vesicles (REV). This method is used for the preparation of LUV and has the ability to encapsulate large macromolecules with high efficiency.
  
  - **Ether Vaporization Method:** This may either be ethanol injection method or ether injection method depending on the type of solvent used. In ethanol injection method, the ethanolic solution of lipid is injected rapidly through a fine needle into an excess of saline or other aqueous medium and is known to yield high proportion of SUVs. Although this method is extremely simple and rapid, it suffers from some drawbacks like the limited solubility of lipids in ethanol. On the other hand, ether injection method involves injecting the immiscible organic solution of the lipid very slowly through a fine needle into an aqueous phase at the temperature of vaporizing the organic solvent. Ether injection method is often time consuming and results in relatively low encapsulation efficiencies.
• **Methods based on size transformation or fusion of prepared vesicle:** In this case the small unilamellar vesicles are first prepared and then the drug loading is done using one of the below mentioned techniques

  - **Dried-reconstituted vesicles (DRV)** – This method starts with freeze-drying the dispersion of empty SUVs and then rehydrating it with the aqueous fluid containing the material to be entrapped. This leads to a dispersion of solid lipids in finely divided form. This method usually produces unilamellar liposomes and the entrapment efficiency is usually in the range of 40%.

  - **Freeze Thaw sonication method** – This method is an extension of the classical DRV method and is based on freezing a dispersion of SUVs and then thawing by standing at room temperature for 15 min. This step is followed by a brief sonication cycle during which the SUVs rupture, refuse and causes the solute to equilibrate inside and outside. The presence of charge is necessary for the formation of ice crystals to aid in rupture/fusion process and therefore neutral lipids cannot be used, which is one of the major drawbacks of this method.

Apart from these most commonly employed methods for the preparation of liposomes, several other methods are also employed like detergent depletion, dialysis, calcium induced fusion etc. However, the selection of the appropriate method of liposomes preparation is dictated by the physicochemical nature of the drug, intended route of administration, kind of end product desired (SUV, LUV, MLV etc) as well as the economic considerations.

**Liposome cell interaction**

Although ligand-mediated binding of liposomes to cell surface receptors can increase the cellular uptake of liposome-encapsulated drugs, the internalization process itself is not sufficient to yield an enhanced therapeutic effect as long as the entrapped drug is not delivered to the (sub) cellular intervention site. In most cases, the drug needs to be delivered into the cytosol in order to become effective. Among
the several mechanisms that have been proposed to explain the process of cytosolic drug delivery, the most important ones are depicted in Figure 2.7.

**Figure 2.7.** Liposome-cell interaction - Drug-loaded liposomes can specifically (a) or nonspecifically (b) adsorb onto the cell surface. Liposomes can also fuse with the cell membrane (c), and release their contents into the cell cytoplasm, or can be destabilized by certain cell membrane components when adsorbed on the surface (d) so that the released drug can enter cell via micropinocytosis. Liposome can undergo the direct or transfer-protein-mediated exchange of lipid components with the cell membrane (e) or be subjected to a specific or nonspecific endocytosis (f). In the case of endocytosis, a liposome can be delivered by the endosome into the lysosome (g) or, en route to the lysosome, the liposome can provoke endosome destabilization (h), which results in drug liberation into the cell cytoplasm (Torchilin, 2005).

**Principles of site-specific drug targeting**

The concept of site-specific delivery was first proposed by Paul Ehrlich and involves the delivery of a larger fraction of drug to the target site and therefore, reducing exposure to normal tissues. Liposomes have been employed for accomplishing both passive and active targeting of drugs. Size characteristic of liposome is known to have a major effect on their *in vivo* behavior.
Passive targeting – Enhanced permeability and retention effect

Passive targeting consists of the transport of nanocarriers through leaky tumor capillary fenestrations into the tumor interstitium and cells either by convection or passive diffusion. The accumulation and retention of nanoparticulate drugs are known to be greatly enhanced in solid tumor tissue compared to those in normal tissue, due to a phenomenon now known as the EPR (enhanced permeability and retention) effect of macromolecules and lipidic particles (Figure 2.8).

