CHAPTER- 1

Introduction

1.1. Cancer

Cancer can be generally described as an uncontrolled growth and spread of abnormal cells in the body. Cancer is not a single disease. It is a group of more than 200 different diseases. The word ‘Cancer’ comes from the Latin word ‘Carcinoma’ meaning crab. Cells are basic units of life. All organisms are composed of one or more cells. Normally, cells divide to produce more cells only when the body needs them. Sometimes cells keep dividing and thus creating more cells even when they are not needed. When this happens; a mass of tissue forms. This mass of extra tissue is called a tumor. During the early years of a person’s life, normal cells divide faster to allow the person to grow. After the person becomes an adult, most cells divide only to replace worn-out or dying cells or to repair injuries. Cancer begins when cells in a part of the body start to grow out of control. There are many kinds of cancer, but they all start because of out-of-control growth of abnormal cells. Once this change takes place, the set of instructions in the gene is changed and the cell becomes abnormal which no longer acts like it normally does. Cancer is actually due to the accumulation of many such errors. Life-threatening cancer develops gradually as a result of a complex mix of factors such as complex interactions of viruses, a person’s genetic make-up, their immune response and their exposure to other risk factors which may favor the cancer. The notion of cancer as a serious, life-threatening disease must be very ancient; and probably for a long time different cultures have speculated that both external and internal factors play a role in the cause of cancer.

Tumors are usually classified as simple (or benign) and malignant (cancer). Benign tumors tend to remain localized are often surrounded by a
capsule and rarely give rise to serious effects. Malignant tumors, on the other hand, do not remain localized but invade other tissues and give rise to secondary tumors (metastases) in other parts of the body, through the bloodstream or lymphatic system. Cells become cancer cells because of damage to DNA. DNA is in every cell and directs all its actions. In a normal cell, when DNA gets damaged the cell either repairs the damage or the cell dies. In cancer cells, the damaged DNA is not repaired, but the cell doesn’t die like it should. Instead, this cell goes on making new cells that the body does not need. These new cells will all have the same damaged DNA as the first cell does.

1.2. Different kinds of cancer

Cancer can originate almost anywhere in the body, and are of different kinds:

- **Carcinomas**: These cancers originate in the epithelium. The epithelium is the lining cells of an organ. Carcinomas are the most common type of cancer. Common sites of carcinomas are the skin, mouth, lung, breast, stomach, colon and uterus (Richard, 1988).

- **Sarcomas**: Sarcomas are cancers of connective and supportive tissues (soft tissues) of all kinds. Sarcomas can be found anywhere in the body, and they often form secondary growths in the lungs. They are 2% of all tumors (Richard, 1988; Anthony and Peter, 1997).

- **Lymphomas**: These cancers arise in the lymph nodes and tissues of the body's immune system. They are 4% of all tumors (Jeffrey, 1990).

- **Leukemias**: Leukemias are cancers of the immature blood cells that grow in the bone marrow and tend to accumulate in large numbers in the bloodstream. They are 4% of all tumors (Alberts et al., 1989).
1.3. Epidemiology of cancer

The International Agency for Research on Cancer (IARC), the specialized cancer agency of the World Health Organization, released the latest data on cancer incidence, mortality and prevalence worldwide (Table 1.1). The new version of IARC’s online database, GLOBOCAN 2012, provides the most recent estimates for 28 types of cancers in 184 countries worldwide and offers a comprehensive overview of the global cancer burden.

**Table 1.1 All Cancers Estimated Incidence, Mortality and Prevalence Worldwide in 2012:**

<table>
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<tr>
<th>Estimated numbers (thousands)</th>
<th>Both sexes</th>
<th>5-year prevalence</th>
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<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Deaths</td>
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<tr>
<td>World</td>
<td>14090</td>
<td>8202</td>
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<tr>
<td>More developed regions</td>
<td>6076</td>
<td>2878</td>
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<tr>
<td>Less developed regions</td>
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<td>WHO Africa region (AFRO)</td>
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<tr>
<td>WHO Americas region (PAHO)</td>
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<tr>
<td>WHO Europe region (EURO)</td>
<td>3737</td>
<td>1933</td>
</tr>
<tr>
<td>WHO South-East Asia region (SEARO)</td>
<td>1724</td>
<td>1171</td>
</tr>
<tr>
<td>WHO Western Pacific region (WPRO)</td>
<td>4543</td>
<td>2978</td>
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<tr>
<td>IARC membership (24 countries)</td>
<td>7060</td>
<td>3470</td>
</tr>
<tr>
<td>United States of America</td>
<td>1604</td>
<td>617</td>
</tr>
<tr>
<td>China</td>
<td>3065</td>
<td>2206</td>
</tr>
<tr>
<td>India</td>
<td>1015</td>
<td>683</td>
</tr>
<tr>
<td>European Union (EU-28)</td>
<td>2657</td>
<td>1276</td>
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Global burden rises to 14.1 million new cases and 8.2 million cancer deaths in 2012. According to GLOBOCAN 2012, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008. Prevalence estimates for 2012 show that there were 32.6 million people (over the age of 15 years) alive who had a cancer diagnosed in the previous five years. Projections based on the GLOBOCAN 2012 estimates predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and ageing of the global population. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in less developed regions of the world, and these proportions will increase further by 2025. Part of this growth in absolute numbers derives from the ageing of populations worldwide. The cancer epidemic in high income countries and increasingly in low and middle-income countries is also due to high or increasing levels of prevalence of cancer risk factors (Stewart and Kleihues, 2003).

1.4. Aetiology of cancer

The cancer could be due to physical (e.g., radiation), chemical (carcinogens) and viral agents (papilloma virus or EB virus acting through oncogenes). Many mechanisms have been postulated in the process of chemical carcinogenesis. Certain chemicals undergo biotransformation to reactive metabolites, which can form adduct with DNA resulting in subsequent mutations. Free radical generation which can damage DNA has also been projected for radiation and chemical carcinogenesis. Oxygen free radicals play an important role in the initiation as well as promotion phase of carcinogenesis. The toxic activity of free radical and peroxides can be counteracted to some extent by the cellular antioxidant defense system present in the body. Superoxide dismutase, catalase and glutathione peroxidase are considered as the primary antioxidant enzymes being
involved in the direct elimination of active oxygen species. Glutathione-S-transferases, Glutathione reductase and glucose-6-phosphate dehydrogenase are secondary antioxidant enzymes. Antioxidant enzymes, which help in detoxification of reactive oxygen species by decreasing peroxide levels or by maintaining a steady supply of metabolic intermediates like glutathione and NADPH for the primary antioxidant enzymes (Palan et al., 1991).

It is estimated that around 43% of cancer deaths are due to tobacco use, unhealthy diets, alcohol consumption, inactive lifestyles and infection. Of these, tobacco use is the world’s most avoidable cause of cancer. In addition to lung cancer, tobacco consumption causes cancer of the oral cavity, pharynx, larynx, oesophagus, stomach, pancreas, liver, kidney, ureter, urinary bladder, uterine cervix and bone marrow (myeloid leukaemia). Exposure to environmental tobacco smoke (passive smoking) also increases lung cancer risk. Tobacco use and alcohol consumption act synergistically to cause cancer of the oral cavity, pharynx, larynx and oesophagus.

Among all types of cancers, oral cancer (OC) is a serious and growing problem in many parts of the globe. Oral and pharyngeal cancer, grouped together, is the sixth most common cancer in the world (Warnakulasurya, 2010). The annual estimated incidence is around 2,75,000 for oral and 1,30,300 for pharyngeal cancers excluding nasopharynx, two thirds of these cases occurring in developing countries (Warnakulasurya, 2010).

