# CHAPTER I

# INTRODUCTION

## 1.1 Statement of the problems

Cancer is becoming a significant health problem, which is the leading cause of death in Thailand. Liver cancer is the third most common cancer in men and the fifth most common in women (Attasara and Buasom, 2009; Srivatanakul, 2001). The most relevants are hepatitis B (HBV), hepatitis C (HCV) viruses, alcohol and environmental chemicals (Wu *et al.*, 2009; Schafer and Sorrel, 1999; Mazzanti *et al.*, 2008; Gish, 2006). Liver cancer remains a major chronic health problem associated with toxicological substances. The long latency period of cancer induction (years in rodents and decades in humans) is a major problem in the evaluation of toxicological hazards and risk assessment.

To evaluate the ability of chemicals induce carcinogenesis which is a complex process, the detection of carcinogenic potential of environmental compounds has been developed. The short-term carcinogenicity tests currently used to predict the ability of chemicals induced DNA damage and mutation. Accordingly, these screening methodologies aim to identify genotoxic agents under the premise that such agents would most likely pose cancer risk in humans (Ellinger-Ziegelbauer *et al.*, 2009; Guyton *et al.*, 2009), while the medium-term carcinogenicity test has been repeatedly used for carcinogenic or chemopreventive studies, based on the two-step initiation and promotion concept of hepatocarcinogenesis (Ichihara *et al.*, 1999; Ito *et al.*, 2003).

Nowadays, the natural dietary constituents from herbs can be considered chemopreventive agents because they have been shown to inhibit carcinogenesis process (Debersac *et al.*, 2001). One of the major mechanisms of chemical protection against mutagenesis, carcinogenesis and other forms of toxicity mediated by electrophiles is the

induction of enzymes involved in their deactivation, particularly phase II xenobiotic-metabolizing enzymes (Tan and Spivack, 2009).

Flavanones are a subclass of flavonoids naturally occurring in several plants and fruits (Aherne and O'Brien, 2002). They influence on several steps in carcinogenesis including protecting DNA from oxidative damage, inhibiting carcinogen activation, and activating carcinogen detoxifying systems. One important mechanism by which flavanones may exert their chemopreventive effects is through their inhibition of phase I metabolizing enzymes, such as cytochrome P450 (CYP), which metabolically activates procarcinogens to reactive intermediates that trigger carcinogenesis. Inhibition of CYP activities by flavanones has been extensively studied because of their potential use as agents blocking the initiation stage of carcinogenesis (Doostdar *et al.*, 2000). Another mechanism claimed to be responsible for the chemopreventive activity of flavanones is the induction of phase II metabolizing enzymes leading to detoxification and accelerated excretion of carcinogens and represents one mechanism of their anticarcinogenic effects (Galati and O'Brien, 2004).

Pinocembrin (5, 7-dihydroxyflavanone) is the most abundant flavonoids in propolis. Previous investigations have revealed the isolation of pinocembrin from the rhizomes of fingerroot *Boesenbergia pandurata* or "Kra-chai" in Thai (Jaipetch *et al.*, 1982; Li Ching *et al.*, 2007), and have been proven to have antioxidant (Santos *et al.*, 1998; Liu *et al.*, 2008; Shindo *et al.*, 2006), antibacterial (Pepeljnjak *et al.*, 1985; Del Rayo Camacho *et al.*, 1991; Hwang *et al.*, 2003) and anti-inflammatory properties (Sala *et al.*, 2003; Tuchinda *et al.*, 2002). Furthermore, it was exhibited a strong antimutagenic activity against mutagenic heterocyclic amines using Ames test (Trakoontivakorn *et al.*, 2001). Our previous study demonstrated that pinocembrin had no toxicity in male rat (Charoensin *et al.*, inpress). Moreover, it could inhibit activities of P450 isozymes involved in carcinogen activation (Siess *et al.*, 1995). However, the *in vivo* carcinogenic and anticarcinogenic effect of pinocembrin has not previously been investigated. Then, the *in vivo* models are needed to determine whether administration of pinocembrin is a practical approach to anticarcinogenesis in rat. Therefore, in this study, the effect of pinocembrin on initiation and promotion stages of rat hepatocarcinogenesis has been investigated. In present study, we used diethylnitrosamine, which is a well-known rodent

liver carcinogen to induced rat hepatocarcinogenesis. To investigate the effect of pinocembrin on the initiation stage of rat hepatocarcinogenesis, the detection of micronucleus formation was used as the endpoint marker. Furthermore, to investigate the effect of pinocembrin on the promotion stage of rat hepatocarcinogenesis induced by diethylnitrosamine, the number of glutathione-*S*-transferase placental form positive foci (GST-P positive foci) was evaluated. In addition, phase I and phase II xenobiotic-metabolizing enzymes, were also measured.

## 1.2 Literature reviews

### 1.2.1 Multistep chemicals carcinogenesis

Cancer has been the leading cause of death in Thailand. Liver cancer is the third most common cancer in men and the fifth most common in women (Attasara and Buasom, 2009; Srivatanakul, 2001). The most relevants are hepatitis B (HBV), hepatitis C (HCV) viruses, alcohol and environmental chemicals (Wu *et al.*, 2009; Schafer and Sorrel, 1999; Mazzanti *et al.*, 2008; Gish, 2006). Liver cancer remains a major chronic health problem associated with toxicological substances.

Chemical carcinogenesis is a complex process that begins with exposure, usually to complex mixtures of chemicals that are found in the human environment (Klaunig and Kamendulis, 2004). Once internalized, carcinogens frequently are subjected to competing metabolic pathways of activation and detoxification, although some reactive environmental chemicals can act directly.

Carcinogens may increase the risk of getting cancer by altering cellular metabolism or damaging DNA directly in cells, which interferes with biological processes, and induces the uncontrolled, malignant division, ultimately leading to the formation of tumors. They can be classified as genotoxic and nongenotoxic carcinogens (Oliveira *et al.*, 2007; Combes, 2000). The genotoxic groups of chemical carcinogens can be divided into direct and non-direct acting species, depending on whether or not they require metabolism for reactive species to be generated (Ito *et al.*, 1995). Genotoxic agents usually refer to chemicals that directly damage genomic DNA, which in turn can result in mutation and/or clastogenic changes (Rundle, 2006; Combes, 2000). Chemicals in this category are frequently activated in the target cell and produce a dose-dependent increase in neoplasm formation (Klaunig and Kamendulis, 2004). Usually DNA damage, if too severe to repair, leads to programmed cell death, but if the programmed cell death pathway is damaged, then the cell cannot prevent itself from becoming a cancer cell, Figure 1-1 (Rundle, 2006).

Nongenotoxic or epigenetic carcinogens lack of chemical reactivity and hence do not form DNA adducts (Mally and Chipman, 2002), but rather lead to effects in their target cells which indirectly lead to neoplastic transformation or which enhance the

development of tumors from preinitiated cells (Klaunig *et al.*, 1998; Ellinger-Ziegelbuer *et al.*, 2005; Hawighorst *et al.*, 2001; Combes, 2000; Hernandez *et al.*, 2009). The possible mechanisms of nongenotoxic carcinogens are including increased production of free radical damaging DNA, lipids, and proteins (Klaunig *et al.*, 1998) or gap junction intercellular communication inhibitors (Mally and Chipman, 2002; Hernandez *et al.*, 2009). Direct or via lipid peroxidation products-mediated oxidative DNA damage may contribute to initiation (Williams, 2001; Klaunig, 1998). Oxidative protein damage can modulate many signaling pathways within cells, including regulation of transcription factors and kinase cascades. This may lead to aberrant gene expression resulting in increased cell proliferation or decreased apoptosis. Nongenotoxic carcinogens may also enhance cell proliferation through either direct mitogenic effect or as regenerative hyperplasia after induction of dose-dependent cytotoxicity, Figure 1-1 (Klein and Costa, 1997; Mally and Chipman, 2002).

Chemicals carcinogenesis is a multi-process which can be divided into three distinct stages, these are: initiation, promotion and progression, Figure 1-2 (Oliveira *et al.*, 2007; Klaunig and Kamendulis, 2004).

