CHAPTER 1

INTRODUCTION

1.1 Statement of the problem

Aflatoxin B_1 (AFB_1), a potent mutagen and carcinogen, is a secondary fungal metabolite commonly found as a contaminant in a variety of foodstuffs. Exposure to aflatoxin has been associated with increases in the incidence of hepatocellular carcinoma (HCC) in several studies. AFB_1 is metabolically activated by the microsomal mixed-function monooxygenase system to 8,9-epoxide (Swenson *et al., 1974*) which binds to nucleophillic sites in DNA to form DNA adducts (Essigmann *et al.*, 1977). The formation of AFB_1-DNA adducts in the target cell gives rise to promutagenic sites in DNA and is considered a critical initiation step in tumor development. The AFB_1-8,9-epoxide can also be hydrolyzed to AFB_1-dihydrodial and bind to protein, particularly albumin, a predominant protein in the body, to form AFB_1-albumin adduct (Skipper *et al.*, 1985). The level of AFB_1-albumin adduct is highly correlated with AFB_1-DNA adduct (Wild *et al.*, 1986). It is well known that single administration of AFB_1 alone is not sufficient to induce liver tumor formation, whereas AFB_1 administration on a daily basis over a 2-8 week period induces 20-100% tumor incidence respectively (Kensler *et al.*, 1986). In addition to both major macromolecular adducts, DNA adducts and albumin adducts, AFB_1 also induced formation of reactive oxygen species (ROS) (Shen *et al.*, 1996) and formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) *in vivo* and *in vitro*. A time- and dose-dependent increase in hepatic levels of 8-OHdG residues in liver DNA treated with AFB_1 has been recently reported (Shen, 1995 ; Yarborough, 1996). Given the importance of oxidative stress in chemically induced carcinogenesis (Klaungig, 1998; Marnett, 2000), it is reasonable to assume that AFB_1-induced ROS formation and oxidative DNA damage may, in addition to the formation of AFB_1-DNA, also have an important role in AFB_1 carcinogenicity. AFB_1 treatment caused significant increase (15-57%) in γ-glutamyl transpeptidase (GGT) activity in liver. The increase in GGT activity indicated the AFB_1 exposure (Rojanapo *et al.*, 1993). In addition to liver parameters, various sera related to hepatic injury were also affected following AFB_1 treatment, such as significant

evaluations in the levels of serum glutamate oxaloacetate transaminase, glutamate pyruvate aminotransferase and lactate dehydrogenase (Rastogi *et al.*, 1995), whereas, glutathione-S-transferase activity was decreased after AFB_1 administration (Kensler *et al.*, 1986 ; Dwivedi *et al.*, 1992 ; Rastogi *et al.*, 1995).

The extent of aflatoxin contamination in foods is a function of the ecology of molds and is not completely preventable. Secondary prevention programs, such as chemoprevention may be useful in this setting. Cancer chemoprevention entails the use of natural or synthetic agents to retard, block, or reverse the carcinogenic process, via mechanisms such as modulation or inhibition of adduct formation to biomolecules in the body or modulation of xenobiotic enzyme activities.

Centella asiatica (L.) Urban (Umbelliferae), a small herbaceous plant that grows predominantly in the Southern Hemisphere, has been commonly used as a traditional herbal medicine in Asia for centuries. In the course of pharmacological studies the plant showed anti-inflammatory activity (Chen *et al.*, 1999), antioxidant activity (Shukla, 1999) and anti-tumor activity (Babu *et al.*, 1995). Moreover, it was found that a titrated extract of *Centella asiatica* could improve the conditions of 5 of 12 clients with chronic hepatic disorders (Darnis *et al.*, 1979). Since this plant has many pharmacological effects, it would be interesting to find out if it has protective effects on AFB_1-induced liver carcinogenesis.

This study was designed to study the modulation effect of water extract of *Centella asiatica* on AFB_1 induced liver carcinogenesis in rat. The chemopreventive ability of the extract will be investigated by focusing on the serum AFB_1-albumin adduct and 8-OHdG formations in liver as well as determining of GGT activities in serum in AFB_1-treated rats, respectively.