1.5. Oral cancer

Oral cancer is usually defined as a neoplastic disorder in the oral cavity, which includes the following areas: lip, buccal mucosa, lower and upper alveolar ridges, retromolar gingiva, oropharynx, floor of the mouth, hard palate and the anterior two thirds of the tongue (Collins, 1996) (Fig. 1.1). About 2% of all malignancies that can occur in the body arise in the oral cavity. In some areas of the world this percentage is higher. The majority of
malignancies consist of squamous cell carcinomas of the covering oral mucosa, while the remaining include malignant tumors of salivary gland, lymphoreticular disorders, bone tumors, malignant melanomas, sarcomas, malignant odontogenic tumors and metastases from tumors elsewhere in the body. Nearly 10% of malignant have their primary site in oral cavity, and it is the sixth leading cause of human cancer worldwide (Warnakulasurya, 2010). Oral cancer might be considered as the most common cancer in head neck region affecting predominantly male with 75% of diagnosed cases around 60 year old, to which 90% are oral squamous cell carcinoma.

![Anatomy of the oral cavity and oropharynx.](image)

Fig. 1.1 Anatomy of the oral cavity and oropharynx. (A) Open-mouth view, (B) Tongue elevated, showing floor of mouth. (Adapted from Abeloff et al., 2008)
1.6. Types of oral cancer

There are several types of malignant oral cancers; more than 90 percent of all diagnosed oral cancers are squamous cell carcinoma.

1.6.1. Squamous cell carcinoma

This type of cancer originates in the squamous cell layer in the lining of the oral cavity and oropharynx. In the early stages, this cancer is present only in the lining layer of cells (called carcinoma *in situ*). When the cancer spreads beyond the lining, it is called invasive squamous cell carcinoma.

Oral squamous cell carcinoma (OSCC) is characterized by multiple genetic alterations that result in clinically known malignant neoplasm. The accumulation of damaged genetic material leads oral keratinocytes in an uncontrolled division of mutant cells. OSCC is a multiple disease and variety of epigenetic and genetic changes have been correlated to malignant transformation of potentially malignant oral lesions. Several genetic alterations have been identified related to OSCC and can be used as diagnostic markers.

1.6.2. Verrucous carcinoma

It is also considered a type of squamous cell carcinoma and this low-grade cancer, rarely metastatic (spreads to distant sites). Comprising less than 5 percent of all diagnosed oral cancers, verrucous carcinoma can spread deeply into surrounding tissues, requiring surgical removal with a wide margin of surrounding tissues.

1.6.3. Minor salivary gland cancers

The lining of the oral cavity and oropharynx contains numerous salivary glands. Sometimes cancer will originate in a salivary gland. Treatment depends on the type and location of the salivary gland cancer as well as the extent of spreading. According to the American Cancer Society,
salivary gland cancers account for less than 1 percent of all cancers (American Cancer Society, 2009).

1.7. Symptoms and signs

- Red, white or red and white patches in the mouth (white patches are most common)
- Changes in the soft tissues of the mouth, including lumps, swelling, crusting and eroded tissue
- Mouth sores that do not heal within two weeks
- Bleeding from the mouth
- Pain or tenderness in the face, mouth or neck
- Persistent ear pain
- Facial numbness
- Difficulty chewing, swallowing of sneaking
- Chronic sore throat
- Hoarseness
- Loose or shirting teeth or a change in the dentures fit
- Unexplained weight loss.

1.8. Epidemiology of oral cancer

Oral cancer constitutes a major health problem being the sixth most common human cancer worldwide, with an incidence of more than 3,00,000 cases annually, with variations between countries and geographical areas (Tanaka et al., 2011). The annual estimated incidence is around 2,75,000 for oral and 1,30,300 for pharyngeal cancers excluding nasopharynx, two-thirds of these cases occurring in developing countries. There is a wide geographical variation in the incidence of oral cancer, with approximately two-thirds of patients in the developing countries of Southeast Asia, Eastern Europe and Latin America (Curado et al., 2009). In India, the gingival–buccal complex (alveolar ridge, gingival- buccal sulcus, buccal mucosa) forms the most
common subsite for cancer of the oral cavity, in contrast to cancer of the tongue that is more common in the western world (Pathak et al., 2005). India has one of the highest incidences of oral cancer (age-standardized rate of 9.8 per 10 000) making it the most common cancer among men (men: women ratio 2:1) and accounts for about 30% of all new cases annually (Sankaranarayanan et al., 2005). A recent survey of cancer mortality in India shows cancer of the oral cavity as the leading cause of mortality in men and responsible for 22.9 % of cancer-related deaths (Dikshit et al., 2012). There is a trend towards increasing incidence and delayed presentation of oral cancer (about 60% patients present at stage III or IV) (Lingen et al., 2008). The Indian national cancer registry data show an increasing incidence as per age. However, the incidence among women is lower than among men. This can be related to differences in lifestyle and behavioral pattern between the two genders (Thorat et al., 2009).

The areas characterized by high incidence rates for oral cancer (excluding lip) are found in the South and Southeast Asia (e.g. Sri Lanka, India, Pakistan and Taiwan), parts of Western (e.g. France) and Eastern Europe (e.g. Hungary, Slovakia and Slovenia), parts of Latin America and the Caribbean (e.g. Brazil, Uruguay and Puerto Rico) and in Pacific regions (e.g. Papua New Guinea and Melanesia) (Fig. 1.2). Scanning through the information, it was possible to extract data either for entire populations or for sub-populations living in some geographic areas. For some countries with several data sets from several regions, two highest rates for the population are extracted. In high-risk countries such as Sri Lanka, India, Pakistan and Bangladesh, oral cancer is the most common cancer in men, and may contribute up to 25% of all new cases of cancer. On a visit to a cancer treatment centre in any of these high-risk countries in South Asia, one may find that at least up to a quarter of the patients warded are suffering from oral cancer. The overall 5-year
survival rate for all stages of oral cancer is 60%. These rates are better for localized tumors (82.8%) as compared to tumors with regional (51.8%) or distant metastases (27.8%) (Warnakulasuriya, 2009).

Fig. 1.2 Countries with high incidence and mortality from oral cancer

**1.9. Aetiology risk factors for oral cancer**

Oral cancer is a multifactorial disease. Exposure to one of three broad groups of carcinogenic stimuli, namely, chemical, physical and viral, is known to induce cancer in genetically and systemically conditioned oral mucosa. Within the oral cavity, it appears that carcinomas are caused predominantly by chemical carcinogens, although evidence implicating viral and physical stimuli in the development of some oral cancers continues to mount (Syrjanen, 2005; Reddout et al., 2007). The pathogenesis of oral cancer is equally complex and exposure to carcinogens does not inevitably result in the development of oral cancer. This is because a number of familial, dietary, hormonal and sex-related factors are known to modulate neoplastic
processes generally. Tobacco and alcohol have emerged as the most important culprits contributing to the aetiology of oral cancers. Other factors frequently cited are ultraviolet light, nutritional and dietary factors, precancerous lesions, immunosuppression, genetic and dental factors.

1.9.1. Tobacco

Smoking and tobacco use are considered the strongest risk factors for oral cancer. Cigarettes, cigars or pipes, tobacco chewing, and dipping snuff are all linked to oral cancer. The uses of other tobacco products also increase the risk of oral cancer. The major carcinogen in tobacco belongs to the family of nicotine derived nitrosoamines collectively called “tobacco specific nitrosamines”. Polynuclear aromatic hydrocarbons such as Benzo[a]pyrene and aromatic amines are the other carcinogens found in tobacco.

1.9.2. Betel quid and Areca nut

Betel chewing is reported to be the most important etiological factor in oral mucous fibrosis. The use of betel quid, containing both areca nut and associated with a much higher relative risk of oral cancer.

1.9.3. Alcohol

Alcohol exerts an independent effect on risk for oral cancer and acts synergistically with tobacco use to increase risks dramatically. The effect of alcohol may be direct e.g N-nitroso compounds, mycotoxins, tannins, aldehydes, among others, as well as systemic. Intermediate metabolites of alcohol, such as acetaldehyde, may be more toxic than the direct action of alcohol on the mucosa. Additionally, the effects of alcohol may be mediated through the production of prostaglandin, lipid peroxidation and the generation of free radicals mediated reactive oxygen species, both may induce specific DNA mutations and cancer. Several studies were reported that alcohol is the major risk factor for oral cancer.
1.9.4. Diet and nutrition

There is a strong epidemiological evidence for a protective effect of fruits and vegetables against most of the important human cancers. Diet high in vegetables, fruits, tea and fiber decreases the risk of oral and pharyngeal cancers because these nutrients can prevent the activation of carcinogens and increase their detoxification. Micronutrients like vitamin C, E, P-carotene, folate and Zinc have important role in prevention of oral cancer. These factors can cause polymorphism in detoxifying enzyme GST and other metabolic genes which modulate the risk of cancer and decrease the genotoxic damage. According to WHO reports 35-55% of human cancers and approximately 15% of oropharyngeal cancers can be attributed to dietary deficiencies or imbalance. Several studies were reported the imbalance of fruits and vegetables to oral cancer.

