Chapter 2

Review of Literature

1. General data of plants called Hua-Khao-Yen

Dioscorea membranacea (a)

Dioscorea burmanica (b)

Pygmaeopremna herbacea (c)

Figure 2.1

Plants called Hua-Khao-Yen; *Dioscorea membranacea* (a), *Dioscorea birmanica* (b), *Pygmaeopremna herbacea* (c), *Smilax corbularia* (d) and *Smilax glabra* (e)

Smilax corbularia (d)

Smilax glabra (e)

Figure 2.1 (continued)

Thai medicinal plants locally known as "Hua-Khao-Yen" have mostly been used in Thai traditional medicines (Ancient Medicine Association, 1962, 1978; Mutita, 1989; Pongbunrod, 1976; Traditional Lanna Thai Medicine, 1982). More than 2400 preparations of Hua-Khao-Yen have been registered at the Ministry of Public Health of Thailand (Division of Medical Research, 1986). These preparations have been used to treat leprosy, venereal diseases, inflammations, bacterial infections and cancers. 'Hua- Khao-Yen' are found as ingredients in almost every traditional drug formula for cancer (Vimolkhunakorn, 1979). Selective interviews of traditional doctors of Southern Thailand (Itharat *et al*, 1998) found that they used Hua-Khao-Yen as one of the ingredients in their drug formulae for cancer, around 60% of the 30 formulae of drugs for cancer listed. Moreover, Thai traditional doctors have used five species of "Hua-Khao-Yen" (Figure 2.1) including *Dioscorea birmanica*, *Smilax corbularia*, *Smilax glabra*, *Pygmaeopremna herbacea* and *Dioscorea membranacea*, to treat cancers, AIDS, septicemia and lymphatic diseases (Itharat, 2002). The extracts of these species are usually prepared by boiling with water or soaking with ethanol (Pongbunrod, 1976; Tungtrongjit, 1978). Among the five species, *Dioscorea membranacea* Pierre, called Hua-Khao-Yen-Tai, showed the highest cytotoxic activity against human cancer cell line but less active for normal cells (Itharat, 2003) and it has also been widely used to prepare Thai traditional anti-cancer medicines.

2. *Dioscorea membranacea* Pierre

2.1 General description of *Dioscorea membranacea* Pierre

Dioscorea membranacea Pierre (Dioscoreaceae), its Thai vernacular names are Phak Lum Phua, Phak Khanong Ma, Khao-Yen-Tai and Khrua That (Supatanakul *et al*., 1985). It is distributed from Thai westwards to north Mynmar and eastwards into Cambodia; southwards passing beyond the Isthmus of Kra into Malaysia, it grows on limestone at its southern limit.(Burkill, 1951) Its rhizome is edible and medicinally used for long time by local people. (Supatanakul *et al*, 1985) It was used to treat cancer. (Subchareon, 1998; Vimolkhunakorn, 1979)

Descriptive of *Dioscorea membranacea* Pierre (Figure 2.2):-rhizome wide-running, perhaps even to 2 m, dark brown, with white flesh. Stem slightly ridged, unarmed. Leaves deeply trifid above a cordate base, shortly acuminate, 9 nerved, two primary nerves reaching the forerunner tip along with the midrib and the second pair reaching the tips of the letheral lobes; petiole 1/2-2/3 the length of the blade. Male flowers in small subsessile cymes with up to 4 flowers, tepals 1 mm long, long-ovate. Stamens all alike, the filaments inserted just below the tepals, 0.3 mm long; anther introse, small. Female flowers on down-wardly directed spike- like racemes. Tube of flower absent. Outer tepals obovate, inner ones lanceolate, a little shorter than the outer. Style short. Capsules 1-2 cm apart. (Burkill, 1951)

Figure 2.2

Dioscorea membranacea Pierre A and B inflorescence C flower D stem (Itharat, 2002)

2.2 Chemical compounds and biological activities of *D. membranacea*

2.2.1 Anticancer activity

Dioscorea membranacea Pierre, called Hua-Khao-Yen-Tai, has been the most widely used to prepare Thai traditional anti-cancer medicines. By following the cancer treatments program of Thai traditional doctors in Songkhla province, it was found that the formula with *D. membranacea* could extend lifespan by 2-3 years for elderly patients and more than ten years for the young patients. The previous research

showed that *D. membranacea* rhizomes was potently cytotoxic against cancer cells but less toxic to normal cells, making it possible to contribute to therapeutic effects.

The ethanolic extract exhibited high cytotoxic activity by SRB assay against COR-L23 lung, cancer cell line, MCF-7 breast cancer cell line and LS-174T colon cancer cell line with IC_{50} of 6.2, 12.0 and 16.7 μg/ml, respectively while its water extract exhibited high cytotoxic activity against breast and colon cancer cell lines with IC_{50} of 5.5 and 15.6 μg/ml, respectively. Both extracts had no cytotoxic activity to SVK-14 keratinocyte normal cell line with IC_{50} more than 70 μg/ml, indicating the specificity of the *D. membranacea* extracts to only cancer cell lines (Itharat *et al*., 2004). Using bioassay-guided isolation the cytotoxic compounds from *D. membranacea* got two novel cytotoxic naphthofuranoxepins, dioscorealides A [1] and B [2], and a new 1, 4-phenanthraquinone, dioscoreanone [3]. The structure determination achieved mainly by means of NMR and CD spectral and X-ray crystallographic analyses. The cytotoxicity of all three compounds was determined using the SRB assay. The target cell lines were large-cell lung carcinoma COR-L23, colon adenocarcinoma LS-174T, breast adenocarcinoma MCF-7, and non-cancer human keratinocyte SVK-14. Among the three compounds, dioscorealides A [1] is slightly active against only MCF-7 (IC_{50} = 27.4 μg/ml), whereas dioscorealides B [2] exhibits the best potency, especially against MCF-7 and COR-L23 (IC_{50} = 0.92 and 1.59 μg/ml, respectively), as well as the best selective discrimination among normal (IC_{50} = 43.5 μg/ml) and cancer cells. On the other hand, dioscoreanone [3] exhibited against MCF-7, COR-L23 and SVK-14 by IC_{50} = 3.76, 2.89 and 16.5 μg/ml, respectively, although active at a similar magnitude, does not show selectivity as good as that of dioscorealide B [2] (Itharat *et al*., 2004). Itharat also studied cytotoxic activity of isolated compounds of *D. membranacea*. Bioassay-guided isolation was used to separate the active ingredients of the ethanolic extract of *D. membranacea* by testing cytotoxic activity against three human cancer cell lines, i.e. large cell lung carcinoma (COR-L23), colon cell line (LS-174T) and breast cancer cell line (MCF-7), and two normal human cell lines, keratinocytes (SVK-14) and normal human fibroblasts (HF), using the SRB assay. Eight compounds were isolated, two naphthofuranoxepins (dioscorealides A [1] and B [2]), a 1, 4-phenanthraquinone (dioscoreanone [3]), three steroids (β-sitosterol [4], stigmasterol [5] and β-D-sitosterol

glucoside [8]) and two steroid saponins diosgenin-(3-*O*-α-L-rhamnopyranosyl (1→2) β-D-glucopyranoside [6] and diosgenin 3-*O*-β-D-glucopyranosyl (1→3)-β-D glucopyranoside [7]). Cytotoxic activity of 2, 3 and 6 showed against all three cancer cell lines. Compound 2 showed selective cytotoxic activity against lung and breast cancer, but was less active against the two normal cells and was not toxic to cell membranes in the LDH assay.