Initiation stage, a rapid and irreversible process, begins with the genotoxic damage of cellular DNA upon exposure to endogenous or exogenous carcinogens. The initiation stage of chemically induced carcinogenesis involves the metabolic activation of carcinogens and subsequent covalent modification of genomic DNA, leading to activation of oncogenes and/or inactivation of tumor suppressor genes (Klaunig and Kamendulis, 2004; Ito *et al.*, 1995; Surh *et al.*, 2008).

Metabolism of genotoxic carcinogens by the P450 system creates electrophilic intermediates that can react with cellular macromolecules (Oliveira *et al.*, 2007). The structures formed through the covalent bonding of these intermediates to DNA bases are referred to as carcinogen-DNA adducts. Failure of the cell to repair these adducts can lead to mutations in the DNA code. Adduct formation and the subsequent induction of mutations is thought to be necessary but not sufficient causes in the pathway from exposure to such chemicals to cancer. However, it can be prevented either by inhibition of these enzymes or by induction of phase II enzymes leading to detoxification and

accelerated excretion of carcinogens. These results can be inhibited initiation stage of carcinogenesis process (Oliveira *et al.*, 2007; Ito *et al.*, 1995).

Promotion stage is recognized as a reversible process characterized by clonal expansion of initiated cells by the induction of cell proliferation and/or inhibition of apoptosis, resulting to form a solid mass of proliferating preneoplastic cells (Klaunig and Kamendulis, 2004; Ito *et al.*, 1995; Surh *et al.*, 2008). Promoter compounds do not interact directly with DNA and unchain biological effects without being metabolically activated. These agents increase cell proliferation in susceptible tissues, contribute towards fixing mutations, enhance alterations in genetic expression and cause changes in cellular growth control. On the other hand, these promoters delay the natural inhibition of the quiescent cells or in G0 by gap junctions (Oliveira *et al.*, 2007). The promotion stage requires the continuous presence of the promoting stimuli, and thus it is a reversible process.

Progression, the third stage, involves cellular and molecular changes that occur from the preneoplastic to the neoplastic cells, this stage is reversible (Klaunig and Kamendulis, 2004). During progression, cell proliferation is independent from the presence of stimulus. Progression is characterized by genetic instability, faster growth, invasion, metastization, and changes in the biochemical, metabolical and morphological characteristics of cells, involves genetic instability, changes in nuclear ploidy, and disruption of chromosome integrity (Oliveira *et al.*, 2007). Angiogenesis, as an epigenetic occurrence, is essential to neoplasic progression (Ito *et al.*, 1995). The acquisition of an angiogenic phenotype precedes the development of characteristics that contribute to malignancy and its inhibition delays neoplasic development (Hawighorst *et al.*, 2001).

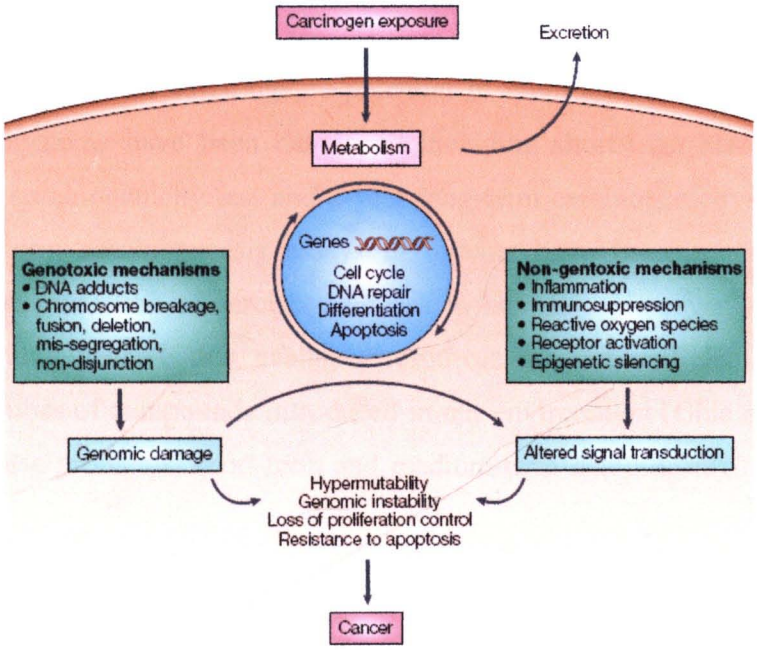


**Figure 1-1** Overview of genotoxic and nongenotoxic effects of carcinogens (Luch, 2005)

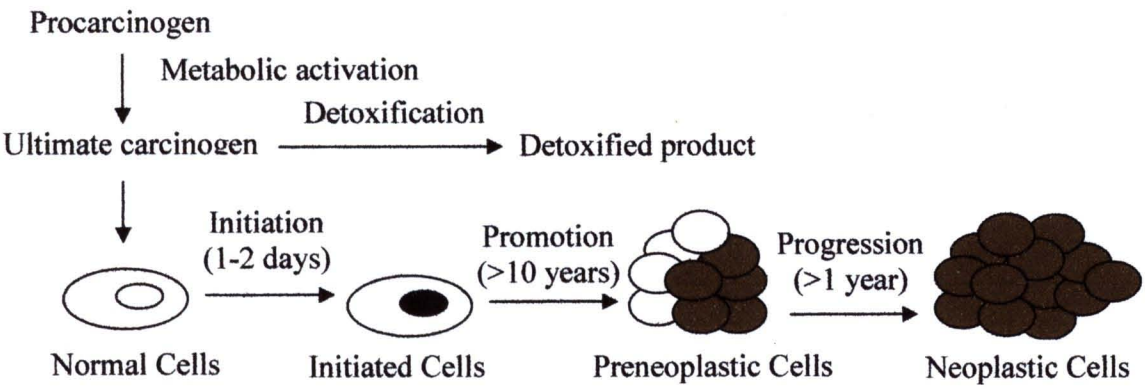


**Figure 1-2** Mechanisms of multistage carcinogenesis (Farombi, 2004)

### 1.2.2 Carcinogenicity test

For the detection of carcinogenic potential of environmental compounds, various experimental systems have been developed including short-term carcinogenicity test, medium-term carcinogenicity test and 2-year long-term carcinogenicity test (Jena *et al.*, 2005). Long-term carcinogenesis assays in rodents have been considered the most reliable for the prediction of carcinogenic risk to humans. However, they are so time consuming and expensive that available resources are not sufficient for testing the increasing number of compounds introduced in our environment (Ghia *et al.*, 1996). To circumvent these problems, short-term and medium-term carcinogenicity test have been developed.

#### 1.2.2.1 Short-term carcinogenicity test

The short-term carcinogenicity tests currently used to predict the ability of chemicals induce DNA damage and mutation. Accordingly, these screening methodologies aim to identify genotoxic agents under the premise that such agents would most likely pose cancer risk in humans. The standard genotoxicity testing battery consists of (a) a bacterial gene mutation assay, (b) an *in vitro* mammalian mutation and/or chromosome damage assay and (c) an *in vivo* chromosome damage assay (Ellinger-Ziegelbauer *et al.*, 2009; Guyton *et al.*, 2009). In addition to their use as a screening tool and surrogate for carcinogenicity data in the development of drugs and other chemicals, genotoxicity data constitute part of the weight of evidence evaluation in regulating environmental chemical. In practice, environmental contaminants have not been regulated as carcinogens on the basis of positive genotoxicity results alone. Nonetheless, chemicals testing positive in standard genotoxicity assays are generally assumed to contribute to cancer induction via a genotoxic or mutagenic mode of action that is indicative of human risk (Brambilla and Martelli, 2004). Since the short-term carcinogenicity studies can be performed in less time and at lower costs than long-term carcinogenicity study. Furthermore, these studies related to long-term carcinogenicity results (Kirkland *et al.*, 2006).