1.9.5. Free radicals

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) can function both as initiators and promoters in carcinogenesis, while antioxidants provide protection against the cellular and molecular damage caused by reactive oxygen species and reactive nitrogen species (Rasheed et al., 2007). The increase in ROS and RNS may have been the event that led to the consumption and reduction of salivary antioxidant systems, thus explaining the oxidative damage to the DNA and proteins, and possibly the promotion of oral cancer (Bahar et al., 2007).

1.9.6. Human papilloma viruses (HPVs)

Human papilloma viruses (HPVs) are the major causes of cervical cancer, a common neoplasm in women from developing countries and may be related to oral, pharyngeal and laryngeal cancer (Levi et al., 1998). HPVs are sexually transmitted and can infect genital and mucous membranes. Some of the genital mucosal type HPVs causes cervical cancer in women.
Approximately 20 million people in the United States are infected with genital HPVs and 5.5 million new infectious occur each year.

1.9.7. Irritation

Although it has been suggested that chronic irritation to the lining of the mouth from poorly fitting or defective complete dentures may be a risk factor for oral cancer, the majority of studies have shown no correlation (Rosenquist et al., 2005).

1.9.8. Dental plaque

Polymicrobial supragingival dental plaque is a possible independent risk factor, as it possesses a relevant mutagenic interaction with saliva, and thus oral health may be a cofactor in the development of oral cancer (Bloching et al., 2007).

1.10. Molecular pathogenesis of oral cancer

Oral carcinogenesis like any other cancer is a progressive disease and normal epithelium passes through stages starting from dysplasia to finally transforming into invasive phenotypes. Although all types of carcinomas are seen in oral cavity, the most common form of OC is squamous cell carcinoma. Use of genetic and proteomic approach in recent years have revealed the molecular pathological picture of OC. There is active search to identify genetic alterations in oncogenes or tumor suppressor genes, role of genomic instability and epigenetic modifications and to generate a gene expression profile in oral oncogenesis (Williams, 2000). Understanding these genetic changes and gene expression patterns are keys to the understanding of molecular pathogenesis of OC. Though, there are some significant leads achieved, the complete understanding of molecular pathology of OC and its association with causative agent will require another decade of intensive research.
1.11. Cancer treatment techniques

Treatment of oral cancer depends on the type of cancer and the stage of the cancer. Currently, there are numerous techniques that are used for cancer treatment. But each technique has its own limitations and adverse effects (Burish and Tope, 1992; Schwartz, 1995). Surgical treatment (excision of the tumor) is usually the first choice of treatment preferred by physicians. However, surgical excision is not effective when the cancer cells have infiltrated the nearby vital organs or have spread to distant parts of the body (metastasis). Surgical excision is preferred for the removal of larger tumors. Cryosurgery is another surgical technique that is used for freezing and killing the tumor cells. It is an alternative to surgical excision and it is used to treat tumors that have not spread to distant organs and to treat pre-cancerous or non-cancerous lesions. Another technique of cancer treatment is radiation therapy, which uses radiation energy to destroy cancer cells and reduce the size of tumors. Radiation is more harmful for cancerous cells than for normal cells because cancerous cells are more unstable in all respects and thus more vulnerable to the damaging radiations. However, the cellular repair mechanism is also not prominently active in the highly dividing cells like cancer cells. However, due to proper functionally active cellular repair mechanism normal cells can recover from the effects of radiation more easily. Radiation therapy equipment is expensive and the treatment expense is higher. The time period of the radiation therapy normally takes 1 to 2 months. Radiation cause more complications and even loss of function. Radiation treatment is not good and cannot completely eradicate cancer cells, cancer cells in a certain period of time will be long. Chemotherapy is the treatment of cancer with anticancer drugs. These drugs are used as pills, intravenous injections, or topical applications. Chemotherapeutic drugs may destroy healthy tissues along with cancer cells and carcinomatous tissues.
(cytotoxicity). The cytotoxic effect of chemotherapeutic drugs is highest in bone marrow, gonads, hair follicles and digestive tract, all of which contain rapidly proliferating cells. The adverse effects of chemotherapy include fatigue, nausea, vomiting, alopecia (loss of hair), gastrointestinal disturbance, impaired fertility, impaired ovarian function and bone marrow suppression resulting in anemia, leucopenia and thrombocytopenia (Tormey et al., 1986; Barton and Waxman, 1990).

Targeted cancer therapy uses target-specific drugs that invade cancer cells and block the growth and metastasis of cancer cells by interfering with specific molecules involved in carcinogenesis and tumor growth (Kim, 2003). To overcome the disadvantages of current cancer treatment techniques, the scientific community has turned toward nanotechnology to develop newer and more effective drug carrier systems to safely shepherd the anticancer drugs to the cancer cells.

1.12. Oral cancer prevention

Oral cancer is sometimes associated with known risk factors for the disease. Many risk factors can be modified but not all can be avoided.

- **Tobacco and alcohol use:** Tobacco use (cigarettes, pipes, cigars and smokeless tobacco) is responsible for most cases of oral cancer. Alcohol, particularly beer and hard liquor, are associated with an increased risk of developing oral cancer. The risk of developing oral cancer is higher in people who use both tobacco and alcohol. Avoiding or stopping the use of tobacco decreases the risk of oral cancer. It is not known if stopping the use of alcohol decreases the risk of oral cancer.

- **Sun exposure:** Exposure to sunlight may increase the risk of lip cancer, which occurs most often on the lower lip. Avoiding the sun and/or using a sunscreen or colored lipstick on the lips may decrease the risk of lip cancer.
- **Other factors**: Some studies suggest that being infected with the human papillomavirus (HPV) may increase the risk of oral cancer.

- **Chemoprevention**: Chemoprevention is the use of drugs, vitamins or other agents to prevent or delay the growth of cancer or to keep it from coming back. Tobacco users who have had oral cancer often develop second cancers in the oral cavity or nearby areas, including the nose, throat, vocal cords, esophagus and windpipe. Studies of chemoprevention in oral cancer are under way, including chemoprevention of leukoplakia and erythroplakia.

### 1.13. Chemoprevention

Chemoprevention may involve perturbation of a variety of steps in tumor initiation, promotion and progression. Numerous potential mechanisms have been described and attempts have been made to broadly classify agents according to the effects they have on different stages of carcinogenesis (De Flora and Ferguson, 2005). The concept of preventive strategies to interrupt these processes has generated a great deal of interest. Chemoprevention, a term first used by Sporn and colleagues, (Sporn et al., 1976) is defined as the use of drugs or other natural, synthetic or biologic agents to inhibit, delay or reverse the stepwise carcinogenic progression to invasive cancer. Chemoprevention studies use these fundamental premises to identify alterations or biomarkers to serve as intermediate endpoints in the trials. Primary chemoprevention refers to the use of an agent that prevents carcinogenesis in healthy individuals who are at high risk. Secondary chemoprevention refers to preventing the full transition to malignancy in a patient who already has developed a pre-malignant lesion. Tertiary chemoprevention refers to the use of an agent that prevents a second primary cancer or metastasis in a patient who had a first malignancy that has been treated.
Accumulating epidemiological and experimental studies suggest that a high consumption of fruits and vegetables and the intake of certain non-nutrients that are present in foods reduce the risk of different cancers (Surh, 2003; Ramos, 2008). Therefore, the identification of dietary components as potential cancer chemopreventive agents in the form of functional foods or nutraceuticals has become an essential subject for study in current research. This is the case for polyphenols, natural dietary compounds present in fruits and vegetables, which have attracted a great deal of interest because of their potential ability to act as highly effective chemopreventive agents (Ramos, 2008). More than 1000 potential chemopreventive agents have been identified in dietary sources and many are being tested in vitro and in vivo systems with a variety of cancer types. Identification and testing of a successful chemopreventive agent is a long process, requiring in vitro studies, animal efficacy and toxicity studies and eventually lengthy human clinical trials.