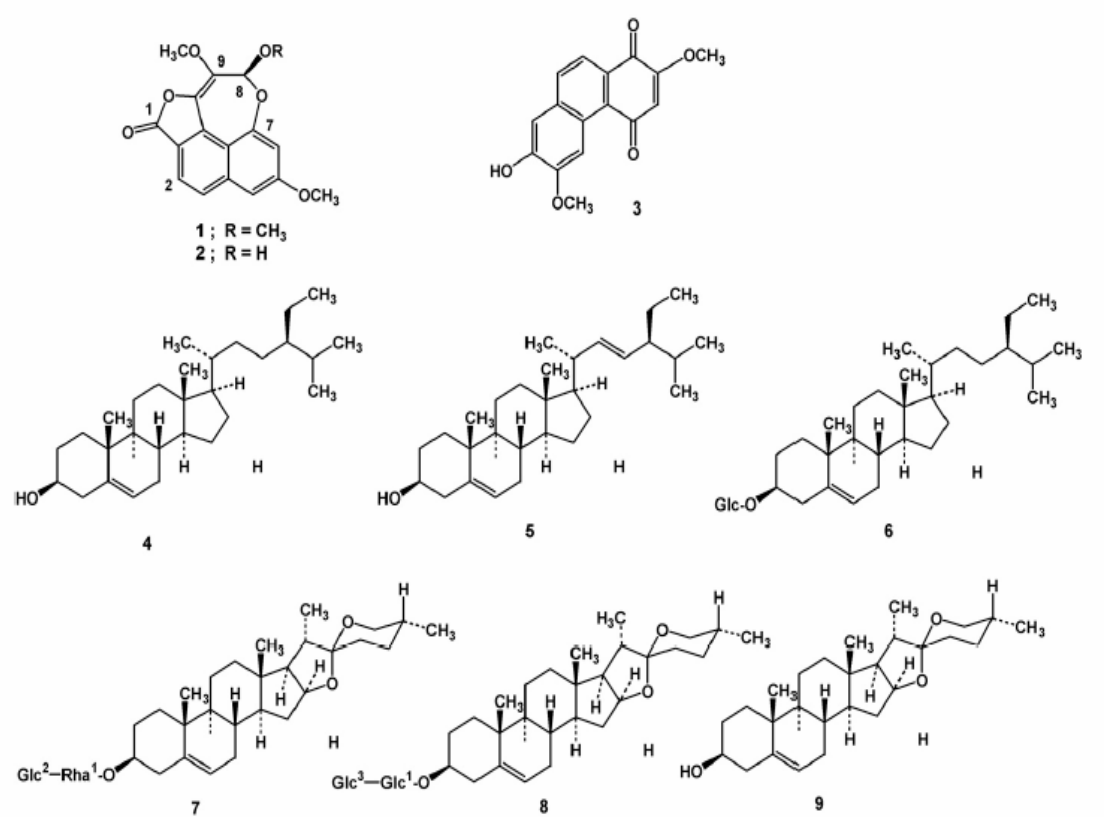

Figure 2.3

Chemical structures of isolated compounds from *D. membranacea*, dioscorealide A [1], dioscorealide B [2], dioscoreanone [3], β-sitosterol [4], stigmasterol [5], diosgenin 3-*O*-β-D-glucopyranosyl (1→3)-β-D glucopyranoside [6], diosgenin-3-*O*-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside [7], β-D-sitosterol glucoside [8], diosgenin [9] (obtained from the acid hydrolysis of 7)

The molecular mechanism of cytotoxic compounds of *D. membranacea* was investigated. It was found that *in vitro* cytotoxic activity of dioscorealide B against human breast cancer cells (MCF-7) with IC_{50} = 0.94 μg/ml. To determine whether this active compound induces apoptosis in MCF-7, Annexin V assay was performed. The results showed that the numbers of apoptotic cells were increased to 7-12 folds of control after treatment with various concentrations of dioscorealide B (1, 2 and 4 μg/ml) for 24 hours. In addition, the data revealed that dioscorealide B induced the activation of caspase-7, -8, and -9. These data suggested that the apoptotic mechanism of this compound might be involved in both intrinsic and extrinsic apoptotic pathway. Moreover, the increase in the proapoptotic protein (Bax) expression was observed at 6 hours while the decrease in the antiapoptotic protein (Bcl-2) expression was observed at 3 hours after the treatment with 1 μg/ml dioscorealide B. Together, the results indicated that dioscorealide B possessed anticarcinogenic effect against human breast cancer cells and involves in apoptosis pathway (Saekoo *et al*.2008).

2.2.2 Antioxidant acyivity

The DPPH test for antioxidant activity was employed, as was a test for LDH release as an indicator of damage to the cell membrane. Eight isolated compounds of *D. membranacea*, two naphthofuranoxepins (dioscorealides A [1] and B [2]), a 1, 4-phenanthraquinone (dioscoreanone [3]), three steroids (β-sitosterol [4], stigmasterol [5] and β-D-sitosterol glucoside [8]) and two steroid saponins (diosgenin-(3-*O*-α-L-rhamnopyranosyl (1→2) β-D-glucopyranoside [6] and diosgenin 3-*O*-β-D-glucopyranosyl (1→3)-β-D glucopyranoside [7]) were determined their antioxidant activity. The highest antioxidant activity was shown by [3] (Itharat *et al*., 2007).

Beside cytotoxic and relative activities, *D. membranacea* extracts were also studied the other biological activities and toxicity.

2.2.3 Anti-HIV activity

Tewtrakul *et al*. (2006) tested ethanolic and water extracts from five species of Hua-Khao-Yen for their inhibitory effects against HIV-1 protease (HIV-

PR) and HIV-1 integrase (HIV-1 IN). The result revealed that the extracts of *D. membranacea* were apparently inactive (IC_{50} > 100 μg/ml) to inhibit HIV-1 IN activity. Interestingly, only the ethanolic extract of *D. membranacea* showed appreciable activity (IC_{50} = 48 μg/ml) against HIV-1 PR, while the other extracts possessed mild activity. This result strongly supported the basis for the use of *Smilax corbularia* and *D. membranacea* for AIDS treatment by Thai traditional doctors.

2.2.4 Anti-inflammatory activity

Tewtrakul and Itharat (2007) examined Hua-Khao-Yen for their inhibitory activities against lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW 264.7 cell lines. Among the plant species studied, an ethanolic extract of *D. membranacea* exhibited the most potent inhibitory activity, with an IC_{50} value of 23.6 μg/ml. From this extract, eight compounds (two naphthofuranoxepins [1, 2], one phenanthraquinone [3], three steroids [4–6] and two steroidal saponins [7, 8]) were isolated and further investigated for their inhibitory properties of NO production. It was found that diosgenin-3-*O*-α-l-rhamnosyl (1 → 2)-β-d-glucopyranoside [7] possessed the highest activity (IC_{50} = 3.5 μM), followed by dioscoreanone [3] (IC_{50} = 9.8 μM) and dioscorealide B [2] (IC_{50} = 24.9 μM). Regarding structural requirements of diosgenin derivatives for NO production inhibitory activity, compound 7 which has a rhamnoglucosyl moiety at C-3 exhibited much higher activity than compounds that have either a diglucosyl substitution [8] or its aglycone [9]; whereas, hydroxyl substitution at position 8 of naphthofuranoxepin derivatives conferred higher activity than the methoxyl group. It is concluded that diosgenin-3-*O*-α-l-rhamnosyl (1 → 2)-β-d-glucopyranoside [7], dioscoreanone [3] and dioscorealide B [2] are active principles for NO inhibitory activity of *D. membranacea*. Compounds 1–3 were also tested for the inhibitory effect on LPS-induced TNF-α release in RAW 264.7 cells. The result revealed that 3 possessed potent activity against TNF-α release with an IC_{50} value of 17.6 μM, whereas, 1 and 2 exhibited mild activity. This study may support the use of *D. membranacea* by Thai traditional doctors for treatment of the inflammatory diseases.

Reanmongkol *et al*. (2007) examined the effects of the ethanolic and aqueous extracts from the rhizome of *D. membranacea* on inflammatory response

using carrageenin-induced paw edema in rats. Antinociceptive activity using writhing, hot plate and formalin test in mice and the antipyretic activity in yeast-induced fever in rats, were also examined. Oral administration of the ethanol extract at the dose of 1600 mg/kg significantly decreased the paw edema induced by carrageenin in rats. The aqueous extract (1600 mg/kg) also significantly suppressed the carrageenin-induced paw edema in rats. The ethanol extract had no significant effects on antinociceptive response in writhing, formalin and hot plate tests and antipyretic activities in yeast-induced fever in rats. No significant effects on writhing test and yeast-induced fever were observed after oral administration of the aqueous extract in experimental animals. These results suggest that the ethanolic and aqueous extracts possess anti-inflammatory action but no analgesic or antipyretic actions and their anti-inflammatory action may act at some site(s) of action or inhibit of some inflammatory mediators, which is different from the action of aspirin.