**Figure 2.8.** Passive and active targeting of tumor cells using liposomes. At sites of pathology (cancer tissue) where the endothelium layer is inflamed, mediators such as bradykinin, vascular endothelium growth factor and prostaglandins cause an increase in the endothelial permeability. Liposomes extravasate through the gaps between cells and enter the interstitial fluid. Active targeting is achieved by conjugating ligands to the liposome that bind to a specific target cell receptor, leading to internalization or release of the drug. Passive targeting can be mediated by internalization or local high-concentration release of the drug (Malam et al., 2009).

This effect is applicable only to macromolecules and lipidic particles, not to low-molecular-weight compounds; the category to which most drugs in use today belong (Maeda et al., 2000; Maeda, 2001). Low-molecular-weight compounds such as Mitomycin C are distributed freely by diffusion to various tissues and organs; the
compounds move against the concentration gradient until finally an equilibrium results. Therefore, their concentration in tumor cannot be higher than that in blood plasma, nor can they be retained at high concentrations in tumor for a significant time period because of rapid excretion (washout) into the blood stream. The plasma concentration also diminishes rapidly as a result of efficient renal clearance via the urine. In contrast, macromolecules and polymeric drugs are retained in tumor tissue at a much higher concentration than that in plasma (Noguchi et al., 1998).

This phenomenon, the EPR effect, is now recognized as a general characteristic of viable and rapidly growing solid tumor. Another general characteristic is the architectural defectiveness of tumor blood vessels, which also causes enhanced leakiness. Several factors including the physiological conditions as well as physicochemical properties of nanocarrier are known to affect the EPR effect (Table 2.5).

**Table 2.5. Factors affecting enhanced permeability and retention (EPR) effect**

**A. Physiological factors affecting the EPR effect in solid tumor (Mäeda, 2001)**

1. Active angiogenesis and high vascular density in tumor
2. Extensive production of vascular mediators that facilitate extravasation, including bradykinin, nitric oxide, VPF/VEGF, prostaglandins, collagenase (MMP)
3. Defective vascular architecture, for example, lack of smooth muscle cells, lack of or reduced numbers of receptors for angiotensin II, large gap in endothelial cell–cell junctions, anomalous vascular conformation (e.g., branching or stretching)
4. Impaired lymphatic clearance of macromolecules and lipids from interstitial tissue (resulting in their retention)

**B. Physicochemical properties of the nanocarrier that affect the EPR effect**

1. Size - The ideal nanocarrier size should be somewhere between 10 and 100 nm. Any size below 10 nm may get filtered through kidneys and anything larger than 100 nm may result in specific capture by the liver.
2. The charge of the particles should be neutral or anionic for efficient evasion of the renal elimination.
3. The nanocarriers must be hidden from the mononuclear phagocyte system, which destroys any foreign material through opsonization followed by phagocytosis.
Active targeting (Danhier et al., 2010)

In active targeting, targeting ligands are attached at the surface of the nanocarrier for binding to appropriate receptors that are overexpressed by tumor cells or tumor vasculature and not expressed by normal cells. Moreover, targeted receptors should be expressed homogeneously on all targeted cells. These targeting ligands may either be monoclonal antibodies (mAbs) and antibody fragments or non-antibody ligands (peptidic or not). The binding affinity of the ligand influences the tumor penetration because of the “binding-site barrier”. For targets in which cells are readily accessible, typically the tumor vasculature, because of the dynamic flow environment of the bloodstream, high affinity binding appears to be preferable.