The micronucleus assay has been widely used to evaluate the genotoxicity of chemicals (Asano *et al.*, 1998). The formation of micronuclei is extensively used in molecular epidemiology as a biomarker of chromosomal damage, genome instability, and eventually of cancer risk (Iarmarcovai *et al.*, 2008). Micronucleus originated from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division (Figure 1-3) due to unrepaired or mis-repaired DNA lesions, or chromosome malsegregation due to mitotic malfunction (Bonassi *et al.*, 2007). Micronucleus tests using bone marrow or peripheral blood cells have been widely used for evaluating the genotoxicity of chemicals *in vivo* (Hakura *et al.*, 2007). However, it is well known that some compounds need metabolic activation in the liver, and it has been pointed out that some pro-mutagens elicit a negative response in bone marrow micronucleus assay (Muller-Tegethoff *et al.*, 1995). It may be considered that some active metabolites have a very short life-span and do not reach the bone marrow at sufficient concentrations to induced micronuclei. In fact, some rodent liver carcinogens, including di-alkyl-nitrosamine, nitro aromatic compounds, gave negative results in a bone marrow assay (Igarashi and Shimada, 1997; Suzuki *et al.*, 2004). In order to evaluate the genotoxic effect of chemicals in the liver, liver micronucleus test using partial hepatectomy has been developed. However, short-term carcinogenicity test has become clear that mutagenicity does not always correlate with carcinogenicity and there are a variety of chemical agents that are nongenotoxic but carcinogenic (Ito *et al.*, 2003), the medium-term carcinogenicity test has been developed.

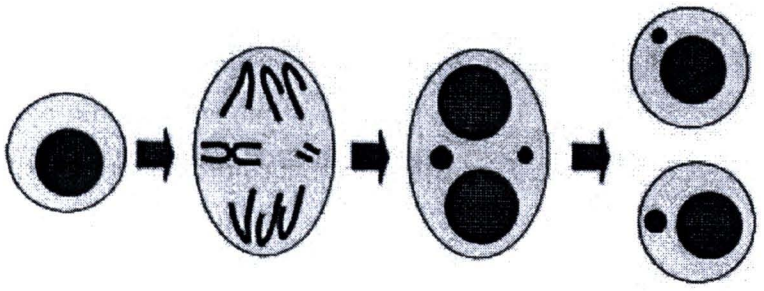

**Figure 1-3** A schematic diagram show the origin of micronucleus (Bonassi *et al.*, 2007)

### 1.2.2.2 Medium-term carcinogenicity test

In the last decade, medium-term carcinogenicity tests have received great attention since short-term mutagenicity tests have been shown to provide substantial rates of false positive and false negative results (Ghia *et al.*, 1996; Ito *et al.*, 2003).

The rat liver medium-term carcinogenicity test first established by Ito *et al.* (1988) has been repeatedly used for carcinogenic or chemopreventive studies (Ichihara *et al.*, 1999) and has great advantages due to reproducibility and reliability for generation of data within 8 weeks (Imaida and Fukushima, 1996), the standard protocol see in Figure 1-4. Based on the two-step initiation and promotion concept of hepatocarcinogenesis, the screening assays have the important advantage of easy detection of the preneoplastic enzyme-altered lesions which are widely accepted as early indicators of neoplastic development (Fukushima *et al.*, 2005; Ito *et al.*, 2003; Tsuda *et al.*, 2003). The protocol utilizes diethylnitrosamine as a carcinogenic initiator, followed by the two-thirds partial hepatectomy (2/3PH) to maximize hepatic regeneration ability. For assessment of promoting or inhibitory effects of the test chemicals, glutathione-*S*-transferase placental form (GST-P) positive foci are used as the endpoint marker. Moreover, production of GST-P foci has been closely correlated with the actual tumor yields (Kushida *et al.*, 2005; Shirai, 1997; Ito *et al.*, 2003; Tsuda *et al.*, 2003), so that they are regarded as reliable preneoplastic lesions in rats (Doi *et al.*, 2009). Moreover, the results obtained in the assays have good correlation with data of 2-year carcinogenicity tests (Ogiso *et al.*, 1990).

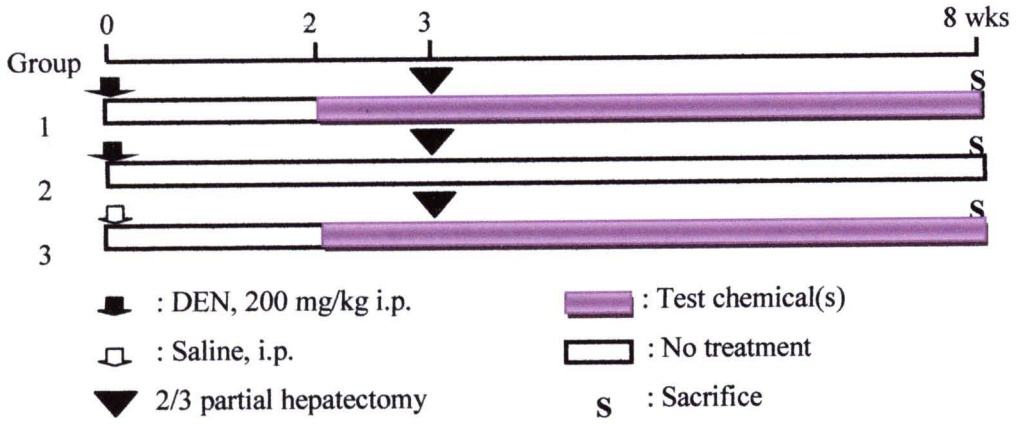


**Figure 1-4** Standard protocol of the medium-term liver bioassay (Ito *et al.*, 2003)

### 1.2.2.3 Glutathione- *S*-transferase placental positive lesion

The rat glutathione- *S*-transferase P-form (GST-P) has been found to be dramatically up-regulated in its expression in preneoplastic and neoplastic cells and is widely used as a specific marker in the basic analysis of chemical carcinogenesis, see in Figure 1-5 (Ito *et al.*, 1996; Higashi *et al.*, 2004). It has been suggested as one of the positive marker enzymes for hepatocellular foci induced by diethylnitrosoamine and other liver carcinogens (Sasagawa *et al.*, 2002). GST-P is the main isoform in normal placental tissue and comprises 67% of the total GST concentration in this phase. During development, GST-P decreases in concentration and is absent in adult tissues (Fatemi *et al.*, 2006). Interestingly, GST-P positive foci have been detected in adult tissues during medium-term hepatocarcinogenesis assay being regarded a suitable biomaker for early detection of liver neoplasms (Silva *et al.*, 2007).

The regulation of the GST-P gene expression in normal liver cells and in the pre-neoplastic lesion, see in Figure 1-6. In the normal liver, C/EBP α binds to GPE1 and represses GST-P gene expression possibly with the C/EBP α co-repressor, CA-150. During chemical carcinogenesis, C/EBPα is released from the GPE1 element and substituted by the Nrf2/MafK heterodimer. MOZ then interacts with MafK and acetylates (-Ac) the histones in the enhancer and promoter regions of the chromatin of the GST-P gene, activating the GST-P gene expression (Sakai and Muramatsu, 2007).