2.2.5 Anti-allergic activity

Tewtrakul *et al.* (2008) tested for anti-allergic activity against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells of of *D. membranacea* extracts. The ethanolic and water extracts possessed anti-allergic activity with IC_{50} of 37.5±2.6 and 33.9±0.6 μg/ml, respectively.

2.2.6 Toxicity

Itharat and Ooraikul (2007) tested for acute toxicity of ethanolic and water extracts of *D. membranacea* at Department of Medical Sciences, Ministry of Public Health. The result showed that both ethanolic and water extracts had no acute toxicity in rats (LD_{50} = 9 g/kg and LD_{50} >25 g/kg, respectively).

3. Cancer

Cancer means any malignant tumour, including carcinoma and sarcoma. It arises from the abnormal and uncontrolled division of cells that then invade and destroy the surrounding tissues. Spread of cancer cells (metastasis) may occur via bloodstream or the lymphatic channels or across body cavities such as the pleural and

peritoneal spaces, thus setting up secondary tumours at sites distant from the original tumour. Each individual primary tumour has its own pattern of local behaviour and metastasis; for example, bone metastasis is very common in breast cancer but very rare in cancer of the ovary. (Elizabeth, 2007)

Cancer is not just one disease but many diseases. There are more than 100 different diseases (Balmer *et al.,* 2005; US National Cancer Institute, 2008), characterized by uncontrolled cellular growth, local tissue invasion, and distant metastases. (Balmer *et al.,* 2005) Most cancers are named for the organ or type of cell in which they start - for example, cancer that begins in the colon is called colon cancer; cancer that begins in basal cells of the skin is called basal cell carcinoma.

Cancer types can be grouped into broader categories. The main categories of cancer include:

- *Carcinoma* - cancer that begins in the skin or in tissues that line or cover internal organs.
- *Sarcoma* - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.
- *Leukemia* - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
- *Lymphoma and myeloma* - cancers that begin in the cells of the immune system.
- *Central nervous system cancers* - cancers that begin in the tissues of the brain and spinal cord.

(US National Cancer Institute, 2008)

3.1 Etiology of cancer

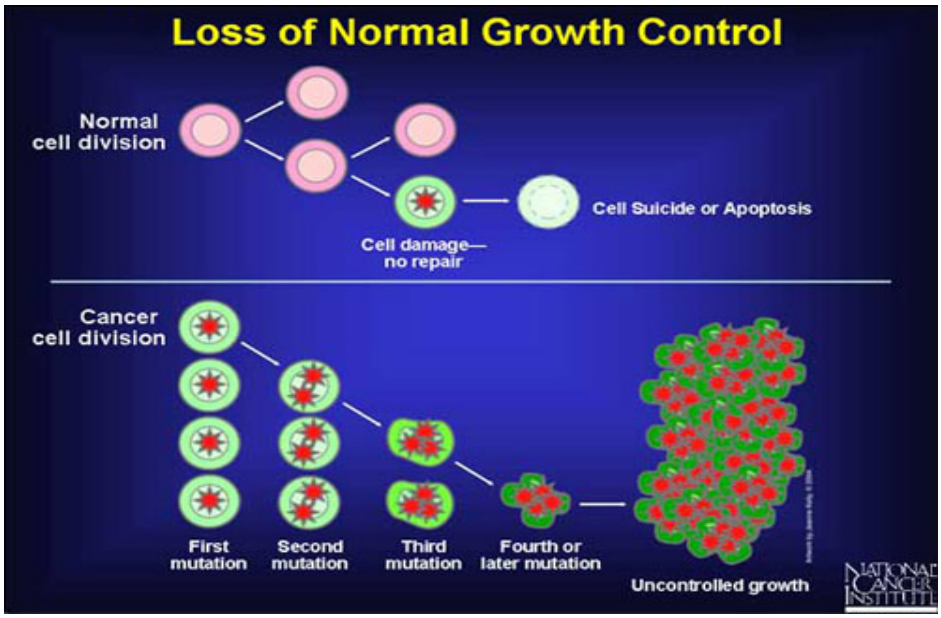

Figure 2.4

Loss of normal cell growth control (US National Cancer Institute, 2008)

Carcinogenesis is a multi-step process that includes initiation, promotion, conversion, and progression. The growth of both normal and cancerous cells is genetically controlled by the balance or imbalance of oncogene, protooncogene, and tumor suppressor gene protein products. Multiple genetic mutations are required to convert normal cells to cancerous cells. Apoptosis and cellular senescence (aging) are normal mechanisms for cell death.

3.1.1 Carcinogenesis

The mechanism by which cancers occur is incompletely understood. A cancer, or neoplasm, is thought to develop from a cell in which the normal mechanisms for control of growth and proliferation are altered. Current evidence supports the concept of carcinogenesis as a multistage process that is genetically regulated. The first step in this process is *initiation*, which requires exposure of normal cells to carcinogenic substances. These carcinogens produce genetic damage

that, if not repaired, results in irreversible cellular mutations. This mutated cell has an altered response to its environment and a selective growth advantage, giving it the potential to develop into a clonal population of neoplastic cells. During the second phase, known as *promotion*, carcinogens or other factors alter the environment to favor growth of the mutated cell population over normal cells. The primary difference between initiation and promotion is that promotion is a reversible process. In fact, because it is reversible, the promotion phase may be the target of future chemoprevention strategies, including changes in lifestyle and diet. At some point, however, the mutated cell becomes cancerous (*conversion* or *transformation*). Depending on the type of cancer, 5 to 20 years may elapse between the carcinogenic phases and the development of a clinically detectable cancer. The final stage of neoplastic growth, called *progression*, involves further genetic changes leading to increased cell proliferation. The critical elements of this phase include tumor invasion into local tissues and the development of metastases.

Substances that may act as carcinogens or initiators include chemical, physical, and biologic agents. Exposure to chemicals may occur by virtue of occupational and environmental means, as well as lifestyle habits. The association of aniline dye exposure and bladder cancer is one such example. Benzene is known to cause some leukemias. Some drugs and hormones used for therapeutic purposes are also classified as carcinogenic chemicals. Physical agents that act as carcinogens include ionizing radiation and ultraviolet light. These types of radiation induce mutations by forming free radicals that damage DNA and other cellular components. Viruses are biologic agents that are associated with certain cancers. The Epstein- Barr virus is believed to be an important factor in the initiation of African Burkitt's lymphoma. Likewise, infection with hepatitis B virus is known to be a major cause of hepatocellular cancer. All the previously mentioned carcinogens, as well as age, gender, diet, growth factors, and chronic irritation, are among the factors considered to be promoters of carcinogenesis.

3.1.2 Genetic basis of cancer

Cancer has been described as "a malady of genes, arising from genetic damage of diverse sorts and leading to distortions of either expression or

biochemical function of genes." In recent years, there has been marked progress in the understanding of the genetic changes that lead to the development of cancer, largely because of improvements in research techniques and new information generated as part of the human genome project. There are two major classes of genes involved in carcinogenesis: oncogenes and tumor-suppressor genes. Oncogenes develop from normal genes, called protooncogenes, and may have important roles in all phases of carcinogenesis. Protooncogenes are present in all cells and are essential regulators of normal cellular functions, including the cell cycle. Genetic alteration of the protooncogene through point mutation, chromosomal rearrangement, or gene amplification activates the oncogene. These genetic alterations may be caused by carcinogenic agents such as radiation, chemicals, or viruses (somatic mutations), or they may be inherited (germ-line mutations). Once activated, the oncogene produces either excessive amounts of the normal gene product or an abnormal gene product. The result is dysregulation of normal cell growth and proliferation, which imparts a distinct growth advantage to the cell and increases the probability of neoplastic transformation. As an oncogene, the gene product is overexpressed or amplified, resulting in excessive cellular proliferation. In contrast, tumor-suppressor genes regulate and inhibit inappropriate cellular growth and proliferation. Gene loss or mutation results in loss of control over normal cell growth.