1.2 Literature review

1.2.1 Aflatoxin B_1

Aflatoxin B_1 (AFB_1) is a potent human liver carcinogen (IARC, 1993) formed as a secondary metabolite of the moulds *Aspergillus flavus* and *Aspergillus parasiticus*. It is found as a food contaminant in a variety of foods including corn, peanuts, soybeans, soy sauce and cereal crops (IARC, 1993; Eaton and Groopman, 1994). A relationship between mycotoxin exposure and human liver cancer incidence is suggested by dose-dependent rates in some areas of Africa and

Asia (Peer and Linsell, 1977). Exposure to aflatoxin has been associated with an increased incidence of Hepatocellular carcinoma (HCC) in several correlation studies in Kenya, Swaziland, Mozambique, Transkei and Thailand (Munoz *et al.*, 1987). Although aflatoxins are not extremely toxic, consumption of aflatoxin contaminated food by animals can lead to decreased weight gain, hemorrhaging, and suppression of the immune system (Miller *et al.*, 1994). It is well known that a single administration of AFB_1 alone is ineffective in the induction of liver tumors, whereas AFB_1 administered on a daily basis over a 2-8 week period induces a 20-100% tumor incidence respectively (Kensler *et al.*, 1986).

1.2.2 Aflatoxin B_1 metabolism

AFB_1, the highest concentration of aflatoxin metabolites, is freely soluble in moderately polar solvents (e.g., chloroform and methanol), and especially in dimethylsulfoxide. It also has some water solubility. This compound cannot be destroyed under ordinary cooking conditions or during pasteurization. AFB_1 in itself is not carcinogenic, but is metabolized by the body to produce an ultimately carcinogenic metabolite. Following transport across the plasma membrane, the AFB_1 molecule is activated by microsomal (smooth/tubular endoplasmic reticulum (ER)-associated) mixed-function monooxygenase, which requires cytochrome P450, NADPH and molecular oxygen to form the highly reactive AFB_1-8,9-epoxide (Swenson *et al.*, 1974). The AFB_1 epoxide reacts with DNA to form the AFB_1-N7-guanine adduct (AFB_1-gua). As shown in Figure 1, the AFB_1 epoxide reacts with guanine to form a number of adducts (Essigmann *et al.*, 1977). AFB_1 may also be reversibly converted by NADPH-reductase to aflatoxicol. The aflatoxicol thus may act both as sink and reservoir for AFB_1 (Patterson, 1993). The microsomal monooxygenase system is also responsible for transforming the AFB_1 into polar molecules such as AFM_1, AFP_1 and AFQ_1. Furthermore, the epoxide is unstable and also undergoes hydrolysis to the AFB_1-8,9-dihydrodiol (AFB_1-diol) followed by rearrangement to a putative dialdehyde phenolate intermediate, which is capable of condensing with the primary amino acid group of proteins and other cellular constituents forming Schiff bases. Albumin, the predominant protein, is adducted by AFB_1 (Wild *et al.*, 1986; Sabbioni *et al.*, 1987). AFB_{2a} is thought to be a hydrolytic product of AFB_1 or its conjugate, in the phenolate form which binds to proteins, forming Schiff bases. The toxicity of AFB_{2a} is decreased when administered orally due to non-

absorption in the gut (Thompson *et al.*, 1992). The AFM_1, AFP_1 and AFQ_1 can be eliminated by the hepatocytes, but the epoxide binds to nucleic acid and proteins are thought to be the carcinogenic form of AFB_1. Humans metabolize AFB_1 to an 8,9-epoxide, forming DNA and albumin adducts by the same activation pathways as susceptible animal species. Humans metabolize AFB_1 to the major AFB_1-*N*7-guanine and-serum albumin adduct at levels comparable to those in susceptible animal species.