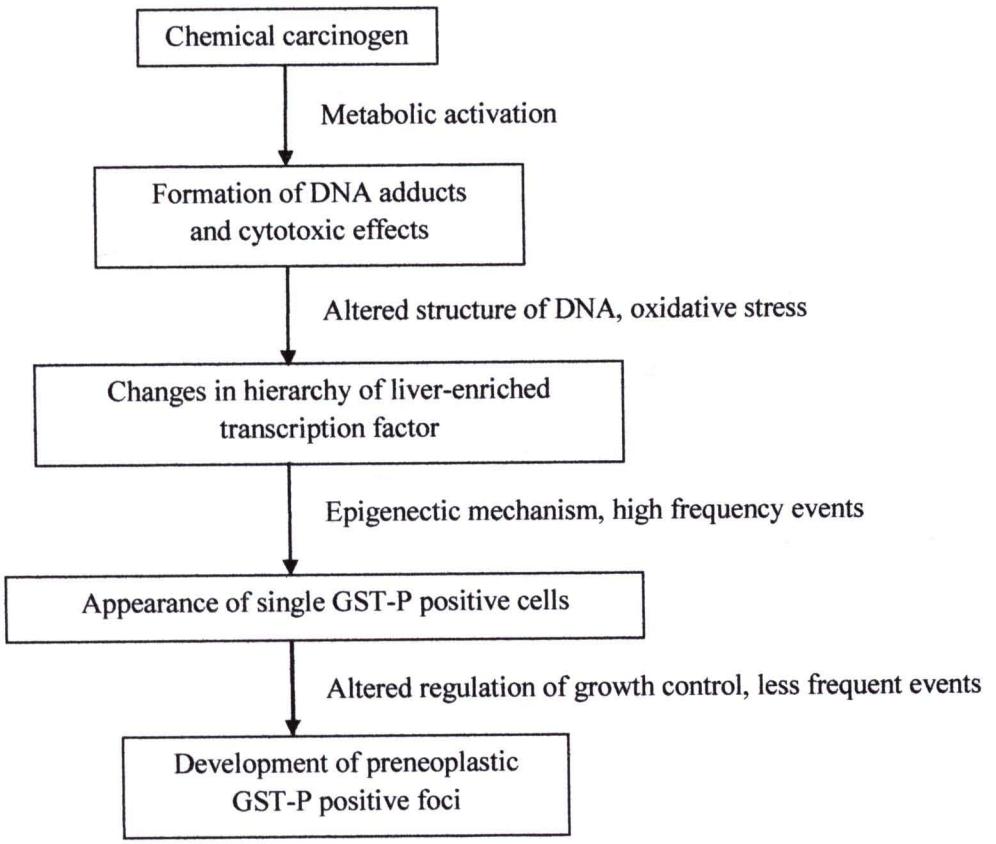


**Figure 1-5** Hypothetical model for the development of GST-P positive lesions (Higashi *et al.*, 2004)

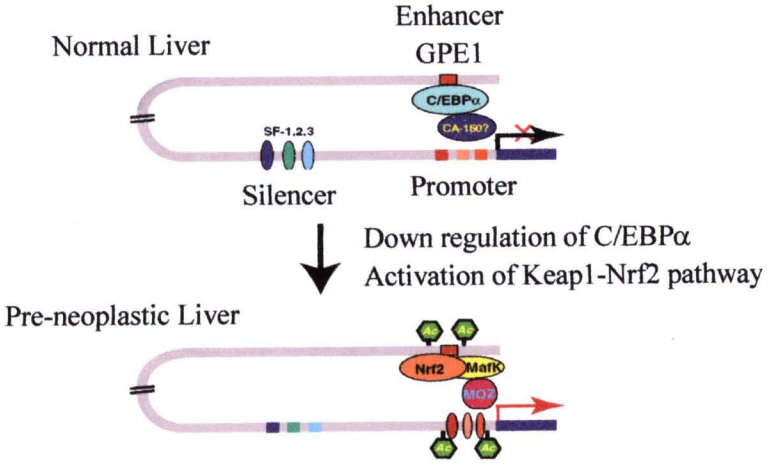


**Figure 1-6** Regulation of the GST-P gene expression in normal liver cells and in the pre-neoplastic lesion (Sakai and Muramatsu, 2007)

### 1.2.3 Diethylnitrosamine and Hepatocarcinogenesis

N-nitroso compounds are known hepatocarcinogenic agents and have been implicated in the etiology of several human cancers (Jagan *et al.*, 2008; Verna *et al.*, 1996). It has been found in a variety of products, including tobacco smoke, meat, and whiskey. Diethylnitrosamine (DEN), one of the most important environmental carcinogens, is known to cause perturbations in the nuclear enzymes involved in DNA repair/ replication and is normally used as a carcinogen to induce liver cancer in animal models. Currently, the mechanism of DEN-induced hepatocarcinogenesis is thought to be as follow in Figure 1-7. DEN is hydroxylated by cytochrome P450 2E1 isozymes in the liver, through an alkylation mechanism, to become bioactive. Subsequently, bioactivated DEN reacts with DNA, causing ethylation of the bases. The ethyl DNA adducts can interrupt base pairing, resulting in mutations and the activation of proto-oncogenes, for example, ras, and inhibition of tumor–suppressor genes, for example, p53, which often result in hepatocellular carcinoma (Sadik *et al.*, 2008; Matsuda *et al.*, 2005).

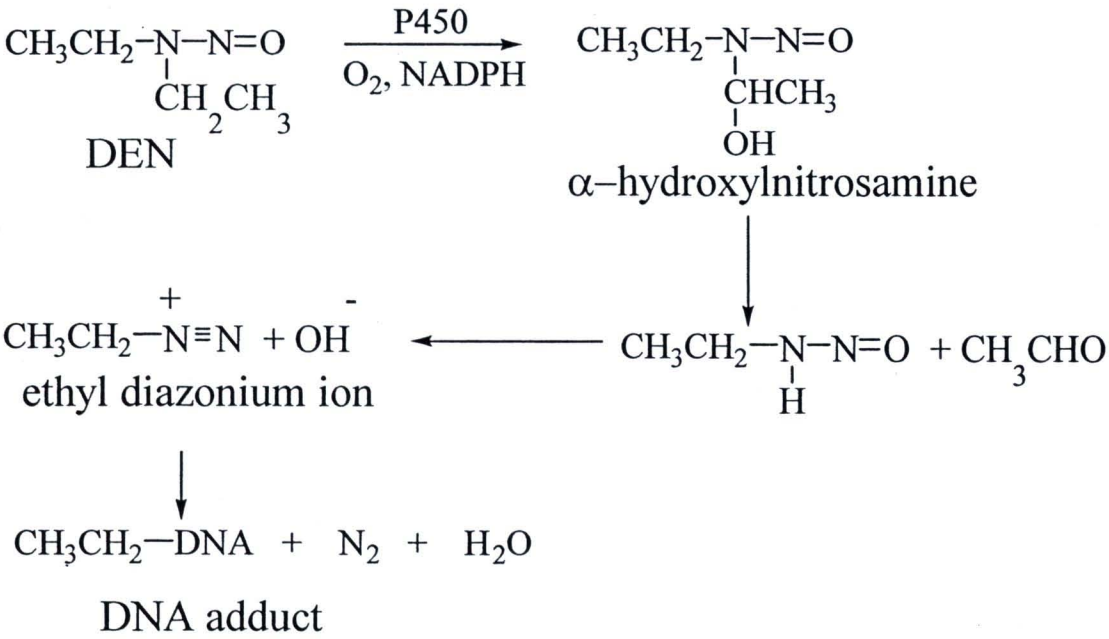


**Figure** 1-7 Biotransformation of diethylnitrosoamine and mechanism of DNA-adduct formation

### 1.2.4 Cancer chemoprevention and carcinogenesis

Cancer chemoprevention has attracted much attention as one of the most practical and realistic strategies in reducing the global burden of cancer (Surh *et al.*, 2008) by slowing the progression of reverse, or inhibiting carcinogenic process (Kwon *et al.*, 2007; Hail. *et al.*, 2008). Chemopreventive agents are important to note that suitable chemopreventive agents should have little or no toxicity, have a high efficacy, be orally administrable, have a known mode of action and low cost. Chemopreventive effects in carcinogenesis are based on the results of *in vitro* studies and animal models (Lee and Park, 2003).

Many natural dietary constituents from herbs can be considered chemopreventive agents because they have been shown to inhibit carcinogenesis process (Debersac *et al.*, 2001). One of the major mechanisms of chemical protection against carcinogenesis, mutagenesis, and other forms of toxicity mediated by electrophiles is the induction of enzymes involved in their deactivation, particularly phase II xenobiotic-metabolizing enzymes (Tan and Spivack, 2009). Dietary chemopreventive compounds functioning as detoxifying enzyme inducers include polyphenols flavonoids, isothiocyanates and organosulfur compounds (Nair *et al.*, 2007).

Chemopreventive agents have been divided into two broad groups, depending on whether or not the compound is perceived to act before, or after, the mutagenic steps of the carcinogenic process. Compounds that prevent mutagenesis have been termed "blocking agents" and compounds that act post-mutagenesis have been termed "suppressing agents" (Henderson *et al.*, 2000; Chen and Kong, 2005; Morse and Stoner, 1993).

Blocking agents, aim to prevent the occurrence of DNA mutation caused by carcinogens (Chen and Kong, 2004). The mechanisms of chemopreventive blocking agents have been assigned to one or more of the following categories: CYP enzyme inhibitors, CYP enzyme inducers, and phase II metabolizing enzyme inducers. The phase I enzymes system is considered to be the main activating machinery, which is capable of converting pro-carcinogens into ultimate carcinogenic forms that can damage DNA, protein and lipids, while phase II enzymes represent the main conjugating and detoxifying machinery (Manson *et al.*, 2000 ; Lee and Park, 2003; Morse and Stoner, 1993).