Another group of genes important in carcinogenesis is the DNA repair genes. The normal function of these genes is to repair DNA that is damaged by environmental factors, or errors in DNA that occur during replication. If not corrected, these errors can result in mutations that activate oncogenes or inactivate tumor suppressor genes. As more mutations in the genome occur, the risk for malignant transformation increases. The DNA repair genes have been classified as tumor suppressor genes, because a loss in their function results in increased risk for carcinogenesis.

Oncogenes and tumor-suppressor genes provide the stimulatory and inhibitory signals that ultimately regulate the cell cycle. These signals converge on a molecular system in the nucleus known as the cell cycle clock. The function of the clock in normal tissue is to integrate the signal input and to determine if the cell cycle should proceed.

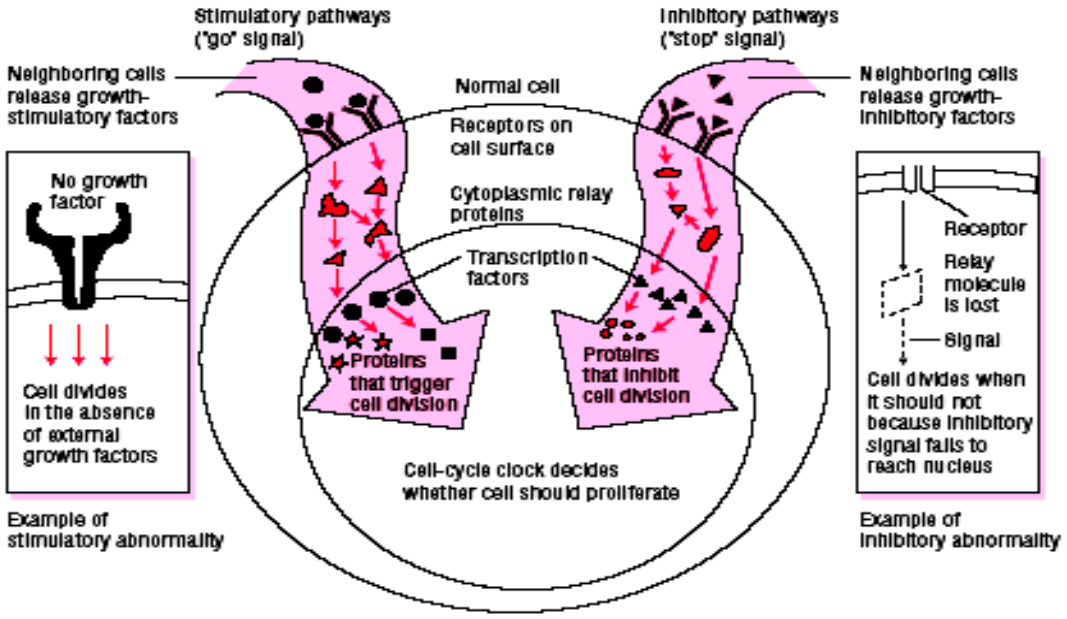

Figure 2.5

The effects of oncogenes and tumorsuppressor genes on cellular function. Signaling pathways in normal cells relay growth-controlling messages from the outer surface to the nucleus, where the cell-cycle clock receives these messages and decides whether the cell should divide. In cancer cells, genetic mutations can either activate oncogenes, resulting in excessive stimulation (too many "go" signals) or inactivate tumor-suppressor genes, resulting in loss of cell-cycle inhibition (no "stop" signals). Examples of abnormal stimulatory or inhibitory processes are provided in the boxes. (Balmer *et al.,* 2005)

3.2 Tumor origin

Tumors may arise from any of four basic tissue types: epithelial tissue, connective tissue (i.e., muscle, bone, and cartilage), lymphoid tissue, and nerve tissue. Although some malignant cells are atypical of their cells of origin, the involved cells usually retain enough of their parent's traits to identify their origin. Benign tumors are named by adding the suffix *-oma* to the name of the cell type. Hence, adenomas are benign growths of glandular origin, or growths that exhibit a glandular pattern. Table 1 lists common tumor nomenclature by tissue type.

Some cancers are preceded by cellular changes that are abnormal, but not yet malignant. Correction of these early changes could potentially prevent the occurrence of a cancer. Precancerous lesions may be described as consisting of either hyperplastic or dysplastic cells. Hyperplasia is an increase in the number of cells in a particular tissue or organ, which results in an increased size of the organ. It should not be confused with hypertrophy, which is an increase in the size of the individual cells. Hyperplasia occurs in response to a stimulus and reverses when the stimulus is removed. Dysplasia is defined as an abnormal change in the size, shape, or organization of cells or tissues. Hyperplasia and dysplasia may precede the appearance of a cancer by several months or years.

Malignant cells are divided into those of epithelial origin or the other tissue types. Carcinomas are malignant growths arising from epithelial cells. Malignant growths of muscle or connective tissue are called sarcomas. Therefore an adenocarcinoma is a malignant tumor arising from glandular tissue. Another term used frequently in the description of malignancy is *carcinoma in situ*. In this instance, the cancer is limited to the epithelial cells of origin; it has not yet invaded the basement membrane. Carcinoma in situ is a preinvasive stage of malignancy, and most tumors have progressed well beyond this stage at diagnosis. Like all classification systems, there are exceptions to these rules. Malignancies of hematologic origin, such as leukemias and lymphomas, are classified separately.

Table 2.1

Tumor classification by tissue type

Tissue of Origin	Benign	Malignant
Epithelial		
Surface epithelium	Papilloma	Carcinoma (squamous, epidermoid)
Glandular tissue	Adenoma	Adenocarcinoma
Connective tissue		
Fibrous tissue	Fibroma	Fibrosarcoma
Bone	Osteoma	Osteosarcoma
Smooth muscle	Leiomyoma	Leiomyosarcoma
Striated muscle	Rhabdomyoma	Rhabdomyosarcoma
Fat	Lipoma	Liposarcoma
Lymphoid tissue and hematopoietic cells		
Bone marrow elements		Leukemias
Lymphoid tissue		Hodgkin's and non-Hodgkin's lymphoma
Plasma cell		Multiple myeloma
Neural tissue		
Glial tissue	"Benign" gliomas	Glioblastoma multiforme, astrocytoma
Nerve sheath	Neurofibroma	Neurofibrosarcoma
Melanocytes	Pigmented nevus (mole)	Malignant melanoma
Mixed tumors		
Gonadal tissue	Teratoma	Teratocarcinoma

(Balmer *et al.*, 2005)

No all tumors are cancerous, tumors can be benign or malignant. Benign tumors aren't cancerous when malignant tumors are cancerous. Cells in benign tumors do not spread to other parts of the body when cells in malignant tumors can invade nearby tissues and spread to other parts of the body. The spread of cancer from one part of the body to another is called metastasis. (US National Cancer Institute, 2008)

3.3 **Diagnosis** (Balmer *et al.*, 2005)

Because patients with clinically evident metastatic cancer can rarely be cured, early detection is critical. Screening programs are designed to detect cancers in asymptomatic people who are at risk of a specific type of cancer. Knowing the early warning signs of cancer is also important in early detection, when cancers are most likely to be localized.

Because cancers are most curable with surgery or radiation before they have metastasized, early detection and treatment have obvious potential benefits. In addition, small tumors are more responsive to chemotherapy, as discussed previously.

Early diagnosis is difficult for many cancers because they do not produce clinical signs or symptoms until they have become large or have metastasized. Cancer screening programs are designed to detect signs of cancer in people who have not yet developed symptoms from cancer. Lack of effective screening methods for some cancers and inaccessibility of some anatomic sites further complicate the process. Education of the public on the early warning signs of common cancers is extremely important for facilitating early detection. For some cancers, effective screening procedures do exist. The Papanicolaou (Pap) smear test, for example, is an effective tool to detect cervical cancer in its early stages. Self-examination of the breasts in women and of the testicles in men may lead to early diagnosis of cancers in these organs. The American Cancer Society has published guidelines for routine screening examinations.