An ideal chemopreventive agent must have (i) little or no toxicity, (ii) a high efficacy in multiple sites, (iii) the capability for oral consumption, (iv) a known mechanism of action, (v) low cost and (vi) human acceptance (Rajamanickam and Agarwal, 2008). Currently, natural products, especially the antioxidants present in common food and beverages, have obtained great attention for cancer prevention owing to their various health benefits, noticeable lack of toxicity/side effects and the limitations of other chemotherapeutic agents (Manson et al., 2005). Several studies have shown that natural products composed of a wide spectrum of biologically active phytochemicals, including phenolics, flavonoids, carotenoids, alkaloids and nitrogen; suppress the early and late stages of carcinogenesis (Nishino et al., 2007).
1.14. Cancer cell lines as a model for cancer study

Cancer cell lines have been widely used for research purposes and proved to be a useful tool in the genetic approach, and its characterization shows that they are, in fact, an excellent model for the study of the biological mechanisms involved in cancer (Louzada et al., 2012). The use of cancer cell lines allowed an increment of the information about the deregulated genes and signalling pathways in this disease (Vargo-Gogola and Rosen, 2007). Furthermore, the use of the cell model was in the origin of the development and testing of anticancer drugs presently used (Nakatsu et al., 2005; Ruhe et al., 2007; Gazdar et al., 2010), and in the development of new therapies (Gazdar et al., 2010; Pfragner et al., 2009; Louzada et al., 2012), but also as an alternative to transplantable animal tumors in chemotherapeutics testing (Shoemaker, 2006). The major advantages of cell lines are:

- Pure population of tumor cells.
- Possibility of wide distribution to investigator worldwide.
- Limited replicative ability.
- Identification and testing of conventional and novel therapeutic approaches.
- Growth as substrate dependent or substrate independent cells.
- Ability to utilize a single passage repeatedly.
- Ability for clonal selection.
- Development of model to study multistage pathogenesis.

1.15. In vivo evaluation by animal models

In vivo animal models are applied to investigate the effects and efficacy of specific delivery system. The effectiveness of a chemopreventive agent can be confirmed in a target organ-specific manner in chemically-induced carcinogenesis models. These experiments provide information not available
in human populations as they are adequate for hazard identification, dose-response modelling, exposure assessment and risk characterization which are the four required steps for quantifying the estimated risk of cancer development associated with toxic chemical exposure. Regardless of a new generic drug or nanoparticle formulation for drug delivery, the activity of a formulation tested against *in vitro* models is sometimes unable to be used to predict its fate in *in vivo* model, not to mention clinical cancers (Johnson et al., 2001). This is due to the much more sophisticated mechanisms that take place within the body compared to the simplified *in vitro* cell line models. Therefore, research involving animals has been essential to evaluate the formulation for efficacy and safety before proceeding to human trials. The main animal models of carcinogenesis using chemical agents, probably because the chemical constituents of tobacco and alcohol were quickly identified as possibly responsible for most human OSCC (Mognetti et al., 2006). Experimental animal models that accurately represent the cellular and molecular changes associated with the initiation and progression of human cancer, are of crucial importance, although some are more suited for certain applications than others. They have many intrinsic advantages for cancer research and for new therapeutic approaches, increasing their value.

Experimental carcinogenesis models are valuable tools to investigate the multistep characteristics of carcinogenesis and to study various modulations that intervene in the development of cancer. These models also have many advantages over simple *in vivo* studies.

**1.16. 7,12-dimethylbenz[a]anthracene (DMBA)**

The classical carcinogens are polycyclic aromatic hydrocarbons (PAHs) generated from the combustion of fossil fuels, and aromatic amines, present in cigarette smoke (Lee et al., 2002). DMBA is a potent and site specific carcinogen (PAH) is metabolically activated by CYP1A1 and CYP1B1
monooxygenase to form electrophilic metabolites such as phenols, diols, diol epoxide and tetrols. The diol epoxide derivatives of DMBA are capable of binding covalently to adenine residues of DNA forming DNA adducts that may eventually culminate in neoplastic transformation. However, subsequent conjugation by phase II enzymes also plays a key role in determining the carcinogenicity of these metabolites (Fig. 1.3).

Fig. 1.3 Activation of DMBA leading to the formation of DNA adducts

1.17. The hamster buccal pouch (HBP model)

Syrian golden hamster (*Mesocricetus auratus*) has become a widely used animal cancer model for the chemical induction of buccal squamous cell carcinoma. This model has been used extensively over the past six decades to study the pathogenesis of oral cancer (Smith and Thomas, 2006). The hamster oral cancer model was developed in 1954 by Sally and co-workers (Eveson, 1981). They reported that application of 7,12-dimethylbenz[a]anthracene (DMBA) three times per week for fourteen weeks, led to the development of tumor in their buccal cavity. In mammalian cells, polycyclic aromatic hydrocarbons are bioactivated through the formation of reactive diolepoxide metabolites that subsequently bind to
adenine and guanine residues in DNA and form adducts (Shklar, et al., 1979; Dipple, et al., 1983; Dipple, et al., 1984). Adduct formation has been implicated in the carcinogenic mechanism of polycyclic aromatic hydrocarbons (Joyce and Daniel, 1982; Bigger et al., 1983). During malignant transformation, varying degrees of hyperkeratosis, basal cell hyperplasia, cellular pleomorphism and nuclear hyperchromatism are observed within 3-9 weeks of DMBA application. In the final stages, exophytic and/or endophytic squamous cell carcinomas are detected in hamster buccal pouch mucosa that have been topically treated with the carcinogen DMBA for 10-14 weeks. A typical golden Syrian hamster is shown in Fig. 1.4.

The major advantage of this model is its similarity in histopathological, molecular and biochemical alterations to human oral cancer, such as changes in p53 and ras genes, expression of glutamyl transpeptidase and overexpression of growth factors like epidermal growth factor (Mognetti et al., 2006). The other advantages include easy accessibility to the cheek pouch, lack of a requirement for anesthesia during animal treatment, simplicity of application and large amount of sample available for various analysis. It has also been used to test the chemopreventive activity of various agents.
1.18. Flavonoids

Flavonoids are a large class of plant secondary metabolites belonging to the wider family of natural polyphenols. They are present in plant-derived food and beverages, such as fruits, vegetables, cereals, legumes, cocoa, olive oil, tea, coffee and wine, thus comprising an important constituent of the human diet. Flavonoids are a group of more than 4000 polyphenolic compounds that occur in nature in plant foundation. These compounds acquire a common phenyl-benzopyrone arrangement (C$_6$-C$_3$-C$_6$), (Fig. 1.5) and they are categorized according to the saturation level and opening of the fundamental pyran ring, mainly into flavones, flavanols, isoflavones, flavonols, flavanones and flavanonols. In recent years, researches about flavonoids are becoming the area under discussion of therapeutic research and flavonoids represent one of the most interesting groups of biologically active compounds. Flavonoids compounds have an amazing variety of biological activity lying on cancer prevention. These comprise, for instance, antimutagenic and anti-carcinogenic activities (Craig, 1999; Middleton et al., 2000; Yang et al., 2001). Flavonoids are concerned in the regulation of cell proliferation and anticancer properties (Kuntz et al., 1999; Wenzel et al., 2000). Flavonoids have been shown as angiogenesis inhibitors derived from natural sources (Paper, 1998; Marais et al., 2006). Therefore, flavonoids compounds may have potential in support of the cure of solid tumors (Kioka et al., 1992; Tosetti et al., 2002).