Conjugating enzymes, such as NADPH quinone reductase, UDP-glucuronosyl transferases and glutathione-*S*-transferase facilitate the elimination of carcinogens (Yates and Kensler, 2007). Moreover, blocking agents can alter rates of DNA repair and scavenging of reactive oxygen and other free radical species. Even if DNA has been damaged, blocking agents can still be effective at limiting further adduct formation (Manson *et al.*, 2000).

Suppressing agents mostly interfere with the promotion and progression of carcinogenesis (Chen and Kong, 2004; Morse and Stoner, 1993). Generally, the chemopreventive activity of suppressing agents is attributed to their influence on cell proliferation, differentiation, senescence, and/or apoptosis (Chen and Kong, 2004) to normal levels, thus preventing the accumulation of damaged cells, Figure 1-8 (Manson *et al.*, 2003).

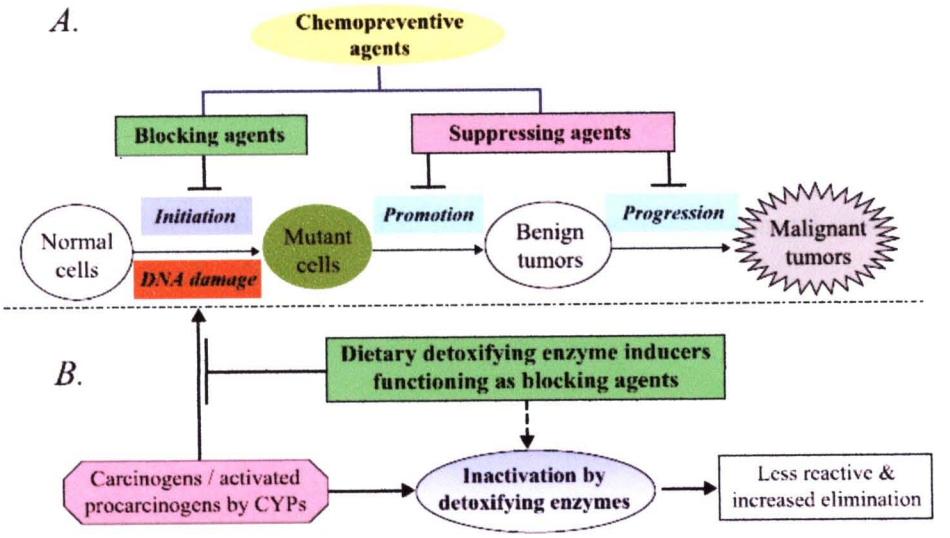


**Figure 1-8** Role of dietary detoxifying enzyme inducers in chemoprevention (A) Concept of chemoprevention (B) Inhibition of carcinogenesis by detoxifying enzyme inducers (Chen and Kong, 2004)

### 1.2.5 Xenobiotic-metabolizing enzymes

The majority of xenobiotics which enters to the body can undergo enzymatic reactions known as "biotransformations". As a result, the xenobiotic molecule generally becomes less lipophilic and more polar so that can be excreted through the normal excretion routes (Nebbia, 2001). Central to the defensive mechanism against toxic chemicals is xenobiotic metabolism, especially, enzymes involved in foreign compound metabolism (Henderson *et al.*, 2000).

Xenobiotic metabolizing enzymes play a major role in regulating toxic, oxidative damaging, mutagenic and neoplastic effect of chemical carcinogen (Dasgupta *et al.*, 2003). There are consisting of phase I and phase II system (McKinney *et al.*, 2004). Many carcinogens are metabolized by phase I enzymes to either biologically inactive metabolites or to chemically reactive electrophilic metabolites that covalently bind to DNA producing carcinogenicity, while phase II enzymes are responsible for detoxification and excretion of activated products. Thus the balance between these plays important roles in preventing initiation of neoplasia because of its role in formation of adducts and mutations and agents inducing drug-metabolizing enzymes are therefore obvious candidates for cancer chemoprevention. Metabolism may lead to an increased rate of chemical detoxification, but in other cases it causes chemical activation to toxic products (Tsuda *et al.*, 2004).

#### 1.2.5.1 Phase I metabolism

##### 1.2.5.1.1 Cytochrome P450

The phase I detoxification system, composed mainly of the cytochrome P450 supergene family of enzymes, is generally the first enzymatic defense against foreign compounds (Liska, 1998). In phase I, a variety of enzymes acts to introduce reactive and polar groups into their substrates. The most common modifications are oxidation, reduction and hydroxylation, usually increasing hydrophilicity (Kaeferstein, 2009). The components of cytochrome P450-dependent mixed-function oxidase system catalyze the splitting of molecular oxygen with one atom being inserted into the drug molecule and the other undergoing reduction to water (Xie *et al.*, 2004).

Cytochromes P450 represents one of the largest and oldest gene superfamilies coding for enzymes present in the genomes of all biological kingdoms (Hannemann *et al.*, 2007). Their name came from their character as hemoproteins and from their unusual spectral properties displaying a typical absorption maximum of the reduced CO-bound complex at 450 nm: cytochrome stands for a hemoprotein, P for pigment and 450 reflects the absorption peak of the CO complex at 450 nm (Bernhardt, 2006). As stated, at least 10 families of Phase I activities have been described in humans. The major P450 enzymes involved in metabolism of drugs or exogenous toxins are the CYP3A4, CYP1A1, CYP1A2, CYP2D6, and the CYP2C enzymes, as shown in Figure 1-9. The amount of each of these enzymes present in the liver reflects their importance in drug metabolism (Liska, 1998).

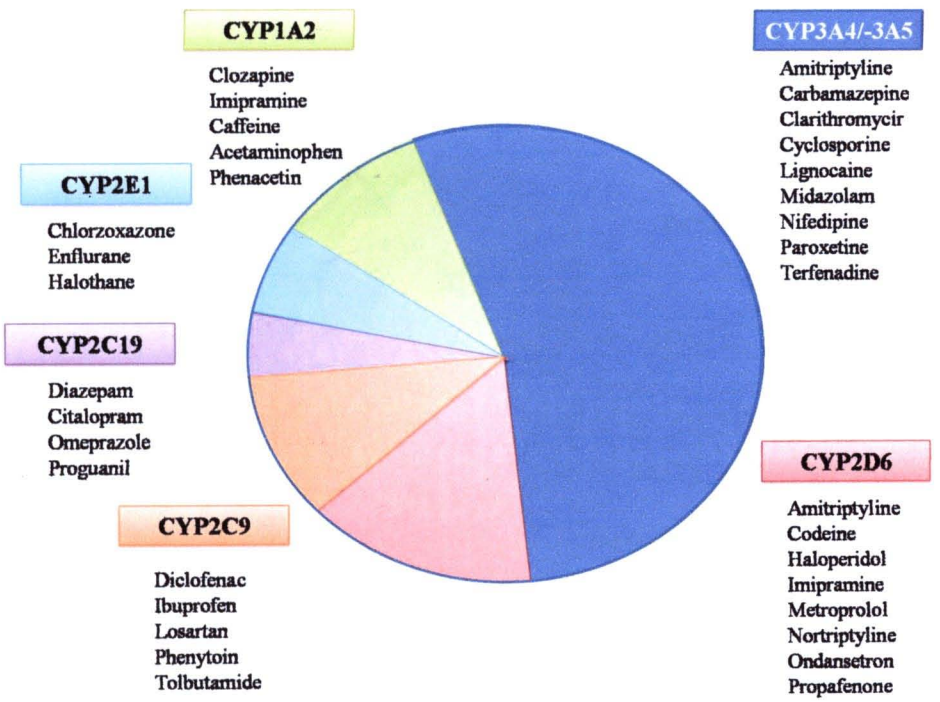


**Figure 1-9** Major detoxification activities in drug metabolism (Liska, 1998)

### 1.2.5.1.2 NADPH cytochrome P450 reductase

The microsomal NADPH-cytochrome P450 reductase (CPR) mediates the transfer of electrons from NADPH to cytochrome P450, other microsomal proteins, and cytochrome *c* (Yim *et al.*, 2005). The microsomal electron transfer system consists of two components; CPR and P450, Figure 1-10. The flavin mononucleotide (FMN) domain of CPR has a similar function to that of the flavodoxins, which contain a single noncovalent-bound FMN prosthetic group, and can substitute for the low-potential ferredoxin during growth under low-iron conditions. The low-potential flavin, flavin adenine dinucleotide (FAD), accepts two reducing equivalents from NADPH (dehydrogenase flavin) and the high-potential flavin, FMN, acts as a one-electron carrier (flavodoxin-type flavin) for the net two-electron transfer from NADPH to P450 (Iyanagi, 2005).