Table 2.2

Seven warning signs of cancer and eight warning signs of cancer in children

Cancer's Seven Warning Signs	**Cancer's Warning Signs in Children**
1. Change in bowel or bladder habits 2. A sore that does not heal 3. Unusual bleeding or discharge 4. Thickening or lump in breast or elsewhere 5. Indigestion or difficulty in swallowing 6. Obvious change in wart or mole 7. Nagging cough or hoarseness If YOU have a warning signal, see your doctor!	1. Continued, unexplained weight loss 2. Headaches with vomiting in the morning 3. Increased swelling or persistent pain in bones or joints 4. Lump or mass in abdomen, neck, or elsewhere 5. Development of a whitish appearance in the pupil of the eye 6. Recurrent fevers not caused by infections 7. Excessive bruising or bleeding 8. Noticeable paleness or prolonged tiredness
From American Cancer Society. Seven warning signs of cancer. Atlanta, American Cancer Society.	*From American Cancer Society. Eight warning signs of cancer in children. Atlanta, American Cancer Society.*

The presenting signs and symptoms of cancer vary widely and depend on the type of cancer. The presentation in adults may include any of cancer's seven warning signs (Table 2.2), as well as pain or loss of appetite. The warning signs of cancer in children are different, and reflect the types of tumors more common in this patient population (Table 2.2). Even with increased public awareness, the fear of a cancer diagnosis can deter patients from seeking medical attention. The definitive diagnosis of cancer relies on the procurement of a sample of the tissue or cells suspected of malignancy and pathologic assessment of this sample. This sample can be obtained by numerous methods, including biopsy, exfoliative cytology, or fine-needle aspiration. A tissue diagnosis is essential, because many benign conditions can masquerade as cancer. Definitive treatment should not begin without a pathologic diagnosis.

3.4 Cancer treatment (Balmer *et al.,* 2005)

Treatment for cancer should not begin until the presence of cancer is confirmed by a tissue (i.e., histologic) diagnosis. Clinical cancer staging provides prognostic information, and in conjunction with the patient's treatment goals, guides the selection of cancer treatment. The goals of cancer treatment include cure, prolongation of life, and relief of symptoms. Surgery and radiation therapy provide the best chance of cure for patients with localized cancers, but systemic treatment methods are required for systemic cancers.

Adjuvant therapy is usually systemic therapy that is administered to treat any existing micrometastases remaining after treatment of localized disease. Because adjuvant therapy is given to patients with no clinical evidence of cancer, the benefit of the treatment cannot be proven for an individual patient, but only for patient populations. Treatment decisions are based largely on an assessment of the presence of risk factors in an individual patient and the patient's estimated risk for cancer recurrence. The effectiveness of adjuvant therapies is measured statistically, by the relative and absolute reduction in the risk of recurrence. In contrast, outcomes can be assessed for individual patients with metastatic disease with carefully defined response criteria. Response criteria are disease-specific and usually include complete response, partial response, stable disease, progression, and clinical benefit.

Cancer cells are genetically unstable, which results in tumor masses of heterogeneous cells, and makes the cancer a “moving target” for drug therapy. Existence of many different clones of cancer cells in most patients provides the rationale for use of cancer drugs in combination, and is the likely reason for failure of cancer drug therapy to cure most patients with advanced cancer.

3.4.1 Modalities of cancer treatment

Four primary modalities are employed in the approach to cancer treatment: surgery, radiation, chemotherapy, and biologic therapy. The oldest of these is surgery, which plays a major role in the diagnosis and treatment of cancer. Surgery remains the treatment of choice for most solid tumors diagnosed in the early stages. Radiation therapy was first used for cancer treatment in the late 1800s and remains a mainstay in the management of cancer. Although very effective for treating many types of cancer, surgery and radiation are local treatments. These modalities are likely to produce a cure in patients with truly localized disease. But because most patients with cancer have metastatic disease at diagnosis, localized therapies often fail to completely eliminate the cancer. In addition, systemic diseases such as leukemia cannot be treated with a localized modality. Chemotherapy (including hormonal therapy) accesses the systemic circulation and can theoretically treat the primary tumor and any metastatic disease. Biologic therapies are currently considered in the broader sense of “biologically directed” therapies. Immunotherapy, the earliest important form of biologic therapy, usually involves stimulating the host’s immune system to fight the cancer. The agents used in immunotherapy are usually naturally occurring cytokines, which have been produced with recombinant DNA technology. Examples of agents used in immunotherapy include interferons (IFNs) and interleukins (ILs). Biologically directed therapies include monoclonal antibodies, other targeted therapies such as tyrosine kinase inhibitors or proteosome inhibitors, and tumor vaccines.

Many cancers appear to be eliminated by surgery or radiation. However, the high incidence of later recurrence implies that the primary tumor began to metastasize before it was removed. These early metastases are too small to detect with currently available diagnostic tests and are known as micrometastases. Adjuvant

therapy is defined as the use of systemic agents to eradicate micrometastatic disease following localized modalities such as surgery or radiation or both. The goal of systemic therapy given in this setting is to reduce subsequent recurrence rates and prolong long-term survival. Thus adjuvant therapy is given to patients with potentially curable malignancies who have no clinically detectable disease after surgery or radiation. Because adjuvant therapy is given at a time that the cancer is undetectable, its effectiveness cannot be measured by response rates; instead it is evaluated by recurrence rates and survival. The value of adjuvant therapy is best established in colorectal and breast cancers. Chemotherapy may also be given in the neoadjuvant or preoperative setting. The goals in this instance are to make other treatment modalities more effective by reducing tumor burden and to destroy micrometastases. For example, in head and neck cancer, neoadjuvant chemotherapy is employed in an attempt to shrink large tumors and to make them more amenable to later surgical resection, and possibly spare critical organs, such as the larynx.

The management of most types of cancer involves the use of combined modalities. Early stage breast cancer is a good example of the use of a combined-modality approach. The primary tumor is removed surgically, and radiation therapy is delivered to the remaining breast (after lumpectomy) or to the axilla (if there is marked lymph node involvement). Adjuvant chemotherapy and/or hormonal therapy is then administered to eradicate any micrometastatic disease.

3.4.2 Purposes of chemotherapy

The era of modern cancer chemotherapy was born in 1941, when Goodman and Gilman first administered nitrogen mustard to patients with lymphoma. Since that time, numerous antineoplastic agents have been developed, and a variety of chemotherapy regimens have been investigated in every type of cancer. Cancer chemotherapy may be indicated as a primary, palliative, adjuvant, or neoadjuvant treatment modality. Treatment with cytotoxic drugs is the primary curative modality for a few diseases, including leukemias, lymphomas, choriocarcinomas, and testicular cancer. Most solid tumors are not curable with chemotherapy alone, either because of the biology of the tumor or because of advanced disease at presentation. Chemotherapy in this setting is often initiated for palliative purposes. It is often

possible to decrease tumor size or to retard growth enough to reduce untoward symptoms caused by the tumor.

4. Breast cancer and MCF-7 cell line

4.1 Breast cancer

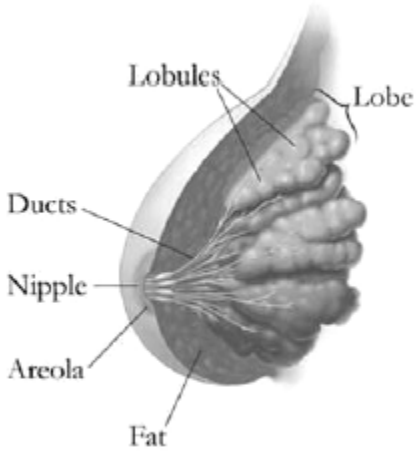

Figure 2.6
The breast

Breast cancer, a common cancer in women, is a disease in which cancer cells are found in the tissues of the breast. Each breast has 15 to 20 sections called lobes. Lobes have many smaller sections called lobules. The lobes and lobules are connected by thin tubes called ducts. The most common type of breast cancer is ductal cancer. It is found in the cells of the ducts. Cancer that begins in the lobes or lobules is called lobular carcinoma. Lobular carcinoma is found in both breasts more often than other types of breast cancer. Inflammatory breast cancer is an uncommon type of breast cancer. In this disease, the breast is warm, red, and swollen. (University of Texas, M. D. Anderson Cancer Center, 2008)

Breast cancer is most commonly diagnosed in early stages, when it is a highly curable malignancy. The etiology of breast cancer is unknown, but a number of factors that increase a woman's chances of developing the disease have been identified. These risk factors, as well as information regarding the biology of the

disease, suggest that a complex interplay between hormones, genetic factors, and environmental and lifestyle influences all contribute to the etiology of this disease. The recent identification of the *BRCA1* and *BRCA2* genes, tumor-suppressor genes important in the development of inherited and perhaps sporadic breast and ovarian cancer, holds promise in identifying patients at high risk, as well as improving our basic understanding of the causes of breast and ovarian cancer.