Flavonoids might act as antioxidants by preventing the DNA damage that can be induced by free radicals or carcinogens, thus blocking the initiation step of cancer (Urquiaga and Leighton, 2000). In vitro and in vivo studies have highlighted the direct and indirect anticarcinogenic effects of flavonoids, which they exercise by quenching oxidative stress and inflammatory response, inducing apoptosis, suppressing mitochondrial
membrane potential (MMP) secretion, inhibiting cell growth, cell invasion and angiogenesis (Kandaswami et al., 2005; Soobrattee et al., 2006; Chen et al., 2008; Rossi et al., 2008; Filip et al., 2009; Ravichandran et al., 2011). A number of flavonoids have been shown to suppress carcinogenesis in various animal models (Yang et al., 2001).

![Basic structure of flavonoid molecule](image)

**Fig. 1.5 Basic structure of flavonoid molecule**

1.19. Silibinin

Silibinin \((\text{C}_{25}\text{H}_{22}\text{O}_{10}, \text{molecular weight, 482.44})\) is isolated from the seeds of *Silybum marianum* (L.) Gaertn (family Asteraceae), which is also known as milk thistle (Kiruthiga et al., 2010; Duan et al., 2011). Milk thistle extracts have been used for centuries in traditional medicine, and they are widely consumed herbal remedies with several putative beneficial effects on health, such as hepatoprotective properties. Silibinin, the major biologically
active compound of milk thistle is a polyphenolic flavonoid, and a strong antioxidant and radical scavenger. The standardized milk thistle extract contains approximately 70-80% of the defined flavonoids and flavonolignans (together known as “silymarin”) and approximately 20-30% chemically undefined fractions, compromising mostly of polyphenols and aliphatic fatty acids. Silibinin is the main component of silymarin complex and constitutes about 50–60% of it depending upon the formulation. The International Union of Pure and Applied Chemistry name for silibinin is (2R, 3R)-3,5,7-trihydroxy-2- [(2R,3R) -3- [4-hydroxy-3-methoxyphenyl] -2- [hydroxymethyl] 2, 3 dihydrobenzo[b] dioxin-6-yl) chroman-4-one. Silibinin is well tolerated and largely free of any adverse effects. Silibinin is non-toxic in acute, sub-chronic and chronic tests even at large doses, and there is no known LD\textsubscript{50} for silibinin in animal studies (Hahn et al., 1968; Flora et al., 1998). The anticancer efficacy of silibinin is clearly evident from the published reports against various cancers including prostate, skin, lung, colon, breast, hepatic, ovarian, cervical, kidney, gastric carcinoma, and the underlying mechanisms are very different in different cancer cells (Deep and Agarwal, 2010). In vitro and in vivo studies have shown that silibinin is a potent sensitizer for apoptosis induced by a range of anticancer drug (Flaig et al., 2007; Sangeetha et al., 2010). Silibinin is known to have poor bioavailability because of two main reasons, namely, it has multiring structure (Fig. 1.6) that is too large to be absorbed by simple diffusion and that silibinin has poor miscibility with oils and other lipids, severely limiting its ability to cross the lipid-rich outer membrane of the enterocytes of the small intestine (Deep and Agarwal, 2010). Therefore, there have been several efforts to prepare silibinin formulations to increase its bioavailability.
1.20. Cancer Nanotechnology

Nanotechnology is an emerging, multidisciplinary field that frequently employs techniques and tools from diverse disciplines, including biology, engineering, chemistry and medicine. Nanotechnology is typically known as the study of the control of matter on an atomic and molecular scale, generally structures in the nanometer ($10^{-9}$ m) range, and involves developing materials or devices on that scale. The basic idea behind nanotechnology is that metal, semiconductor and polymeric nanoparticles have novel optical, electronic, magnetic and structural properties that are often not available from individual molecules and bulk solids (Niemeyer, 2001; Nie et al., 2007). In recent years, nanotechnology has been assessed and implemented in different areas of cancer management and therapeutics with the hope that it will lead to major advances in cancer diagnosis and treatment (Niemeyer, 2001; Ferrari, 2005; Cuenca et al., 2006; Nishiyama, 2007; Wang, 2008).
Most biological processes, including those that are cancer-related, occur on a nanometer scale, and thus nanoparticle technology has been greatly appreciated as a potential tool for cancer diagnosis and treatment, a field of science generally referred to as ‘cancer nanotechnology’. Cancer nanotechnology is emerging as a new field of interdisciplinary research cutting across the disciplines of biology, chemistry, engineering and medicine and it offers nanoparticles designed to target tumors and increase the solubility and bioavailability of attached drugs in order to administer novel therapies (McNeil, 2009). Scientists in this field seek to describe the relation of nanoscale materials and devices to cellular and molecular components specifically related to cancer. Nanoscale materials and devices with unique therapeutic properties can be engineered to deeply infiltrate tumors with a high level of specificity. Cancer nanotechnology is acknowledged by the National Cancer Institute, which considers that nanotechnology offers an extraordinary, paradigm-shifting opportunity to make significant advances in cancer diagnosis and treatment (Cuenca et al., 2006). The idea of crafting more effective cancer treatments by engineering matter at the nanoscale provides a compelling panacea for preferential elimination of cancer cells without serious damage to normal cells. Targeting cancer cell using nanoparticles loaded with anticancer agents is a promising tactic that could meet these challenges (Yih and Fandi, 2006).

1.21. Nanoparticulate-based drug delivery in cancer treatment

The critical step in cancer treatment is the detection of cancer at its initial stage of carcinogenesis. If the tumor has not been detected in its early stage, treatment methods should be devised to eradicate the fully developed cancer cells without harming the normal, healthy cells of human body. Results of numerous scientific research studies done in nanotechnology and nanomedicine are inspiring the scientific community to discover new,
innovative, non-invasive tools at the nano-scale level for such purposes because of its unique properties such as the small size, controlled release of drugs and reduced toxic side-effects. Nanomedicine refers to the research and development of technologies, devices and drug delivery systems for prevention, diagnosis and treatment of disease at the nano-scale. Delivering therapeutic compound to the target site is a major problem in treatment of many diseases. A conventional application of drugs is characterized by limited effectiveness, poor biodistribution and lack of selectivity (Nevozhay et al., 2007). These limitations and draw-backs can be overcome by controlling drug delivery. In controlled drug delivery systems (DDS) the drug is transported to the place of action, thus, its influence on vital tissues and undesirable side effects can be minimized. In addition, DDS protects the drug from rapid degradation or clearance and enhances drug concentration in target tissues; therefore, lower doses of drug are required (Nevozhay et al., 2007). This modern form of therapy is especially important when there is a discrepancy between a dose or concentration of a drug and its therapeutic results or toxic effects. Cell-specific targeting can be achieved by attaching drugs to individually designed carriers. Recent developments in nanotechnology have shown that nanoparticles (structures smaller than 200 nm in at least one dimension) have a great potential as drug carriers. Due to their small sizes, the nanostructures exhibit unique physicochemical and biological properties (e.g., an enhanced reactive area as well as an ability to cross cell and tissue barriers) that make them a favorable material for biomedical applications.

The whole system leads to a special function related to treating, preventing or diagnosing diseases sometimes called smart-drugs or theragnostics (La Van et al., 2003). The primary goals for research of nanobio-technologies in drug delivery include:
More specific drug targeting and delivery,
- Reduction in toxicity while maintaining therapeutic effects,
- Greater safety and biocompatibility and
- Faster development of new safe medicines.

The main issues in the search for appropriate carriers as drug delivery systems pertain to the following topics that are basic prerequisites for design of new materials. They comprise knowledge on (i) drug incorporation and release, (ii) formulation stability and shelf life, (iii) biocompatibility, (iv) biodistribution and targeting and (v) functionality. In addition, when used solely as carrier the possible adverse effects of residual material after the drug delivery should be considered as well. In this respect biodegradable nanoparticles with a limited life span as long as therapeutically needed would be optimal.