Various anti-cancer therapeutics are grouped under the name “ligand targeted therapeutics,” and share the common basic principle viz., the specific delivery of drugs to cancer cells. Antibodies (monoclonal antibody or fragments) target a specific antigenic receptor, interfering with signal-transduction pathways, regulating proto-oncogenes involved in cancer cells proliferation — such as trastuzumab (anti-ERBB2, Herceptin®), bevacizumab (anti-VEGF, Avastin®) or etaracizumab, a humanized anti-αvβ3 antibody (Abegrin). In the active targeting strategy, two main cellular targets can be distinguished:

(i) targeting of cancer cell - as in the case of transferrin receptor, folate receptor, Glycoproteins/lectins, epidermal growth factor receptor (EGFR) etc

(ii) targeting of tumoral endothelium - as in the case of vascular endothelial growth factors (VEGF) and their receptors, αβ3 endothelial cell receptor, Vascular cell adhesion molecule-1 (VCAM-1) – a glycoprotein expressed on endothelial cell surface, matrix metalloproteins (MMPs) etc.

Apart from these ligand couple liposomes, several other approaches have been attempted in order to actively target the chemotherapeutic agents to the cancer cells. The most important ones include acid triggered release (using pH-sensitive liposomes – based on the acidic microenvironment in various tumors), heat triggered release
(using thermo sensitive liposomes - basic strategy was to design liposomes using lipids with the phase transition just above physiological temperature so that local tumor hyperthermia will trigger the drug release from such liposomes), light triggered release (Liposomes can be made photosensitive by the use of lipids that either isomerizes, fragments or polymerizes upon photoexcitation) and enzyme triggered release (involves the use of enzymes, that are upregulated in tumor tissue for site-specific drug release).

**Table 2.6. Approved liposomal formulations (Immordino et al., 2006)**

<table>
<thead>
<tr>
<th>Active agent (product name)</th>
<th>Stealth</th>
<th>Company, year of product marketing</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daunorubicin (DaunoXome®)</td>
<td>No</td>
<td>Nexstar Pharma., 1995</td>
<td>Kaposi sarcoma</td>
</tr>
<tr>
<td>Doxorubicin (DOXIL®/Caelyx®)</td>
<td>Yes</td>
<td>Sequus Pharma., 1997</td>
<td>Kaposi sarcoma</td>
</tr>
<tr>
<td>Doxorubicin (Myocet®/Evacet®)</td>
<td>No</td>
<td>Elan Pharma, 2000</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>Cytarabine (Depocyt)</td>
<td>No</td>
<td>SkyePharma, 1999</td>
<td>Lymphomatous meningitis</td>
</tr>
<tr>
<td>Amphotericin B (Ambisome®)</td>
<td>No</td>
<td>Fujisawa Inc. &amp; Nexstar Pharm, 1997</td>
<td>Fungal infections</td>
</tr>
</tbody>
</table>

**Importance of long circulating/stealth liposomes**

One of the important barriers limiting the application of liposomes for intravenous drug targeting has been a short blood circulation time resulting from rapid and efficient recognition and removal from blood by cells of the mononuclear phagocyte system (MPS), particularly those in the liver and spleen (Torchilin, 2005). This immune system’s first line of defense consists of macrophages specialized in nonspecific elimination (phagocytosis) of all exogenous material in the circulation, including liposome particles. Plasma proteins like antibodies and other so-called opsonins recognize and adhere to liposomal bilayers, provoking the uptake of liposomes by MPS-macrophages (Yan et al., 2005). This MPS-directed behavior of
liposomes has been successfully exploited to achieve selective delivery of antimicrobials in models of intracellular infections caused by pathogens localized in MPS cells (Bakker-Woudenberg et al., 1994). However, in the majority of diseases, the rapid sequestration by the MPS often eliminates the intended beneficial effects and moreover can pose considerable risk of toxicity to these cells (Storm et al., 1998).

The introduction of liposomes exhibiting prolonged circulation by virtue of their capability to oppose rapid MPS uptake represents a milestone in liposomal drug delivery research. Grafting of the liposome with the inert and biocompatible polymer, polyethylene glycol (PEG), leads to the formation of a protective, hydrophilic layer on the surface of the liposomes. This modification prevents the recognition of liposomes by opsonins (ie, antibodies or components of the complement system) and therefore reduces their clearance by cells of the MPS (Huwyler et al., 2008). Such pegylated liposomes are therefore often referred to as ‘sterically stabilized’ or ‘stealth’ liposomes (Lasic and Papahadjopoulos, 1995). In humans, pegylation of liposomes results in an up to 50-fold decrease in the volume of distribution, a 200-fold decrease in systemic plasma clearance and a nearly 100-fold increase in area under the time-concentration curve (Allen, 1994). Using pegylated phospholipids, the apparent terminal half-life of such long-circulating liposomes can be extended in humans from a time-scale in minutes to days (Lasic, 1996).