Conjugation of the reactive epoxide to glutathione mediated by glutathione-*S*-transferase (GST) was the major detoxification reaction of AFB_1 (Degen, 1978). The AFB_1-glutathione conjugate is secreted primary through the bile. The conjugate reduced DNA damage, and this mechanism is important in reducing the tumor burden in experimental animals. Animal species such as the mouse, which are resistant to aflatoxin carcinogenesis, have three to five times more glutathione *S*-transferase activity than susceptible species, such as rat. Humans have less glutathione *S*-transferase activity for 8,9-epoxide conjugation than rats or mice, suggesting that humans are less capable of detoxifying this important metabolite. The conjugate, however, is reported to have the potential to be hydrolyzed by the intestinal microflora, to release the AFB_1 for reabsorption in enterohepatic circulation (Hsieh and Wong, 1982). AFB_1-epoxide might also be detoxified by UDP-glucuronyl-transferase, sulfotransferase and possibly the epoxide hydrolase system (Hayes, 1978).

1.2.3 Integration of AFB_1 biotransformation pathways : Activation versus Inactivation

The fate of AFB_1 is dependent on the relative activity of several biotransformation pathways, in addition to other factors such as DNA repair rates. The amount of mycotoxin that is going to exert carcinogenic or toxic effects will depend on the amount converted to various metabolites as well as on the biological activity of those metabolites. With respect to carcinogenicity, AFB_1-8,9-epoxide is the key active metabolite. As indicated in Figure 2, hydroxylated metabolites of AFB_1 (AFM_1, AFP_1, AFQ_1) are assumed to represent detoxification products. Detoxification of the reactive epoxide forms a dihydrodial that is probably still capable of causing toxic effects (via binding to protein) but presumably is a less potent carcinogenic species than the epoxide.