Most breast cancers are diagnosed in early stages before the disease has disseminated to sites distant from the breast. Treatment consists of local management, as well as systemic adjuvant therapy with chemotherapy, hormonal therapy, or a combination of these. Breast conservation therapy, which consists of complete removal of the tumor (lumpectomy), combined with breast irradiation and axillary lymph node sampling, is currently the preferred method of treatment for most patients with localized breast cancer. Patients who are not candidates for breast conservation or who do not choose this local therapy will generally receive the modified radical mastectomy.

It is apparent from clinical and laboratory experiments and observation that the spread of breast cancer via the bloodstream occurs early in the course of the disease. This results in patients relapsing with systemic metastatic disease following local curative therapy. The likelihood of later development of metastatic disease is related to the size of the primary tumor, presence of lymph node involvement and number of nodes affected, and a number of additional pathologic prognostic factors, which include proliferative capacity, nuclear grade, hormone receptor status, and presence or absence of oncogenes and other protein products. Systemic adjuvant therapy is commonly administered to patients with localized breast cancer following surgical procedures to diminish the risk of or delay disease recurrence. The NIH and other groups have developed guidelines for adjuvant systemic therapy for early-stage breast cancer based on expert consensus, and these treatment recommendations continue to evolve as new data become available.

Advanced breast cancer includes locally advanced breast cancer (stage III) and metastatic breast cancer (stage IV). Treatment of stage III breast cancer generally consists of a combination of surgery, radiation, and chemotherapy administered in an aggressive approach. Although response rates and survival have

improved, there is still much progress to be made in stage III breast cancer. Metastatic breast cancer is usually incurable. The only exception to this is that some promising long-term response rates have been observed in a subset of patients with metastatic disease who have a complete or near complete response to conventional chemotherapy. Unfortunately, this represents a small number of the total population of patients with metastatic breast cancer. Metastatic breast cancer is treated with endocrine therapy or chemotherapy. Patients who are hormone receptor–positive will generally receive initial endocrine therapy followed by combination chemotherapy when endocrine therapy fails. Patients who are hormone receptor–negative or who have symptomatic disease involving the liver, lung, or central nervous system will generally receive chemotherapy as first-line therapy of metastatic disease. Chemotherapy will result in an objective response in about 50% to 60% of patients previously unexposed to chemotherapy. Most patients have partial response, and complete disappearance of disease occurs in fewer than 20% of patients treated. Median duration of response is 5 to 12 months; although some patients will have an excellent response to an initial course of chemotherapy and may live 5 to 10 years without evidence of disease. In general, survival of patients after treatment with commonly used drug combinations for metastatic breast cancer is a median of 14 to 33 months. The response rate to secondand third-line combination chemotherapy varies from 20% to 40%, depending on the previous chemotherapy regimens the patient has received. The availability of capecitabine, vinorelbine, and gemcitabine offers the promise of more successful second- and third-line treatments for metastatic breast cancer in the future. Development of novel targeted agents (e.g., trastuzumab) has also changed the outlook for patients whose tumors overexpress *HER2/neu*.

Current efforts at breast cancer prevention are directed toward the identification and removal of risk factors. In addition, two classes of agents, the retinoids and SERMs, are being evaluated for their ability to prevent breast cancer. Any statement regarding the value of these modalities awaits the results of ongoing clinical trials. Early detection of breast cancer remains important for decreasing breast cancer mortality. The rationale for early detection of breast cancer is based on the clear relationship between stage of breast cancer at diagnosis and the probability of a cure. The American Cancer Society, the U.S. Preventive Services Task Force,

and the National Cancer Institute have developed screening guidelines for early detection of breast cancer. While these guidelines differ in their nuances, the overall benefits of screening mammography are apparent in their recommendations. However, the debate continues as to the absolute benefits and risks of these procedures.

(Lindley and Michaud, 2005)

4.2 MCF-7 cell line

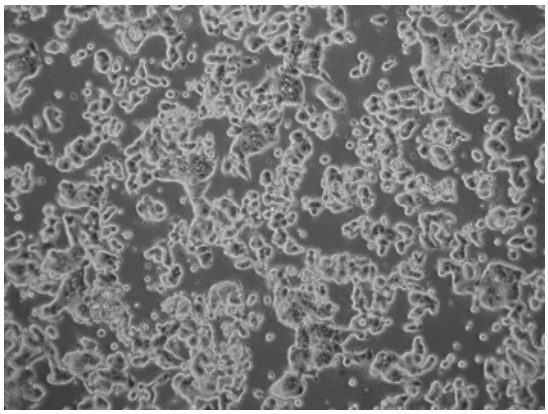

Figure 2.7

MCF-7 human breast cancer cell line

MCF-7 is a breast cancer cell line was isolated in 1970 from a 69-year-old Caucasian woman who previously underwent two mastectomies in a five-year span.. The tissue removed at the first mastectomy was benign, but the second operation found a malignant adenocarcinoma. Over a period of three years the woman was treated for local recurrences by radiotherapy and hormonotherapy. After the removal of nodules in the chest wall, a pleural effusion was discovered. The MCF-7 cell line was derived from this pleural effusion in 1970. (Dickson *et al*., 1986)

The original karyotype of the MCF-7 cell line displayed 85 chromosomes (Dickson *et al*., 1986). Later passages (around 200) have a mode of 69 (Osborne *et al*., 1987). . Karyotypes done more recently have shown discrepancies between the MCF-7 derived from the Michigan Cancer Foundation and the MCF-7 cells supplied

by the ATCC (Graham *et al*., 1986). By comparing the karyotypes, their study concluded that the MCF-7 cells obtained from the ATCC could not have been derived from the same individual as the original MCF-7 cells (Graham *et al*., 1986).

Controversies in the characteristics of the MCF-7 cell line lead one group to compare several MCF-7 cell lines obtained from different sources including two lines from the Michigan Cancer Foundation, one from the NIH, and one from the ATCC (Osborne *et al*., 1987). They compared karyotypes, growth curves, growth in soft agarose, estrogen and progesterone receptor content, and tumor growth in nude mice, all of which differed with each cell line tested. Although a heterozygous population may account for some of the differences, this study emphasizes the importance of knowing the source of your cells.

The data supplied on the MCF-7 cells in this database is sited from a variety of publications that use MCF-7 cells from different sources, and may therefore, deviate from the original MCF-7 cell line characteristics. The MCF-7 cells tend to grow in colonies. They can be passaged with 5% FBS-CMEM.

The MCF-7 cell line can grow in soft agar (Osborne *et al*., 1987). The cells are not able to penetrate a collagen fibroblast matrix (Tong *et al*., 1999). In a Boyden chamber chemoinvasion assay, the cells have low-to-moderate activity (Thompson *et al*., 1992). This data suggests that the MCF-7 cells have a low invasive capability *in vitro*. *In vivo*, the MCF-7 cells are capable of forming tumors in nude mice, but grow substantially better when the mouse is treated with 17 b-estradiol (Shafie and Liotta, 1980). A recomended tumorigenic dose for MCF-7 cells is $5x10^6$ cells (Mukhopadhyay *et al*., 1999). One publication reports that the MCF-7 cells do not form metastasis in nude mice, but another claims that both spontaneous and experimental metastasis are observed with the MCF-7 cells (experimental metastasis were only achievable with estrogen supplementation) (Mukhopadhyay *et al*., 1999), (Shafie and Liotta, 1980).

The MCF-7 cells express E-cadherin, epidermal growth factor receptor, estrogen receptor, and progesterone receptor. They lack the expression of basic fibroblast growth factor, many of the matrix metalloproteases and vimentin (University of Texas, M. D. Anderson Cancer Center, 2009).