The protective effect of pegylation and the resulting extension of the plasma half-life in vivo is found to correlate well with the thickness of the PEG-coating. On theoretical grounds, a thickness of a PEG coating of 5 to 10 per cent of the particle diameter is needed to achieve effective steric stabilization (Lasic, 1996). Other studies explored the thickness of a PEG coating by direct measurement of PEG-tethered ligand-receptor interaction potentials using a surface forces apparatus (Wong et al., 1997) and based on these studies, it may be concluded that coating of 100 nm liposomes with PEG-2000 should lead to effective steric stabilization in vivo. Considering their tremendous clinical potential, these newer forms of liposomes are actively being investigated worldwide and the results have substantially expanded the role of liposomes in developing new therapeutics (Table 2.6 and Table 2.7).
<table>
<thead>
<tr>
<th>Active agent (product name)</th>
<th>Stealth</th>
<th>Company</th>
<th>Application</th>
<th>Trial phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin (SPI-077)</td>
<td>Yes</td>
<td>Sequus Pharma.</td>
<td>Head and neck, Lung cancer</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Cisplatin (Lipoplatin™)</td>
<td>Yes</td>
<td>Regulon Inc.</td>
<td>Several cancer types</td>
<td>Phase II/III</td>
</tr>
<tr>
<td>Camptothecin analog (S-CKD602)</td>
<td>Yes</td>
<td>Alza Co.</td>
<td>Several cancer types</td>
<td>Phase I</td>
</tr>
<tr>
<td>Oxaliplatin analog (Aroplatin)</td>
<td>No</td>
<td>Antigenics Inc.</td>
<td>Colorectal cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>Paclitaxel (LEP-ETU)</td>
<td>No</td>
<td>NeoPharm Inc.</td>
<td>Ovarian &amp; breast cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td>Mitoxantrone (LEM-ETU)</td>
<td>No</td>
<td>NeoPharm Inc.</td>
<td>leukemia, breast, stomach, liver cancers</td>
<td>Phase I</td>
</tr>
<tr>
<td>Irinotecan (LE-SN38)</td>
<td>No</td>
<td>NeoPharm Inc.</td>
<td>advanced cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td>Paclitaxel (MBT-0206)</td>
<td>No</td>
<td>MediGene AG</td>
<td>Anti-angiogenic, breast cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td>Lurtotecan (OSI-211)</td>
<td>No</td>
<td>Enzon Co.</td>
<td>Ovarian, Head and neck cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>Vincristine (Marqibo®)</td>
<td>No</td>
<td>Inex Pharm</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>Phase II/III</td>
</tr>
<tr>
<td>Topotecan (INX-0076)</td>
<td>No</td>
<td>Inex Pharm</td>
<td>advanced cancer</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Liposomal Annamycin®</td>
<td>No</td>
<td>MD Anderson</td>
<td>breast cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>Nistatin (Nyotran®)</td>
<td>No</td>
<td>Aronex Pharm</td>
<td>Fungal infections</td>
<td>Phase II/III</td>
</tr>
</tbody>
</table>
Juglone, an overview

Occurrence

Juglone, a napthoquinone pigment that occurs as a natural product in the roots, leaves, nut-hulls, bark and wood of black walnut (*Juglans nigra*), European walnut (*Juglans regia*) and butternut (*Juglans cinerea*) belonging to family *Juglandaceae* (Funt and Martin, 1993; Botanical Dermatology Database, 1999).