1.22. Importance of nanoparticles in drug delivery

When drugs are loaded into nanoparticles through physical encapsulation, adsorption or chemical conjugation, the pharmacokinetics and therapeutic index of the drugs can be significantly improved in contrast to the free drug counterparts. Nanostructures biomaterials and nanoparticles have unique physicochemical properties such as ultra small and controllable size, large surface area to mass ratio, high reactivity and functionalizable structure. Particle size and size distribution is one of the most widely accepted defining characteristic of nanoparticle-based medicines because size can significantly impact the pharmacokinetics, biodistribution and safety. The size of nanoparticles used in a drug delivery system should be large enough to prevent their rapid leakage into blood capillaries, but small enough to escape capture by fixed macrophages that are lodged in the reticuloendothelial system, such as the liver and spleen. The size of the sinusoid in the spleen and fenestra of the Kuffer cells in the liver varies from 150 to 200 nm and the size
of gap junction between endothelial cells of the leaky tumor vasculature may vary from 100 to 600 nm (Cho et al., 2008), therefore nanoparticles with size below 200 nm can take advantage of the EPR effect for enhanced drug accumulation in tumors (Haley and Frankel, 2008). The EPR effect refers to the accumulation of nanoparticles (NPs) in tumor facilitated by the highly permeable nature of the tumor vasculature and poor lymphatic drainage of the interstitial fluid surrounding a tumor. Particles will always exist in a range of sizes, therefore, size distribution must also be taken into account when designing a nanomedicine. Considering a normal size distribution, for the vast majority of particles to be below 200 nm in size, the mean nanoparticle may need to be well below 200 nm to confer the full “benefits” of a nanomedicine. Biological membranes and access cells, tissues and organs are eligible for entrance of nanoparticles. These cells are not crossed by the larger-sized particles easily (Rao et al., 2010) i.e. by conventional medicine.

There are numerous nano-based drug delivery systems; lipid based (liposomes and lipid nanoparticles with a solid matrix), cyclodextrins and polymeric based (polymeric nanoparticles) nanocarriers, which are considered to be among the most suitable systems for oral delivery (Fig. 1.7). Various techniques have been used to increase the solubility of silibinin, such as complexation with cyclodextrin and liposomes. However, the use of cyclodextrin is associated with a risk of nephrotoxicity and liposomes are not stable during long-term storage. Therefore, it is clear that more efficient and robust methods of increasing the solubility of silibinin are needed.

In recent years, polymeric nano-sized carriers have shown a high tumor targeting ability at tumor tissues and the nano-sized drug carriers were minimally found at normal tissue sites, leading to high antitumoral therapeutic efficacies. The preferential accumulation of polymeric
nano-sized drug carriers at tumor sites is explained by the so-called “enhanced permeability and retention (EPR)” effect, which is caused by the disorganized vascularization and defective vascular architecture of tumors.

![Diagram of nanosized delivery systems for oral route](Adapted from Bilia and Isachhi, 2014)

**Fig. 1.7** Nanosized delivery systems for oral route (Adapted from Bilia and Isachhi, 2014)

### 1.23. Polymeric nanoparticles

Polymeric nanoparticles (PNPs) include solid particles ranging in size from 10 nm to 1000 nm (1 μm) and can be fabricated as nanospheres or nanocapsules. Nanospheres are solid spherical structures composed of a polymeric matrix. Drugs can be conjugated to the nanosphere surface or
adsorbed within the bulk of the polymeric matrix. Nanocapsules are vesicular hollow polymers that encapsulate a drug solution within a polymeric membrane. Polymeric particles consist of a polymeric backbone that is typically composed of a biodegradable monomer (Malam et al., 2009). Polymer particles provide flexibility in design because they can either be biodegradable or non-biodegradable, natural or synthetic (Alexis et al., 2010). PNPs are promising vehicles for drug delivery by easy manipulation to prepare carriers with the objective of delivering the drugs to specific target; such an advantage improves the drug safety (Shokri et al., 2011). Polymer-based nanoparticles effectively carry drugs, proteins and DNA to target cells and organs. Their nanometer-size promotes effective permeation through cell membranes and stability in the blood stream.

Polymeric nanoparticles provide a common platform for inclusion of a drug of therapeutic potential, an imaging agent and an appropriate targeting moiety to end up with a perfect nanotheranostic drug delivery system. Their controlled release properties and the protection they offer to the compound of interest make these nanosystems very advantageous in the scope of drug delivery applications (Zigoneanu et al., 2008), particularly in the gastrointestinal (GI) tract, where conditions are very harsh.

In addition, encapsulation of such nanotheranostic systems within polymer alters bio-distribution by making the uptake and distribution properties primarily those of the carrier rather than those of the neat therapeutic, thereby increasing circulating half-life, avoiding degradation of therapeutic in transit to the delivery site. Therefore, drug encapsulation in protective synthetic colloidal carriers, such as nanoparticles, which can also deliver it in a controlled manner, represents an attractive strategy to successfully orally deliver therapeutic peptides and proteins. A schematic representation of polymeric nanoparticles is shown in Fig. 1.8.
Fig. 1.8 Structure of the polymeric nanospheres and nanocapsules (Vauthier and Bouchemal, 2009)

1.24. Advantages of polymeric nanoparticles

Polymeric Nanoparticles offer a number of advantages making it an ideal drug delivery vehicle, which are as follows

- Nanoparticles can be administered in high concentration of the drug locally which reduces systemic toxicity, complication and allergic reaction to many drugs.
- No follow up surgical removal is required once drug supply is depleted as nanoparticles are formulated with biodegradable and biocompatible polymer.
• Delivers a higher concentration of pharmaceutical agent to a desired location.
• Polymeric nanoparticles can be easily incorporated into other activities related to drug delivery, such as tissue engineering.
• Polymer nanoparticles could also reduce the multi drug resistance that characterizes many anticancer drugs by a mechanism of increasing uptake of drug, reducing its efflux from cells mediated by the p-glycoprotein (Brigger and couvreur, 2002; Mu, and Feng, 2003a,b).

1.25. Routes of Administration

In recent years, significant research has been done using nanoparticles as oral drug delivery vehicles. Oral delivery of drugs using nanoparticles has been shown to be far superior to the delivery of free drugs in terms of bioavailability, residence time and biodistribution (Damge et al., 1990). Oral drug delivery is the choicest route for drug administration because of its non-invasive nature (Rieux et al., 2006). The drugs may also be susceptible to gastrointestinal degradation by digestive enzymes. The advantage of using polymeric nanoparticles is to permit encapsulation of bioactive molecules and maintain them against enzymatic and hydrolytic degradation (Damge et al., 1990). The use of submicron-size particular systems in oral drug delivery, especially peptide drugs, has attracted considerable pharmaceutical interest (Olbrich et al., 2002). The efficacy or proficiency of the orally administered drug commonly depends on its solubility and absorption through the gastrointestinal tract. Therefore, a drug candidate that represents poor aqueous solubility and/or decomposition-rate limited absorption is believed to possess low and/or highly variable oral bioavailability (Aggarwal et al., 2009).
Intravenous route (IV) of administration allows for concentrating the nanoparticles at the target area[s] and rerouting drugs away from the sites where they cause toxicity and increase the circulation time of drugs with short circulation times. IV administration of chemotherapeutics is a major source of discomfort and stress to patients and high costs due to multiple hospitalizations required to complete the multiple IV sessions of chemotherapeutic regimens (Liu et al., 1997). The availability of suitable and effective oral therapeutic agents would make a significant contribution to patients’ quality of life, may significantly reduce costs and may prove more effective than current treatment modalities. Nanoparticles have been used as oral drug carriers for several reasons:

- Improvement of the bioavailability of drugs with poor absorption characteristics.
- Prolongation of the residence time of drugs in the intestine.
- High dispersion at the molecular level and consequently increases of absorption.
- Delivery of vaccine antigens to gut-associated lymphoid tissue.
- Control of the release of the drugs.
- Targeting of therapeutic agents to a particular organ and thus reducing toxicity.
- Reduction of the GI mucosal irritation caused by drugs.
- Assurance of the stability of drugs in the GIT.

1.26. Principal mechanisms of drug targeting to tumors

When treating cancer, the therapeutic agent must reach specifically the tumor tissue at a precise concentration, after having penetrated diverse biological barriers present in the body. Once there, the anticancer drug should have the capacity to selectively destroy cancer cells, avoiding the healthy ones. Therefore, the intracellular concentration of the drug will be
increased and the adverse side effects and toxicity decreased, leading to improvements in patient compliance, quality of life and survival. To efficiently achieve these goals, three mechanisms of drug targeting to tumors have been described.