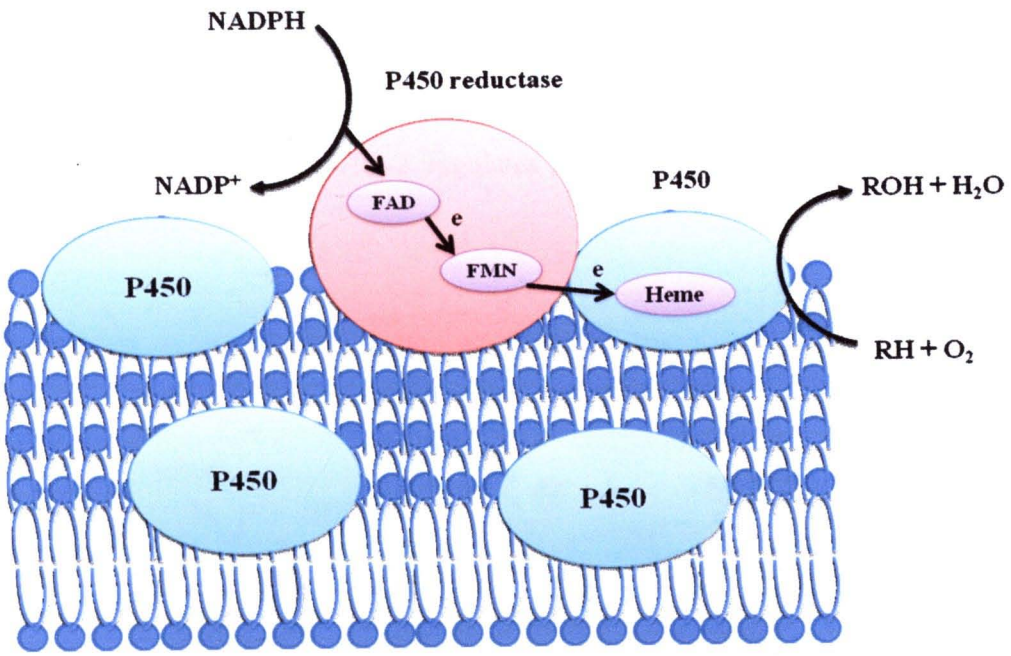


**Figure 1-10** The microsomal NADPH-cytochrome P450 reductase system

### 1.2.5.1.3 Heme oxygenase

The heme oxygenases (HOs) play critical roles in physiological iron homeostasis, antioxidant defense, and as shown from the accumulating evidence in signaling pathways that employ carbon monoxide (CO) as a messenger (Ortiz de Montellano, 2000). Heme oxygenase is the first and the rate-limiting enzyme of the microsomal heme degradation pathway that yields biliverdin, CO, and iron as the final products, Figure 1-11. The microsomal HO system consists of HO and NADPH–cytochrome P450 reductase, and HO was initially supposed to involve cytochrome P450 as a terminal oxidase (Kikuchi *et al.*, 2005).

The isoforms of HO have been reported. Among them, HO-1 is highly inducible by heme itself and several other stimuli including nitric oxide or oxidative stress. It is ubiquitously induced in mammalian tissues and is localized to the endoplasmic reticulum, caveoli, and mitochondria. HO-2 is constitutively expressed that expressed in the brain, endothelium, testis, distal nephron segments, and liver. A third isoform, HO-3, was also described but later shown to be a pseudogene. The most widely studied of these proteins is HO-1, which has been reported as an important cytoprotective enzyme modulating tissue response to injury, while HO-2 regulates normal physiological cell activities (Majewska *et al.*, 2008).

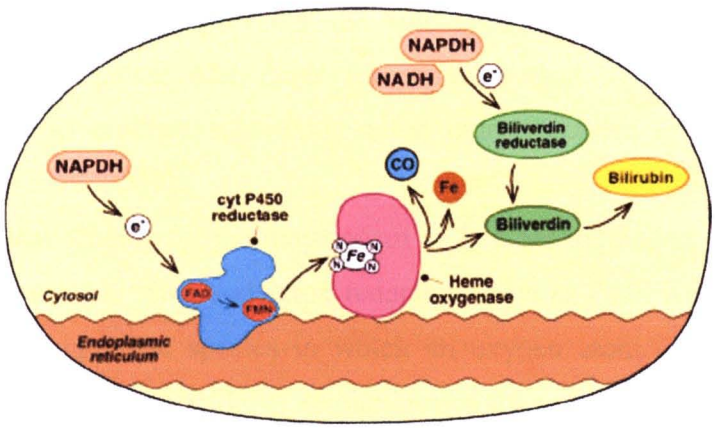


**Figure 1-11** The pathway of heme degradation in mammalian cells (Maines and Gibbs, 2005)

### 1.2.5.2 Phase II metabolism

Phase II metabolism involves conjugation with endogenous hydrophilic compounds with alcoholic or phenolic hydroxyl and amino groups of suitable drugs or phase I metabolites (Kaeferstein, 2009) to further increase polarity and water solubility and therefore drug excretion (Xie *et al.*, 2004). Phase II reactions catalyze conjugation by sulfation, glucuronidation, or glutathioylation and neutralize electrophilic chemicals resulting in a xenobiotic that has been transformed into a water-soluble compound that can be excreted through urine or bile (Tan and Spivack, 2009; Liska, 1998).

#### 1.2.5.2.1 NAD(P)H: quinone oxidoreductase 1

NAD(P)H:quinone oxidoreductase 1 (NQO1: DT-diaphorase, Figure 1-12) plays a pivotal role in detoxifying quinones to their corresponding hydroquinone derivatives. Such an effect helps in maintaining endogenous antioxidants like ubiquinone and vitamin E in their reduced and active forms, thus protecting tissues from mutagens, carcinogens, and oxidative stress damage (Bianco *et al.*, 2005; Anwar-Mohamed and El-Kadi, 2009). NQO1 is a homodimeric flavoenzyme that present in many tissues but is most abundant in liver and can also be elevated in colon, liver, and breast tumors relative to surrounding normal tissue (Misra *et al.*, 2000). NQO1 is considered to be a deactivation enzyme, because it catalyzes the two-electron reduction of quinones, including membrane ubiquinone. This reaction prevents the one-electron reduction of quinones by cytochrome P450 reductase and other flavoproteins that would redox cycle with molecular oxygen to generate superoxide anion radical (Cullen *et al.*, 2003; Anwar-Mohamed and El-Kadi, 2009).

A hypothetical scheme for the metabolism of 1, 4-benzoquinone, a simple quinone is shown in Figure 1-13. Single-electron reduction, such as P450 reductase produces a highly reactive semiquinone species in which an oxygen atom contains an unpaired electron. Due to this electrophilicity, semiquinones are capable of direct reaction with cellular macromolecules including protein and DNA, and this may ultimately lead to neoplasia. Redox-cycling may also occur; the unpaired electron from the semiquinone can be used to reduce $Fe^{3+}$ to $Fe^{2+,}$ which in turn drives the Fenton reaction leading to hydroxyl radical production. Alternatively, a semiquinone may reduce molecular

oxygen, which can lead to generation of singlet oxygen and superoxide-driven Fenton reactions. ROS can cause lipid peroxidation, enzyme inactivation and they can attack DNA directly. The exact consequences of semiquinone production will depend upon the chemical in question. NQO1 catalyses the obligatory two-electron reduction of quinones, thus bypassing the semiquinone intermediate. Fully reduced hydroquinones are generally less toxic to the cell and are more readily conjugated and excreted (Nioi and Hayes, 2004).