Walnut chemistry

Several phytochemical studies have shown walnut to possess a wide array of pharmacologically active compounds. Koyuncu and Askin (Koyuncu and Askin, 1995) investigated the chemical composition of 12 walnut genotypes and reported the composition as follows – Protein, 20.92 – 25.95 %; ash, 1.68 – 2.6 %; fat, 66.30 – 74.95 %. In addition, Zwarts and co-workers (1999) evaluated the fatty acid composition of two US commercial cultivars (Tehama and Vina), three European commercial cultivars (Esterhazy, 139, G120) and five New Zealand selections (Rex, Dublin’s Glory, Meyric, McKinster, Stanley) and reported that the total oil content of the nuts ranged from 62.4 – 68.7 %, with the oleic acid content of the oils ranged from 14.3 – 26.2 % of the total fatty acids, while the linoleic acid content ranged from 49.3 – 62.3 % and the linolenic contents from 8.0 – 13.8 %. As a result, walnuts are a rich source of n-3 and n-6 polyunsaturated fatty acids because of which walnut has been used as lipid lowering agents and many studies suggest that frequent consumption of nuts may provide some protection against coronary heart diseases (Sabate *et al.*, 1993; Hu *et al.*, 1998). Replacement of dietary saturated fats with either monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs) decreases plasma total and LDL cholesterol concentrations (Dattilo and Kris-Etherton, 1992). In addition, walnuts are found to contain significant amounts of macronutrients like potassium, phosphorous, calcium, magnesium, sodium, iron, zinc, manganese which may improve the blood pressure and may contribute to its overall nutritional value (Elin and Hosseini, 1993; Muradoglu *et al.*, 2010). Chemical and mineral contents of
Walnuts are known to vary based on the variety, genotype, ecology, technical and cultural practices, climate, and soil conditions.

However, the chief constituent in walnut is juglone (5-hydroxy-1,4-naphtho[1,2-d]pyran-3-one). Also present are alpha-hydrojuglone (1,4,5-trihydroxynaphthalene) and its glycoside beta-hydrojuglone, along with caffeic acid, ellagic acid, hyperin, and kaempferol. Walnuts also contain tannins like galloylglucose and ellagitannins (Ensminger, 1994). Minerals in walnut include iron and zinc (approximately 3 mg/100 g each), sodium (2 mg/100 g), selenium (19 µg/100 g), calcium, magnesium, potassium, copper, and phosphorus. Apart from these, walnuts are also known to possess vitamin E and vitamin C (Murray, 1993; Zwarts et al., 1999).

**Medicinal uses of walnut**

Crushed unripe walnut hulls have been used for generations in various types of folk medicine. The hulls are made into poultices and rubbed into the skin to treat fungal, bacterial, or viral infections such as herpes or warts. External applications of black walnut also kill ringworm as well as tapeworm. In certain regions of the United States, fresh walnut hulls have been employed illegally to immobilize fish (Westfall et al., 1961; Viable herbal solutions, 1997; VegTalk, 2007).

Recently, herbal remedies containing black walnut have been marketed as dietary supplements. Viable Herbal Solutions (1997) sells an extract of black walnut that can be used in one of two ways: it can be taken orally by mixing 10-20 drops in water or juice daily, or it can be used externally by rubbing the extract directly on the skin twice daily. Chinese Tiao He Cleanse is an herbal cleansing program marketed through the Internet for acne, allergies, body odor, constipation, dry stools, fatigue, gastrointestinal disorders, halitosis, headaches, hemorrhoids, inflammatory skin conditions, intestinal parasites and worms, lymphatic inflammation, menstrual problems, and swollen abdomen. Chinese Tiao He Cleanse consists of 30 packets, one of which is a black walnut hull preparation (Greatest herbs on earth, 2004).

According to the German Commission E Report, walnut hull preparations containing juglone are used for catarrhs of the gastrointestinal tract, skin diseases,
abscesses, inflammation of the eyes, in combinations for diabetes, gastritis, for “blood purification,” blood poisoning, and anemia. According to Commission E, the effectiveness for the claimed applications is not documented and the risks of juglone are known, so that the application of walnut hull preparations cannot be justified (Blumenthal, 1998).