**1.26.1. Passive targeting**

“Passive targeting” exploits the unique features of tumor biology that allow nanosized objects to accumulate in the tumor by the EPR effect. Unlike free drugs that may diffuse randomly, nanosized objects can penetrate into the tumor tissue via the leaky vessels (extravasation). The porous blood vessels enables rapid fluid intake into the tumor interstitium from the blood vessels. The dysfunctional lymphatic drainage traps the influx fluid and the nanosized objects within the tumor interstitium, providing sufficient time for nanosized objects to engage the tumor cells (Fig. 1.9). The threshold particle size for extravasation into tumors is <400 nm, but other studies have shown that particles with diameters <200 nm are more effective (Torchilin, 2005).

Since the early works of Matsumura and Maeda in the mid 1980s, the EPR effect has been comprehensively documented using various tumor types and animal models. The parameters which affect the distribution of macromolecules and NPs to the tumor are better understood, and we are slowly unraveling the subtleties of the EPR effect (Matsumura and Maeda, 1986; Jain and Stylianopoulos, 2010; Rabanel et al., 2012). Importantly, it is now recognized that lymphatic drainage is not homogenous throughout the cancerous mass. Vessels in the bulk of the tumor experience higher mechanical stress, and the functional loss in the intratumoral regions is therefore more important than in the margin (Padera et al., 2004). In fact, residual lymphatic activity and de novo lymphangiogenesis are believed to be in part responsible for the progress and dissemination of metastases (Rabanel et al., 2012). The heterogeneity of lymphatic function within the
tumor is therefore a factor that should be considered when addressing tumor NP accumulation.

1.26.2. Active targeting

Active targeting is based on the conjugation of receptors to specific ligands, like peptides or monoclonal antibodies, localised on the surface of the nanoparticles (Byrne et al., 2008; Torchilin, 2010). Some of the receptors typically overexpressed on cancer cells bind to the ligands folate, transferrin or galactosamine. Hence, by appropriately attaching the corresponding moiety to the surface of the nanoparticles, active targeting can be achieved. To effectively target the nanoparticles to their desired sites, it is essential to select the adequate targeting moiety, to be present in a sufficient quantity and to have high affinity and specificity of coupling to cell surface receptors (Parveen and Sahoo, 2011). Currently, actively targeted NPs are envisioned as a promising complementary strategy to EPR to further augment the efficiency of cancer nanomedicines.

1.26.3. Triggered drug targeting

Another promising mechanism of drug targeting to tumors is triggered targeting, where the nanoparticles release their payloads by exposing them to an external stimulus, such as an electric or magnetic field, ultrasound, hyperthermia or light (Lammers et al., 2011). The main disadvantage of these kinds of nano-formulations is that they are not easy to prepare. Moreover, they can release amounts of drug without being triggered or even fail to release their load at the conditions required to induce drug release. However, much effort is being made to solve these limiting shortcomings (Tagami et al., 2011).
Fig. 1.9 (a) Passive, (b) Active and (c) Triggered targeting of nanocarriers (Adapted from Zhang et al., 2014)

1.27. Synthesis of Nanoparticles

Synthesis methods for nanoparticles are typically grouped into two categories: (i) top-down and (ii) bottom-up approaches. The top-down approach involves division of a bulky solid into smaller portions, using milling, chemical methods and volatilization of a solid followed by condensation of the volatilized components. The bottom-up approach uses condensation of atoms or molecular species from the gas phase or the solution. Both methods have advantages and disadvantages in different aspects. The top-down approach employing lithography and etching techniques can advantageously be used to generate required nanostructures in a spatially controlled manner. This property is important for integration and interconnection of nanoparticles into circuit elements and/or to design for other specific applications. On the other hand, the bottom-up approach is very powerful in creating monodisperse nanoparticles with atomic precision and this precise synthesis is important for applications in need of well-defined nanoparticles. Also the machinery and the costs of both
approaches differ considerably. For the top-down techniques generally expensive machinery and careful maintenance are needed. While for the bottom-up techniques, reactions generally take place in a test tube and the cost of reagents are a lot cheaper compared to the costs of machinery used in the top-down approach. There are strong and weak sides of both approaches and one would select the most suitable method for their own application.

1.28. Preparation of polymer nanoparticles

There are several methods by which polymer nanoparticles can be prepared, a few of which are listed below

- Nanoprecipitation Method
- Emulsion Diffusion Method
- Double emulsification Method
- Polymer Coating Method
- Polymerization method (emulsification polymerization)
- Salting out technique
- Dialysis technique
- Nano spray drying technique
- Ionic gelation technique
- Supercritical fluid technique

Among them, solvent displacement or interfacial deposition method (nanoprecipitation method) presents numerous advantages, in that it is a straightforward technique, rapid and easy to perform.

1.29. Nanoprecipitation method

Nanoprecipitation is a simple, reproducible method that comprises of an interfacial deposition process. This method is very commonly used to prepare nanocapsules, which was first developed by Fessi et al., (1989). In the nanoprecipitation method, the nanoparticles are obtained as a colloidal suspension formed when the organic phase is added slowly and with
moderate stirring to the aqueous phase (Fig.1.10). The key variables of the procedure are those associated with the conditions of adding the organic phase to the aqueous phase, such as organic phase injection rate, aqueous phase agitation rate, the method of organic phase addition and the organic phase/aqueous phase ratio. On the basis of Sugimoto’s theory on polymer precipitation (Sugimoto, 1987; Lince et al., 2008), the process of particle formation in the nanoprecipitation method comprises three stages: nucleation, growth and aggregation.

The rate of each step determines the particle size and the driving force of these phenomena is supersaturation, which is defined as the ratio of polymer concentration over the solubility of the polymer in the solvent mixture. The separation between the nucleation and the growth stages is the key factor for uniform particle formation. Ideally, operating conditions should allow a high nucleation rate strongly dependent on supersaturation and low growth rate.

On the other hand, in line with the research carried out by Davies on mass transfer between two liquids and the Gibbs-Marangoni effect (McManamey et al., 1973; Davies, 1975; Quintanar et al., 1998 explained rapid nanoparticle formation as a process due to differences in surface tension. Since a liquid with a high surface tension (aqueous phase) pulls more strongly on the surrounding liquid than one with a low surface tension (organic phase solvent) (Fig. 1.10). This difference between surface tensions causes interfacial turbulence and thermal inequalities in the system, leading to the continuous formation of eddies of solvent at the interface of both liquids. Consequently, violent spreading is observed due to mutual miscibility between the solvents, the solvent flows away from regions of low surface tension and the polymer tends to aggregate on the oil surface and forms nanocapsules.
The Eudragit® E cationic copolymer has been widely used to improve the solubility of poorly water-soluble drugs. It has a basic site containing tertiary amine groups which are ionized in gastric fluid. Therefore, Eudragit®E is easy to dissolve in the gastric environment. Eudragit®E is one type of high polymer material, and is non-toxic, easily absorbed orally, and is widely used in coating and film forming. In recent years, it has been used to prepare microcapsules and nanoformulations to improve the solubility of poorly water-soluble drugs (Fig. 1.11). Eudragit offers valuable advantages for enteric coatings: pH-dependent drug release, protection of actives sensitive, protection of gastric mucosa, increase in drug effectiveness, good storage stability, GI and colon targeting.
The current identification and diagnosis of precancerous and cancerous lesions relies on the histological and cytological examination performed by a pathologist after suspicious tissue is biopsied. Although these methods represent the gold standard for cancer diagnosis, they have several limitations in oral screening. Tissue biopsy is invasive, expensive and often time-consuming. The diagnostic interpretation of the tissue sample has been shown to vary among pathologists, and the pathologic criteria for the identification of precancerous lesions are not well defined. In addition, early precancerous changes are frequently undetectable by conventional visual inspection, leading to missed opportunities for diagnosis. The disadvantages of the traditional biopsy form a strong clinical rationale for developing other techniques to improve the detection of early premalignant changes in the oral mucosa. Therefore, more rapid, objective technologies are needed, which optimally could be executed in the operating suite by the surgeon. Recently,
optical spectroscopic technologies have been under investigation for improving cancer diagnosis and treatment, as they can provide rapid, real-time tissue evaluation with a high degree of spatial resolution (Keereweer et al., 2011). Optical spectroscopy techniques have been used to enable the identification of early neoplastic changes by probing the tissue biochemically, providing detailed information about biochemical tissue components. Optical diagnostic techniques have shown a considerable promise as complementary and possible alternatives to the current diagnostic technique (Hutchings, 2009). These techniques could potentially complement the conventional histopathology by guiding the biopsy and assessing the resection margins during surgery. Alternatively, they may be used as a non-invasive, real time in situ tool for tissue diagnosis through a fibre optic device. Optical spectrum from the tissue yield diagnostic information based on the biochemical composition and structure of the tissue. It not only indicates the presence and location of cancer but also may indicate where cancer originated (Fig. 1.12).