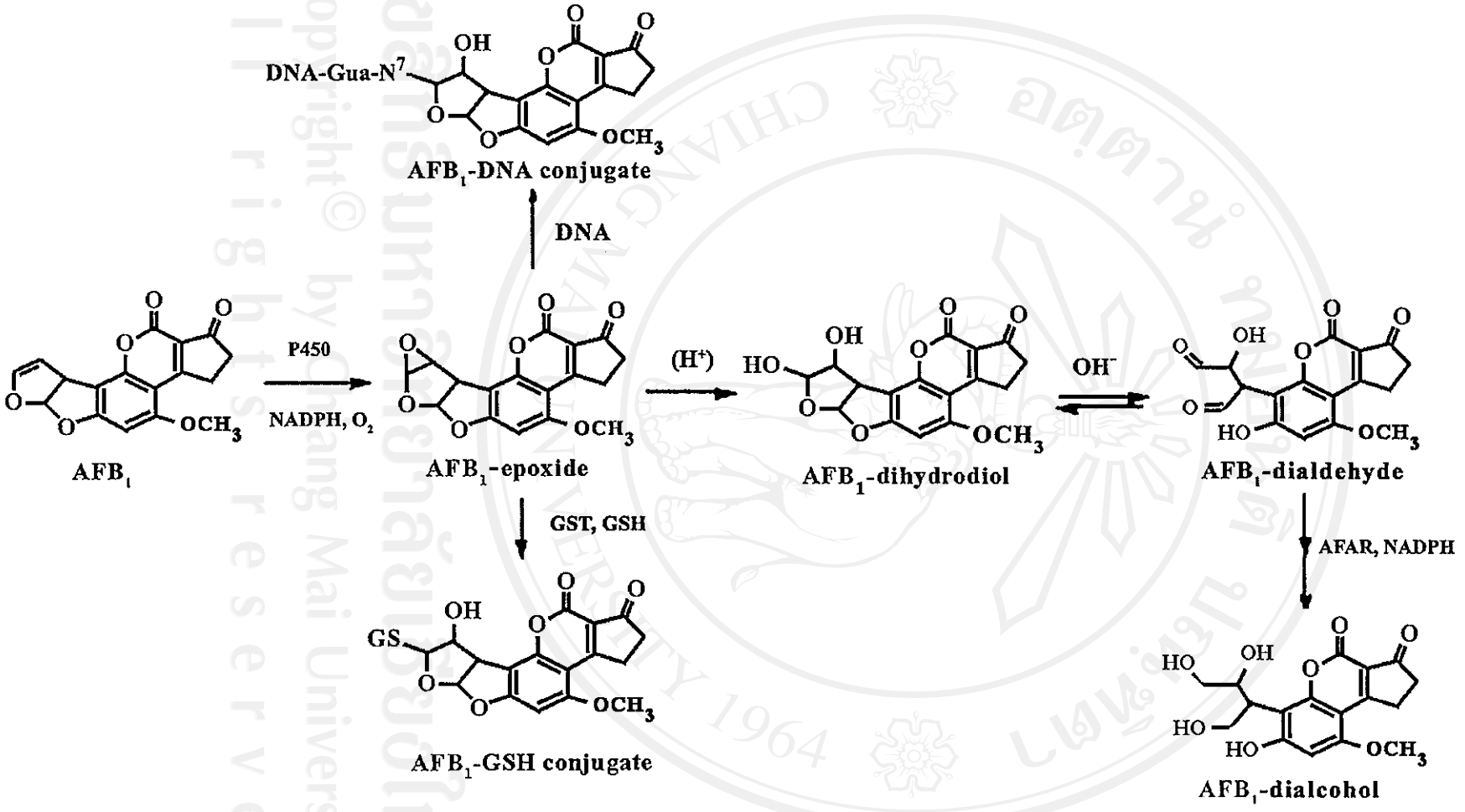

Figure 1 Metabolism of AFB_1. (Derived from Guengerich, 2001)