Walnut extracts are known to possess potent antioxidant properties that have been directly correlated to the large amounts of total phenols, total tannins and non-tannin phenolics (Alamprese et al., 2005). These polyphenols are reported to inhibit in vitro human plasma and LDL oxidation and to reduce the incidence of cardiovascular mortality (Anderson et al., 2001). Haque and co-workers reported protective potential of walnut extracts against cyclophosphamide induced biochemical toxicity (Haque et al., 2003). In another similar study, aqueous extracts of walnut were shown to exert potent antioxidant properties (owing to its high phenolic content) and render protection against cyclophosphamide induced urotoxicity in mice models (Bhatia et al., 2006).

Further, herbal preparations of walnut have also been used in traditional folk medicine for the treatment of cancer. In order to evaluate its potential as anticancer agent, Bhargava and Westfall (Bhargava and Westfall, 1968) treated Swiss mice intraperitoneally for 9 days with extracts isolated from Juglans nigra leaves. They observed a significant reduction in the growth rate of spontaneous mammary adenocarcinomas \( (P<0.001) \). However, based on their observation that long term treatment caused significant decrease in the body weight of the animals \( (P = 0.003) \), the authors considered the effectiveness of juglone to be questionable.

**Chemical identification of juglone (O’neil et al., 2001)**

**CAS Registry Number:** 481-39-0

**Chemical Abstracts Service Name:** 1,4-Napthoquinone, 5-hydroxy-(8CI)

**Synonyms and Trade Names:** Akhrot; C.I. 75500; C.I. Natural Brown 7; 5-hydroxy-1,4-naphthalenedione; 5-hydroxynaphthoquinone; juglone; regianin; walnut extract
**Structural Class:** Bicyclic; naphthoquinone

**Structure, Molecular Formula:** $C_{10}H_6O_3$

![Juglone Structure]

**Juglone (5-hydroxy, 1,4 naphthoquinone)**

**Molecular Weight:** 174.16

**Chemical and Physical Properties**

**Description:** Yellow needles from benzene plus petroleum ether; gives purplish-red solution in aqueous solutions of alkalis

**Melting Point:** 155°C

**Density:** 1.47 g/cm$^3$

**Log P:** 1.86 ± 0.77

**pKa:** 6.96 ± 0.20

**Solubility:** Slightly soluble in hot water; soluble in alcohol, acetone, chloroform, benzene, and acetic acid.

**Pharmacology properties of juglone**

**Antimutagenicity**

Several naphthoquinones (juglone, plumbagin, menadione, 5,8-hydroxy-1,4-naphthoquinone, chimaphilin) were tested in *Salmonella typhimurium* TA98 for their activities against mutagenicities induced by 2-nitrofluorene (2-NF), 3-
nitrofluoranthene (3-NFA), and 1-nitropyrene (1-NP). All of the naphthoquinones tested were potent antimutagens irrespective of the presence of methyl or hydroxyl functions (Edenharder and Tang, 1997).

**Effects on other cellular macromolecules**

Biologically active naphthoquinones readily pass through the cellular membranes where their electrophilicity enables them to conjugate with other compounds. This reaction has been implicated in the toxicity of quinones. Nucleophilic targets include thiol groups (Gant et al., 1986) which results in inhibition of enzymes such as parvulin-like peptidyl-prolyl cis/trans isomerases (Hennig et al., 1998), glutathione-S-transferase (Vos et al., 1989), and cardiac sarcoplasmic reticulum Ca$^{2+}$ ATPase (Floreani et al., 1995). Recent studies have shown that juglone can penetrate the plasma membrane and induce polarization by blocking K+ channels (Varga et al., 1996). As part of a study to identify novel plant-derived inhibitors of signaling kinases, Frew and coworkers (Frew et al., 1995) discovered that juglone and methyljuglone are potent inhibitors of protein kinase C (PKC).