Essentially, all spectroscopy techniques have the same mode of action, based on the interaction of light with matter and dependent on the fact that the optical spectrum displays biochemical constitutes of tissue under examination by measuring the signals of fluorescence, absorption and scattering (Bigio and Bown, 2004; Schwarz et al., 2008). Qualitative and quantitative analysis of the biochemical changes can be performed by studying spectral features and measuring their intensities over the spectral range providing important information about disease diagnosis and disease stages (Manoharan et al., 1996; Mourant et al., 2005).

The advantages of optical spectroscopy

- Reduce pathological observers’ disagreement by providing reliable biochemical tissue measurements used as standard for diagnosis of dysplasia and cancer stages.
Reduce missed lesions (sampling errors) by guiding biopsy location.

Reduce the number of multiple biopsies required for patient follow-up which will probably reduce both costs and patient anxiety.

Provide a simple portable tool with a reduced need for skilled interpretation.

Provide diagnosis for lesions in higher risk areas such as the central nervous system and vascular system, in which surgical biopsy is dangerous.

Increase the ability to assess resection margins during surgical operation.

There are many types of optical spectroscopy systems which have been used in cancer research, such as, elastic scattering spectroscopy, fluorescence spectroscopy and Raman spectroscopy. Each of the modalities provides some information about the tissue that may be exploited for differentiating between different tissues types or for diagnostic purposes.

1.31. **Autofluorescence spectroscopy**

Autofluorescence is one of optical spectroscopic techniques which has the potential to monitor metabolic and morphological changes in various tissue types/conditions and is a minimally invasive or non-invasive technique. Fluorescence detection has advantages over other light based investigation methods: high sensitivity, high speed, safety and the possibility of the use of real-time diagnosis (Mallia et al., 2008). Autofluorescence of tissues is caused by several endogenous fluorophores. These include fluorophores from tissue matrix molecules and intracellular molecules like collagen, elastin, keratin, nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) and porphyrins present in human tissues. Hence, the autofluorescence signal represents a superposition of diverse fluorescence emissions. Therefore, many researchers have successfully used...
the potential of fluorescence spectroscopy for non invasive investigation of malignant lesion under *in vivo* condition (Mallia et al., 2008; Farewell et al., 2010).

1.32. Vibrational spectroscopy

Vibrational spectroscopy is one of the most widely used techniques for the determination of molecular structures, for the identification and characterization of molecules, and for reaction control. These transitions appear in the range $10^2$ to $10^4 \text{ cm}^{-1}$ and originate from the vibrations of nuclei constituting the molecule. The vibrational energies of the molecule can be studied by infrared and Raman spectroscopy. The infrared and Raman spectroscopic methods frequently yield complementary types of information. For a complete vibrational analysis, both methods should be necessarily used
The study of vibrational spectroscopy has resulted in a large volume of data on the vibrations of polyatomic molecules. Vibrational spectroscopy gives a dynamic picture of the molecule. With the introduction of Fourier transform infrared spectrometers and lasers as source for recording Raman spectra, vibrational spectroscopy has become an effective tool for the elucidation of molecular structure (Aamouche et al., 2011).

Vibrational spectroscopy has contributed significantly to the growth of different areas as polymer chemistry, catalysis, fast reaction dynamics, charge-transfer complexes etc. The use of spectroscopy as a means of probing the structure of simple and even complex molecules has been of inestimable value in the field of structural study of organic, inorganic and organometallic compounds, biological molecules, polymers and minerals.

Among various spectroscopic techniques that have been applied to the study of structural and biochemical alterations that occur during diseased changes in complex materials, vibrational spectroscopy is extremely useful. Bands in vibrational spectra are molecule specific and provide direct information about biochemical composition.

The vibrational spectroscopy is a technique to achieve important information on molecular and supramolecular structures of biological samples without tedious and invasive sampling procedures. In recent years, vibrational spectroscopy has been studied for the diagnosis of diverse diseases, especially for various cancers.

1.3.2.1. **Raman Spectroscopy**

Raman spectroscopy is a vibrational spectroscopic technique that can detect the changes of chemical constituents and shows the alterations of molecular structures in the biological tissues, which is named the ‘molecular fingerprint’. Molecular fingerprinting is achieved and quantitative
information can be acquired from the spectrum of sample through the number of molecular bonds that are present. The specific and characteristic peaks or bands in the Raman spectrum can be assigned to alterations of DNA/RNA, proteins, and lipids in the tissues, and can be used for qualitative and quantitative analyses. When Raman effect is produced by frequency shifts of re-emitted photons, information about vibrational, rotational, and other low frequency transitions in molecules is obtained. Compared with other optical techniques, Raman spectroscopy has its own advantages such as label-free detection, non-invasive process, and rapid analysis (Yan et al., 2011). Since Raman spectroscopy has bands with very low intensity values, label-free detection, non-invasive process, its investigation requires high-quality instrumentation and high signal-to-noise ratios to identify subtle spectral differences. With the invention of lasers and sensitive detectors such as charged-coupled-devices (CCDs), Raman spectroscopy of weakly scattering substances such as tissues is now possible. Further, use of excitation photons in near infrared region reduces fluorescence interference from biological tissues. Use of optical fibers for guiding laser light to the desired site and to collect Raman photons facilitates in vivo measurements.

1.32.2. **Fourier Transform Infrared (FT-IR) Spectroscopy**

Fourier transform infrared (FT-IR) spectroscopy is also a vibrational spectroscopic technique used to study molecular structure and structural interactions in biological systems in a non-destructive manner. It measures absorption of vibrating molecules that have resulted from the energy transmissions of vibrating dipoles. An infrared spectrum of a biological sample is composed of characteristic absorption bands originating from all infrared-active vibrational modes of biomolecules present in the tissue, such as proteins, lipids, and nucleic acids (Parker, 1971). Each of these biomolecules absorbs infrared light at certain frequencies over entire
infrared spectrum. The transformation of tissues from normal to cancer is characterized by molecular changes in tissue composition (Beljebbar, et al., 2008). The identification and quantification of these specific molecular changes within tissue can provide diagnostic information for aiding in early detection of tumors.

1.30. Objectives of the present study

The present study mainly focused on the following objectives to explore the optical spectroscopic detection and evaluation of the antitumor efficacy of SILNPs in comparison with free SIL against *in vitro* and *in vivo* oral carcinogenesis.

- Preparation of SILNPs using nanoprecipitation technique.
- Characterization of SILNPs using dynamic light scattering (DLS), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD) and differential scanning calorimeter (DSC) techniques.
- Determination of drug encapsulation efficiency (EE) and *in vitro* drug release pattern of SILNPs using UV spectrometer.
- Study of the changes in cell viability, intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), apoptotic morphological changes and DNA damage in human oral carcinoma (KB) cells.
- Investigation of body weight, tumor incidence, tumor burden, tumor volume and histopathological changes in DMBA-induced oral carcinogenesis.
Determination of cyclin D1 (a marker used for cell proliferation) expression in DMBA-induced oral carcinogenesis by immunohistochemical study.

Study of the metabolic changes in the various endogenous fluorophores like collagen, NADH, FAD and porphyrin during DMBA-induced oral carcinogenesis by autofluorescence (AF) spectroscopy.

Investigation of the alterations in the biochemical and structural changes at the molecular level during DMBA-induced oral carcinogenesis by vibrational spectroscopic techniques such as Raman and FT-IR spectroscopy.