5. Cytotoxic acitivity test

A very large number of plant extracts have been screened for cytotoxic effects against cancer cell lines over the last twenty-five years and have resulted in some significant drugs being introduced, paclitaxel probably taking pride of place. In addition, the traditional use of a considerable number of plants for cancer has been justified to some extent by the findings that have shown that their extracts are cytotoxic, especially if selectivity is demonstrated, either between different cancer cell lines or between cancer and non-cancer cell lines. (Houghton *et al*., 2007)

Cytotoxicity testing is based on one or more mammalian cell lines being grown under conditions where they are actively growing and undergoing mitotic division. Cells are cultured in a microtitre well plate and the rate of multiplication and growth is measured indirectly by formation of a colour, the intensity of which is directly proportional to the number of cells present. A variety of experiments can be used and the most basic is to compare the rate of proliferation of a cancer cell line in the presence and absence of the test substance, usually after a specified time. Ideally several different cancer cell lines can be used so that selectivity can be assessed and the addition of normal cell lines to the battery enables selectivity between cancer cell lines and normal cell lines to be determined. This gives an indication of potential usefulness in a clinical setting, for which a selectivity of at least two orders of magnitude in favour of the cancer cell line being the more susceptible is required. (Houghton *et al*., 2007)

Such tests can also be used to determine whether the cytotoxic effect is merely cytostatic i.e. it stops cells growing or dividing, or cytocidal, where the cells are killed. For such a determination, two sets of identical cells are both exposed to the test agent under identical conditions and for the same period of time. At the end of the exposure period, one set of cells is assayed whilst, for the other set, the medium containing the test substance is discarded and replaced by fresh medium alone. The cells are then incubated for a fixed time before the assay for cell growth is conducted. If the agent has only a cytostatic effect, the cells will grow and undergo mitosis in the fresh medium but, if they have been killed during the initial exposure time, no such increase in number of cells will be observed. (Houghton *et al*., 2007)

Two major techniques are used to assess the cell growth. The first one uses either 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) or 2,3-bis(2-methoxy- 4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT). The MTT method was the first developed and was introduced in 1986 followed by the use of XTT in 1988. Both of these reagents are metabolically reduced by the mitochondria in viable cells to a coloured formazan product, the intensity of which can be measured spectrophotometrically in a plate reader. The use of XTT is preferred since the formazan produced is soluble in water and the solubilisation step required if MTT is used is eliminated. However many cell lines were not so efficient at reducing XTT compared with MTT but the addition of phenazine methosulphate (PMS) showed that reduction was much better. (Houghton *et al*., 2007)

With both of these reagents the formation of colour relies on the activity of the mitochondria so, if the function of these is inhibited by variations in cellular levels of NADH, glucose and other factors, variable results are obtained and a similar result may be given as if the cells were not alive or not proliferating. (Houghton *et al*., 2007)

Because of these limitations, the second major technique for testing cytotoxicity is the more preferred i.e. the sulphorhodamine B (SRB) assay. This relies on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B (2-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-5-sulfo-benzenesulfonate) by basic amino acids in the cells. The greater the number of cells, the greater amount of dye is taken up and, after fixing, when the cells are lysed, the released dye will give a more intense colour and greater absorbance. The SRB assay is sensitive, simple, reproducible and more rapid than the formazan- based assays and gives better linearity, a good signal-to-noise ratio and has a stable end-point that does not require a time-sensitive measurement, as do the MTT or XTT assays. (Houghton *et al*., 2007)

The SRB assays have been used extensively, the latter being that used in the NCI screen and the preferred method in our laboratory.

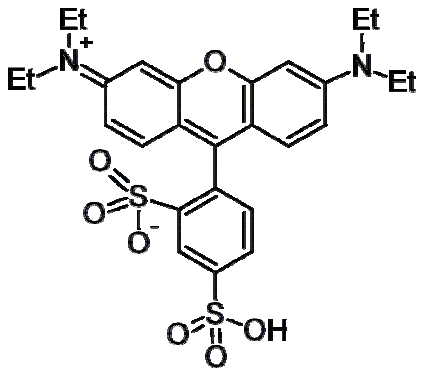

Figure 2.8

Sulphorhodamine B

The sulforhodamine B (SRB) assay, which was developed in 1990, remains one of the most widely used methods for *in vitro* cytotoxic screening. The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA). SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino acid residues under mild acidic conditions, and dissociate under basic conditions. As the binding of SRB is stoichiometric, the amout of dye extracted from stained cells is directly proposional to the cell mass. (Skehan *et al*., 1990)

6. High performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, purification and quantify compounds. HPLC is a powerful technology that is capable of separating complex mixtures into individual components that can then be quantified. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvents used. A well-developed HPLC method resolves and

quantifies components from an analyte of interest in a reproducible, rugged, precise, and accurate fashion. (Thompson and LoBrutto, 2007)

6.1 Chromatography in the pharmaceutical world

In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production. The development of new chemical entities (NCEs) is comprised of two major activities: drug discovery and drug development. The goal of the drug discovery program is to investigate a plethora of compounds employing fast screening approaches, leading to generation of lead compounds and then narrowing the selection through targeted synthesis and selective screening (lead optimization). This lead to the final selection of the most potentially viable therapeutic candidates that are taken forward to drug development. The main functions of drug development are to completely characterize candidate compounds by performing drug metabolism, preclinical and clinical screening, and clinical trials. Concomitantly with the drug development process, the optimization of drug synthesis and formulation are performed which eventually lead to a sound and robust manufacturing process for the active pharmaceutical ingredient and drug product. Throughout this drug discovery and drug development paradigm, rugged analytical HPLC separation methods are developed and are tailored by each development group (i.e., early drug discovery, drug metabolism, pharmokinetics, process research, preformulation, and formulation). At each phase of development the analyses of a myriad of samples are performed to adequately control and monitor the quality of the prospective drug candidates, excipients, and final products. Effective and fast method development is of paramount importance throughout this drug development life cycle. This requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization. (Kazakevich and LoBrutto, 2007)

6.2 Chromatographic process

Chromatographic separations are based on a forced transport of the liquid (mobile phase) carrying the analyte mixture through the porous media and the differences in the interactions at analytes with the surface of this porous media resulting in different migration times for a mixture components. In the above definition the presence of two different phases is stated and consequently there is an interface between them. One of these phases provides the analyte transport and is usually referred to as the mobile phase, and the other phase is immobile and is typically referred to as the stationary phase. A mixture of components, usually called analytes, are dispersed in the mobile phase at the molecular level allowing for their uniform transport and interactions with the mobile and stationary phases. High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture. Analyte molecules undergo multiple phase transitions between mobile phase and adsorbent surface. Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant surface is critical since the higher the number of phase transitions that analyte molecules undergo while moving through the chromatographic column, the higher the difference in their retention. The nature of the stationary and the mobile phases, together with the mode of the transport through the column, is the basis for the classification of chromatographic methods. (Kazakevich and LoBrutto, 2007)

6.3 General separation process

M. S. Tswet defined the fractional adsorption process, with the explanation that molecules of different analytes have different affinity (interactions) with the adsorbent surface, and analytes with weaker interactions are less retained. In modern high-performance liquid chromatography the separation of the analytes is still based on the differences in the analyte affinity for the stationary phase surface, and the original definition of the separation process given at its inception almost 100 years ago still holds true. Liquid chromatography has come a long way with regard to the practical development of HPLC instrumentation and the theoretical understanding of

different mechanisms involved in the analyte retention as well as the development of adsorbents with different geometries and surface chemistry.

6.3.1 Modern HPLC column

The separation of analyte mixtures in modern HPLC is performed in the device called the "column." Current HPLC columns in most cases are a stainless steel tube packed with very small (1–5μm) particles of rigid porous material. Packing material is retained inside the column with special end-fittings equipped with porous frits allowing for liquid line connection (to deliver mobile phase to the column). Stainless steel or titanium frits have a pore size on the level of 0.2–0.5μm, which allows for the mobile phase to pass through while small particles of packing material are retained inside the column. The column is the "heart" of the chromatographic system; and it is the only device where actual separation of the analyte mixture takes place.

6.3.2 HPLC system

Typical HPLC system consists of the following main components:

Solvent reservoirs Storage of sufficient amount of HPLC solvents for continuous operation of the system. Could be equipped with an online degassing system and special filters to isolate the solvent from the influence of the environment.