Juglone also showed potent inhibitory activity against aromatase cytochrome P450 in human placental microsomes in a dose dependent manner. The inhibitory effects of juglone were thought to be due to direct binding of the naphthoquinone to cytochrome P450 rather than an interaction with the thiol groups or formation of superoxide radicals (Muto et al., 1987).

Between 1976 and 1999, 73 patents involving juglone were obtained in the United States (US Patents and Trademarks Office, 1999). These patents demonstrate a variety of potential uses for juglone, for example, to prepare antiviral naphthoquinone derivatives useful for AIDS treatment, in skin-coloring preparations, and in hair dyes (Kurz et al., 1996; Boyd et al., 1999; Schmitt et al., 1999). According to Hocquaux and coworkers at L’Oreal (Hocquaux et al., 1990), juglone has a tinctorial strength in the right range of hues for hair dyes, but has the disadvantage that its resistance to oxidation is low. Further, some derivatives of juglone have been studied for their
potential as antitubercular agents (Sharma et al., 2009). In addition, several groups have also studied the cytotoxic potential of juglone against various cancer models both in vitro as well as in vivo.

**Effect of juglone on in vitro tumor models**

Segura-Aguilar and coworkers (Segura-Aguilar et al., 1992) compared the cytotoxic effect of juglone on human leukemia (HL-60) cells and doxorubicin-resistant human leukemia (HL-60R) cells. The cell-killing effect after incubation of HL-60 cells with juglone was similar to the effect seen for doxorubicin. As can be expected, the HL-60R cells were much more resistant to doxorubicin treatment. On the other hand, the HL-60R cells showed remarkable sensitivity to juglone (as much as HL-60 cells). Thus, the multidrug resistance that develops in the doxorubicin-resistant HL-60R cell line did not prevent the cytotoxic effect of juglone, indicating its therapeutic potential.

Juglone along with other structurally related quinones were studied for their growth inhibitory effect on cultured human colon carcinoma (HCT-15) cells. They observed that the number of hydroxyl groups on the quinone ring played an important role in the toxicity of quinones. They also observed that juglone treatment in these cells caused the cell cycle to arrest in the S phase (Kamei et al., 1998).

Cenas and co-workers (2006) evaluated the effect of juglone on human cancer cells (HeLa cells and/or A549 cells) where juglone was found to be highly cytotoxic and potent inducer of apoptosis (caspase-3/7 activation) in HeLa cells.

More recently, the effect of juglone on the apoptosis induction in human gastric cancer (SGC-7901) cells was investigated. Juglone treatment caused elevated ROS levels, down-regulation in the Bcl-2 expression as well as up-regulation in Bax in comparison to control. Based on these findings, they concluded that juglone can induce apoptosis in SGC-7901 cells through a mitochondrial pathway that seemed to be mediated by the generation of ROS and a reduction in the Bcl-2/Bax ratio (Ji et al., 2011).
Effect of juglone on in vivo tumor models

Apart from these studies, several studies have also been carried out in order to evaluate the in vivo anticancer potential of juglone. Okada and coworkers (Okada et al., 1967) examined the cytological effects of juglone on Ehrlich ascites tumor cells transmitted in Swiss/HaICR mice. Mitotic abnormalities in the tumor cells were noted 6-12 h after intraperitoneal (ip) injections at doses as low as 0.25 mg. The most prominent effects observed were a decrease in the percentage of mitotic figures with a concomitant accumulation of cells in metaphase indicating that juglone appeared to be preventing cells from entering mitosis. Chromosomes in prophase from juglone treated tumors appeared diffuse and sticky and accumulation of abnormal metaphase figures occurred indicating its anticancer potential.

A decade later, de Oliveira and co-workers (1978) studied the effect some quinones including juglone on the growth of experimental tumors and reported that juglone induced 100 % tumor inhibition of Ehrlich ascites cells transplanted in swiss mice.