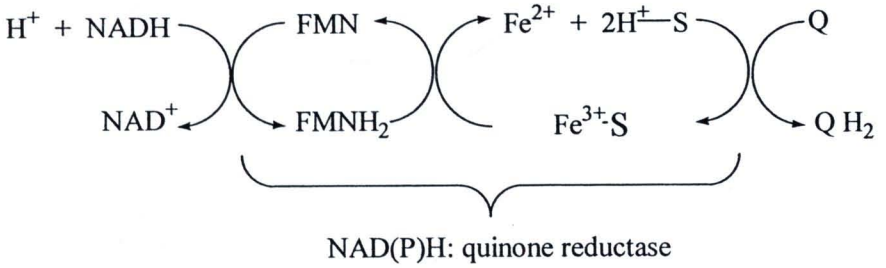


**Figure 1-12** NADPH: quinone oxidoreductase 1

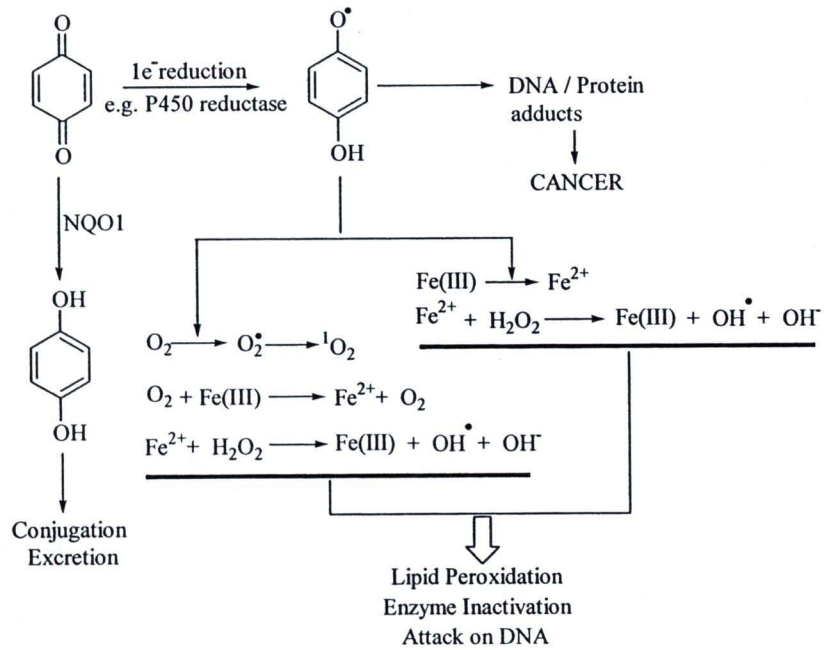


**Figure 1-13** Consequences of quinone metabolism (Nioi and Hayes, 2004)

### 1.2.5.2.2 UDP-glucuronyltransferase

UDP-glucuronyltransferase (UGTs) isoenzymes comprise a superfamily of integral microsomal glycoproteins with their catalytic domain located in the lumen of the endoplasmic reticulum. These enzymes catalyze the conjugation of glucuronic acid with different substrates, including many structurally different compounds, such as phenols, carboxylic acids, aliphatic and aromatic alcohols, aromatic amines, and physiological molecules, including bile acids, sex hormones, and serotonin (Letelier *et al.*, 2005; Saracino and Lampe, 2007). It is the most important phase II reaction of xenobiotic metabolism. One example is this N-glucuronidation of an aromatic amine, 4-aminobiphenyl, by UGT1A4 or UGT1A9 from human, rat, or mouse liver, as shown in Figure 1-14. The products of this reaction biologically become inactive products, more water soluble and are easily eliminated from the organism.

Many substrates of UDP-glucuronyltransferase are metabolites of phase I reactions, some of which are highly carcinogenic, mutagenic and teratogenic (Bock *et al.*, 1979). UGT isoenzymes may also play important roles in controlling endogenous signal compounds such as hormones. Direct inhibition of UGTs by competitive xenobiotics could significantly affect the production of steroid glucuronides possessing pharmacological activity and, as a result, the physiological function of the responsive tissue (Grancharov *et al.*, 2001).

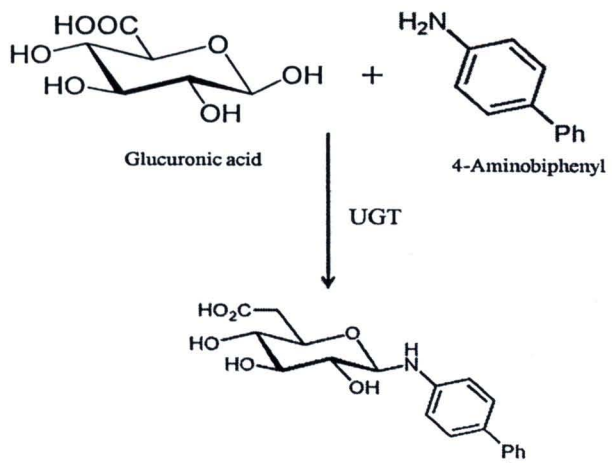


**Figure 1-14** Role of UDP-glucuronyltransferase

### 1.2.5.2.3 Glutathione-*S*-transferase (GST)

Glutathione-*S*-transferases (GSTs) are a family of multi-functional enzymes involved in the cellular detoxification and excretion of many physiological and xenobiotic substances (Wilce and Parker, 1994). The mammalian cytosolic GSTs are divided into 5 subfamilies (Alpha, Mu, Pi, Theta and Zeta) on the basis of similarity in primary structure (Hudson *et al.*, 1998). They catalyse the nucleophilic addition of the thiol of reduced glutathione (γ-glutamyl-cysteinyl-glycine) to electrophilic compounds. The glutathione conjugates so-formed are rendered more water-soluble, thus facilitating their eventual elimination, Figure 1-15 (Wilce and Parker, 1994).

GSTs are known to protect against the toxicity of electrophiles and products of oxidative stress. These enzymes are induced by many foreign and endogenous chemicals, supporting the idea that expression of these enzymes is an adaptive response to potential cellular damage. Substrates of the enzymes also induce GSTs, and it is possible that these substrates and glutathione conjugates may induce other phase II and/or drug metabolizing enzymes, resulting in a multi-faceted response mechanism. Many tumors in humans have been found to over expressed GSTs, yielding resistance to chemotherapeutic drugs. These observations have led to the hypothesis that, at either the cellular level or in whole organisms, the resistance or sensitivity to endogenous genotoxic compounds or therapeutic drugs is a function of the relative expression of GST enzymes (Hudson *et al.*, 1998).

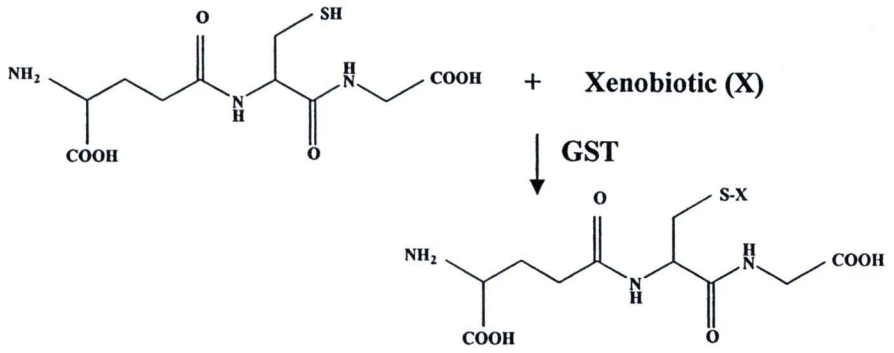


**Figure 1-15** Role of glutathione-*S*-transferase (Townsend and Tew, 2003)

### 1.2.6 Effect of flavanones on xenobiotic-metabolizing enzymes

Flavonoids are a large group of phenolic plant constituents. The structure of flavonoids are consists of 2 benzene rings (A and B), which are connected by an oxygen-containing pyrene ring (C), as shown in Figure 1-16 (Erlund, 2004). Based on their skeleton, flavonoids are classified into 8 groups: flavans, flavanones, isoflavanones, flavones, isoflavones, anthocyanidines, chalcones and flavonolignans (Hodek *et al.*, 2002).

Flavanones are a subclass of flavonoids and rich in seeds, fruit skin, bark, and flowers of most plants. The chemical nature of the flavanones depends on structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (Aherne and O'Brien, 2002). The chemical structures of some representative flavanones are shown in Figure 1-17. Several lines have shown their pharmacological and biochemical functions including anti-oxidative, anti-inflammatory, and anti-bacterial bioactivities (Manthey *et al.*, 2001; Moulari *et al.*, 2006). Hesperidin, a flavanone glycoside in orange juice, suppressed chemically induced carcinogenesis of colon, urinary-bladder, oesophageal, pulmonary, and oral cancers in rodent models (Kohno *et al.*, 2001; Tanaka *et al.*, 1997; Yang *et al.*, 1997). Silibinin, a flavanone isolated from the fruits of milks thistle, is used clinically as an antihepatotoxic agent and recent studies have revealed its anti-metastasis effects on several cancer cells (Chen *et al.*, 2005; Chen *et al.*, 2006). Some studies have indicated flavanones containing none or single hydroxyl group show greater anti-proliferation potential in colorectal carcinoma cells and mouse fibroblast NIH3T3 cells than flavanones with more hydroxyl group substitutions (Ko *et al.*, 2002).