Pump This provides the constant and continuous flow of the mobile phase through the system; most modern pumps allow controlled mixing of different solvents from different reservoirs.

Injector This allows an introduction (injection) of the analytes mixture into the stream of the mobile phase before it enters the column; most modern injectors are autosamplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the autosampler tray.

Column This is the heart of HPLC system; it actually produces a separation of the analytes in the mixture.A column is the place where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface. Most of the chromatography development in recent years went toward the design of many different ways to enhance this interfacial contact.

Detector This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV (ultraviolet), which allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection). Appearance of the analyte in the detector flow- cell causes the change of the absorbance. If the analyte absorbs greater than the background (mobile phase), a positive signal is obtained.

Data acquisition and control system Computer-based system that controls all parameters of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance (continuous monitoring of the mobile-phase composition, temperature, backpressure, etc.).

(Kazakevich and LoBrutto, 2007)

6.4 HPLC separation modes

6.4.1 Reversed-phase chromatography

The most popular system, with over 50% of separations, is reversed-phase liquid chromatography on octadecyl-bonded silica gel. Analytes are retained on the stationary phase materials based on their hydrophobicity. This means that polar compounds are eluted faster than non-polar compounds. The higher the hydrophobicity of the stationary phase surface, the longer will be the retention times of the analytes. Bonding of donor-electron groups onto the stationary phase surface results in a stronger retention of compounds containing dipoles. In some cases, interactions between untreated silanol groups on the stationary phase and hydroxy groups on the analyte increase the selectivity; however, polar groups on the stationary phase are generally eliminated to obtain inert stationary phase materials, which are more stable in long-term operation.

6.4.2 Chromatography of ionic compounds

Neutral compounds are retained by hydrophobicity in reversed-phase liquid chromatography, and by hydrogen bonding and/or ¶-¶ interactions on

hydrophilic phases in normal-phase liquid chromatography. Ionization of the analyte reduces the retention time in reversed-phase liquid chromatography, but in some cases increases the retention time on ion-exchangers. Retentions on ionexchange resins made from polystyrene gel are based both on ion-ion interactions with ion-exchange groups and on hydrophobic interactions with the polystyrene gel matrix. The addition of counter-ions can improve the resolution.

6.4.3 Normal-phase chromatography

Normal-phase liquid chromatography was formerly called adsorption liquid chromatography. Pure or mixed organic solvents are used as the eluent and stationary phase adsorbent is more polar than the eluent. Hydrogen bonding is one of the important molecular interactions between sample molecules and the adsorbent. When no molecular interaction is recognized, such chromatography is called size-exclusion liquid chromatography.

The basic molecular interaction in normal-phase liquid chromatography is electrostatic forces. The sample molecules are retained strongly by hydrogen bonding when the sample molecules themselves or the adsorbent act as both a hydrogen-bond acceptor and donor. The specificity of normal-phase liquid chromatography is the result of the direct formation of a strong molecular interaction between sample molecules and the adsorbent. The position of substitution of sample molecules directly affects the separation of isomers. The substituent effect is weak in reversed-phase liquid chromatography. Therefore, normal-phase liquid chromatography is suitable for the separation of isomers, such as *cis, trans, ortho, meta, para,* and steric isomers. The steric effect is especially important for chiral separations on suitable chiral columns. A variety of chiral separation phases have been synthesized; however, one phase cannot separate many different types of optical isomers, because the steric effect also depends on the molecular structure of the analytes.

In ligand-exchange liquid chromatography, the separation is performed by the replacement of analytes, which form a complex with stationary phase materials or a reagent trapped on the stationary phase surface, by components in the eluent, which act as similar ligands to the analytes. In charge-transfer complex

liquid chromatography, analytes are retained by charge-transfer complex formation on the surface of stationary phase material and are replaced by other components in the eluent which form a charge-transfer complex with the stationary phase materials or with reagents trapped on the stationary phase surface.

Normal-phase liquid chromatography is thus a steric-selective separation method. The molecular properties of steric isomers are not easily obtained and the molecular properties of optical isomers estimated by computational chemical calculation are the same. Therefore, the development of prediction methods for retention times in normal-phase liquid chromatography is difficult compared with reversed-phase liquid chromatography, where the hydrophobicity of the molecule is the predominant determinant of retention differences. When the molecular structure is known, the separation conditions in normal-phase LC can be estimated. A small-scale thin-layer liquid chromatographic separation is often a good tool to find a suitable eluent. When a silica gel column is used, the formation of a monolayer of water on the surface of the silica gel is an important technique. A water saturated very non-polar solvent should be used as the base solvent, such as water-saturated n-hexane or isooctane.

6.4.4 Size-exclusion liquid chromatography (SEC)

This separation method is based on the molecular size of analytes. Analytes pass through porous stationary phase materials having different pore sizes, and molecular interactions between analytes and the stationary phase surface must be eliminated. A very strong solvent is therefore required in this system. This system is also called gel filtration liquid chromatography, gel-permeation liquid chromatography, or molecular sieve chromatography. This system is used to measure the average molecular mass and for the purification of large- from small-size molecules.

(Hanai**,** 1999)

7. Stability Testing

7.1 General principles

The purpose of stability testing is to provide evidence on how the quality of an active substance or pharmaceutical product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light. In addition, product-related factors influence the stability, e.g. the chemical and physical properties of the active substance and the pharmaceutical excipients, the dosage form and its composition, the manufacturing process, the nature of the container-closure system, and the properties of the packaging materials. Also, the stability of excipients that may contain or form reactive degradation products, have to be considered. As a result of stability testing a re-test period for the active substance or a shelf life for the pharmaceutical product can be established, and storage conditions can be recommended. (WHO, 2006a)

7.2 Storage conditions

In general, an active substance should be evaluated under storage conditions (with appropriate tolerances) that test its thermal stability and, if applicable, its sensitivity to moisture. The storage conditions and the lengths of studies chosen should be sufficient to cover storage, shipment, and subsequent use with due regard to the climatic zone(s) in which the active substance is intended to be stored. The long-term testing should cover a minimum of 12 months' duration on at least three primary batches at the time of submission and should be continued for a period of time sufficient to cover the proposed re-test period. Additional data accumulated during the assessment period of the registration application should be submitted to the authorities if requested. Data from the accelerated storage condition and, if appropriate, from the intermediate storage condition can be used to evaluate the effect of short term excursions outside the label storage conditions (such as might occur during shipping). The general case applies if the active substance is not specifically covered by a subsequent section. Alternative storage conditions can be used if justified. (WHO, 2006b)

In order to identify adequate testing conditions, the climate in which the active substance is intended to be stored has been analysed using the climatic data. The Mean Kinetic Temperature (MKT), which includes the reaction rate constants in the evaluation of heat effects on pharmaceutical products, and the mean partial water vapour pressure in any part of the region have been calculated. (WHO, 2006b)

Long-term, accelerated, and, where appropriate, room temperature storage conditions for active substances according to the guideline of The Drug Stability Analysis Side, Division of Drug Analysis Department of Medical Sciences, Ministry of Public Health, Thailand are detailed in the sections below.

Table 2.3

Storage conditions of stability testing of active substances and pharmaceutical products

Study	**Storage conditions**		**Minimum time period covered by data at submission (month)**	**Sampling time (month)**
	Temperature	**Relative humidity**		
Long term	Labeled temperature	Labeled humidity	-	0, 3, 6, 9, 12, 18, 24 and every year until over expired date
Room temperature	30 ± 2°C	70 ± 5 %RH	-	-
Accelerated	40 ± 2°C	75 ± 5 %RH	6	0, 1, 3, 6
	45 ± 2°C	75 ± 5 %RH	4	0, 1, 2, 3, 4
	50 ± 2°C	75 ± 5 %RH	3	0, 1, 2, 3

Stability testing under accelerated conditions of drug preparation could be used to settle temporary age of drug preparation. In the end of studied period, if the drug has the qualities following by its standard regulation, its shelf-life will be established for two years. Besides that long term stability has to be tested couple with accelerated test for settling real shelf life of drug. (Division of Drug Analysis, 1992; Rakvathin, 1995)