Similarly, Sugie and co-workers (1998) examined the effect of juglone (as dietary exposure) on azoxymethane-induced intestinal carcinogenesis in male F344 rats. Starting at 5 weeks of age, male F344 rats were fed with diets containing juglone (200 ppm) or the control diet (without the compound). At 6 weeks of age, all animals were concurrently treated with subcutaneous (sc) injections of azoxymethane (AOM) (15 mg/kg body weight, once weekly for 3 weeks). Animals fed juglone diets were changed to the control diet 1 week after the last carcinogen treatment. They observed that the incidence and multiplicity of tumors in the small intestine (7 % and 0.07 ± 0.25) and the multiplicity of tumors in the entire intestine (0.60 ± 0.76) of animals treated with juglone plus the carcinogen were significantly less than those of animals treated with carcinogen alone ($P < 0.05$). Based on these data, the authors were of the opinion that juglone could be a promising chemopreventive agent for human intestinal neoplasia.
More recently, the anti-tumor effect and mechanism of juglone was evaluated \textit{in vivo} on the mice xenotransplant model of sarcoma 180. Juglone treatment resulted in typical ultrastructural changes in the morphology of tumor cells that resemble apoptosis (as observed by transmission electron microscopy). Also, juglone treatment caused a decrease in the percentage of G1 phase cells along with an increase in the G2/M phase cells. The authors concluded that G2/M phase cycle arrest and apoptosis play important roles in the anti-tumor effect of juglone (Ji \textit{et al.}, 2009).

On the contrary, juglone was found to promote 7,12-dimethylbenz[a]anthracene (DMBA)-initiated skin carcinomas and papillomas in Sencar mice when applied dermally at 440, 880 or 1760 nmol/mouse once a week for 40 weeks. Tumor incidence and tumor multiplicity were both dose dependent. Several other structurally related quinones were also examined, and a good correlation between the ability to induce epidermal ornithine decarboxylase and the ability to behave as a tumor promoter was noted (Monks \textit{et al.}, 1990).

Likewise, in another study, juglone treatment (dermal application of juglone at a dose of 62 µg thrice weekly for 52 weeks) was found to promote skin tumors in female ICR/Ha Swiss mice (30 per group) pretreated with a subcarcinogenic dose of DMBA (20 µg single treatment). However, skin tumors were not observed in mice treated with the same regimen of juglone alone (without pretreatment with initiator - DMBA) (Van Duuren \textit{et al.}, 1978).

Based on the existing literature, the uncertainty regarding the potential of juglone as an anticancer agent is clearly evident. Apart from the potent pharmacological effects, juglone is known to cause some toxicity to normal tissues as well including acute contact irritant dermatitis (Neri \textit{et al.}, 2006).

Therefore, in the present study, an attempt was made to evaluate the \textit{in vitro} cytotoxic potential of juglone against cell lines of murine and human origin and to elucidate its underlying mechanisms. Given that the structure of juglone resembles that of plumbagin, we also aimed to evaluate the radiosensitizing potential of juglone against a chemo- and radio-resistant B16F1 melanoma cell using both \textit{in vitro} as well
as *in vivo* tumor models. In addition, the present investigation was intended to formulate juglone as sterically stabilized liposomal forms aiming enhanced antitumor efficacy and reducing the toxicity associated with juglone.

**AIMS & OBJECTIVES OF THE STUDY**

- To study the cytotoxic potential of juglone *in vitro* against a panel of human and murine tumor cell lines and to understand the mechanisms underlying the cytotoxic potential against B16F1 melanoma cells grown *in vitro*.

- To evaluate the *in vivo* anticancer and radiosensitizing potential of juglone against B16F1 melanoma cells grown as solid tumor on C57BL/6J mice and also to evaluate the radiosensitizing potential of juglone against B16F1 melanoma cells grown *in vitro*.

- To formulate, characterize and optimize sterically stabilized liposomes of juglone with an aim of improving the anticancer efficacy and to reduce the toxicity associated with juglone.

- To comparatively evaluate the optimized sterically stabilized liposomal formulation of juglone against free juglone for the pharmacokinetic, biodistribution, pharmacodynamic as well as toxicity profiles of juglone.
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