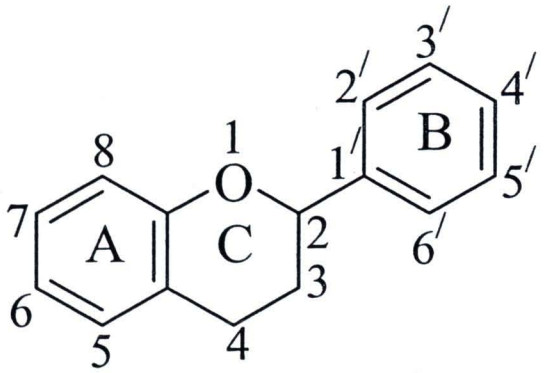


**Figure 1-16** The basic structure of flavonoids (Cook and Samman, 1996)

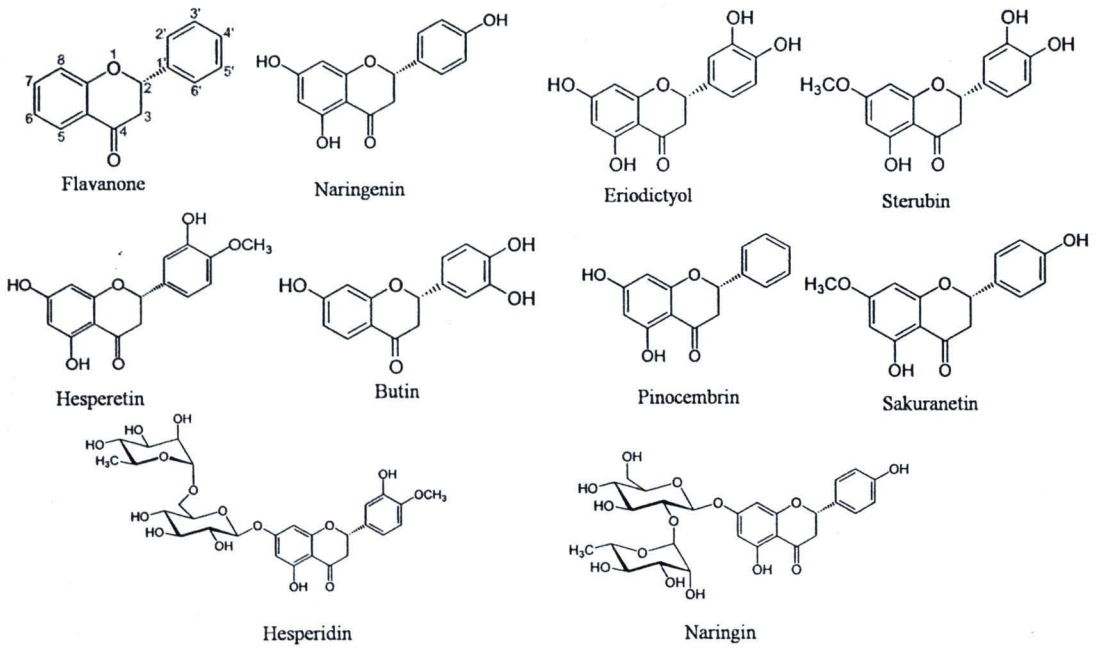


**Figure 1-17** Chemical structures of some representative flavanone

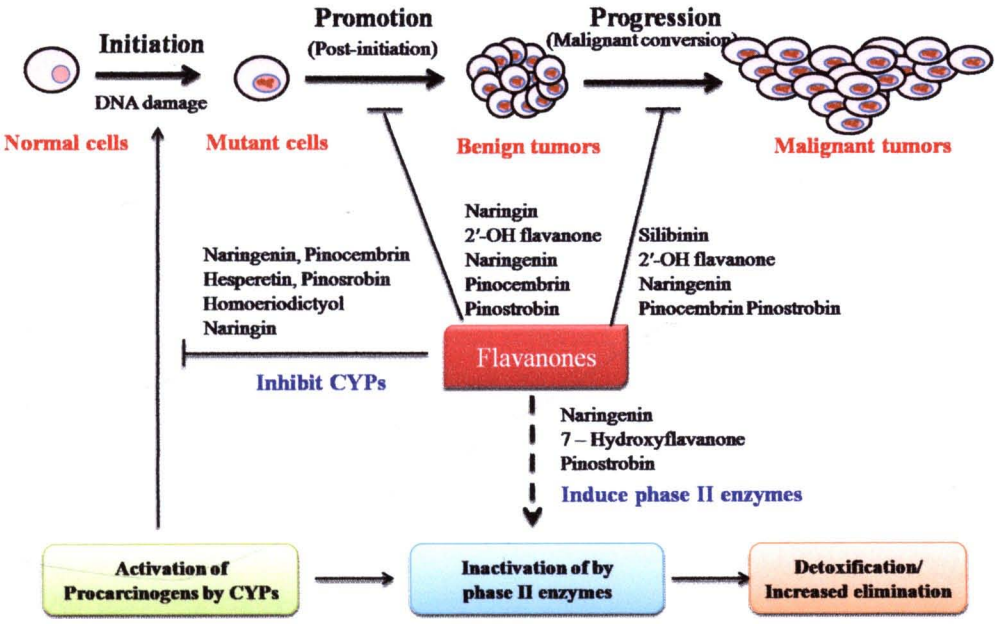


**Figure 1-18** Flavanones that block or suppress multistage carcinogenesis

### 1.2.7 Pinocembrin

Pinocembrin (5, 7-dihydroxyflavanone, Figure 1-19) is the most abundant flavonoids in propolis. Previous investigations have revealed pinocembrin isolated from the rhizomes of fingerroot (*Boesenbergia pandurata* (Roxb.) Schltr.) or "Kra-chai" in Thai which give higher than yield than in propolis (Jaipetch *et al.*, 1982; Li Ching *et al.*, 2007). In this study, we used pinocembrin isolated from rhizome of fingerroot, which is a medicinal plant used in cooking and also in folk medicine in Thailand. Several biological actions of pinocembrin have been reported, including antimicrobial (Pepeljnjak *et al.*, 1985; Del Rayo Camacho *et al.*, 1991; Hwang *et al.*, 2003), antioxidant (Santos *et al.*, 1998; Liu *et al.*, 2008; Shindo *et al.*, 2006), and anti-inflammatory effects (Sala *et al.*, 2003; Tuchinda *et al.*, 2002). Furthermore, it was exhibited a strong antimutagenic activity against mutagenic heterocyclic amines using Ames test (Trakoontivakorn *et al.*, 2001). Our previous study demonstrated that pinocembrin had no toxicity in male rat (Charoensin *et al.*, inpress). Moreover, it could inhibit activities of P450 isozymes involved in carcinogen activation (Siess *et al.*, 1995). Base on these observations, we hypothesized that pinocembrin may protect liver cancer formation in diethylnitrosamine-induced rat.

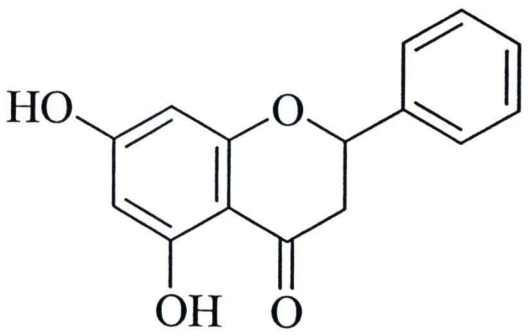


**Figure 1-19** Structure of pinocembrin

## 1.3 Objectives

1. To study the mutagenicity and antimutagenicity of pinocembrin in using rat liver micronucleus test
2. To evaluate the effect of pinocembrin on xenobiotic – metabolizing enzymes
3. To determine the effect of pinocembrin on promotion stage in diethylnitrosamine-induced rat hepatocarcinogenesis