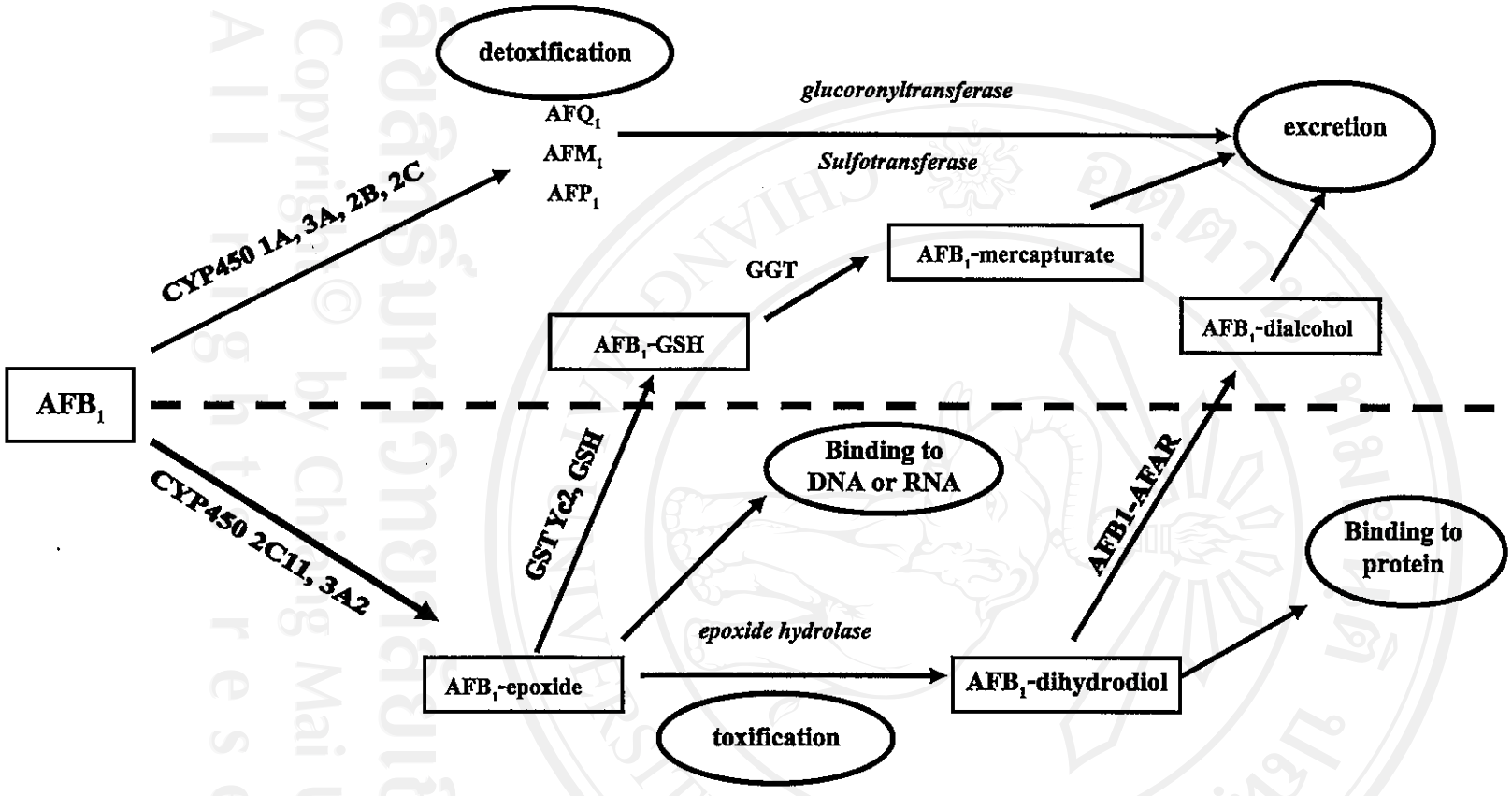

Figure 2 Integration of AFB_1 biotransformation pathway. AFB_1-AFAR, aflatoxin B_1 aldehyde reductase; GGT, γ-glutamyltranspeptidase; GSTYc2, glutathine *S*-transferase containing the Yc2 subunit; GSH, glutathione. (Derived from Manson *et al.*, 1997)

1.2.4 Aflatoxin B_1 exposure and biomarker

The development of molecular biomarkers for aflatoxin is based on the knowledge of metabolism and critical genetic macromolecular adduct formation of these compounds and the possible target sites. These molecular biomarkers may help to identify interindividual differences in susceptibility, so they can be account as cofounders or effect modifiers in data interpretation. The basic mechanism of action of the aflatoxins occurs after they are metabolized by the microsomal mixed function oxygenase system. These enzymes catalyze the oxidative metabolism of aflatoxin B_1, resulting in the formation of various hydroxylated derivatives as well as unstable, highly reactive epoxide metabolites. Synthesis of the 8,9-epoxide has been accomplished, confirming this structure (Baertschi *et al.*, 1988). Microsomes from human and rat livers have been demonstrated to catalyze the production of both the exo and endo form of 8,9-epoxide (Raney *et al.*, 1992). Detoxification of aflatoxin is accomplished by enzymatic conjugation of the hydroxylated metabolites with sulfate or glucuronic acid to form water-soluble sulfate or glucuronide esters that are excreted in urine or bile in conjunction with the unconjugated compounds such as AFQ_1, AFM_1 and AFP_1. An alternative route for removal of AFB_1 from the organism involves the enzyme-catalyzed reaction of the epoxide metabolite with glutathione and its subsequent excretion in the bile. Some of the known detoxification pathways of AFB_1 metabolism have been summarized in Figure 3.

1.2.4.1 AFB_1-murcapturic acid

Detoxification of the AFB_1-epoxide can be mediated in part by glutathione *S*-transferases whose induction could be important in chemoprotection intervention. Thus, biomarkers of the enzymatic conjugation of AFB_1-epoxide with glutathione may be important indices of protection against the toxic effects of this agent. Since glutathione conjugates undergo further metabolic processing *in vivo* to yield mercapturic acid, increased urinary excretion of AFB_1-mercapturate could be expected during chemoprotection intervention. In rats, 1% of the aflatoxin dose was excreted as AFB_1-mercapturate within 24 h. The finding that AFB_1-mercapturate was excreted in urine in a dose-dependent manner provides the basis for investigating its applicability as a biomarker of glutathione *S*-transferase status in aflatoxin chemoprotection studies (Scholl *et al.*, 1997).

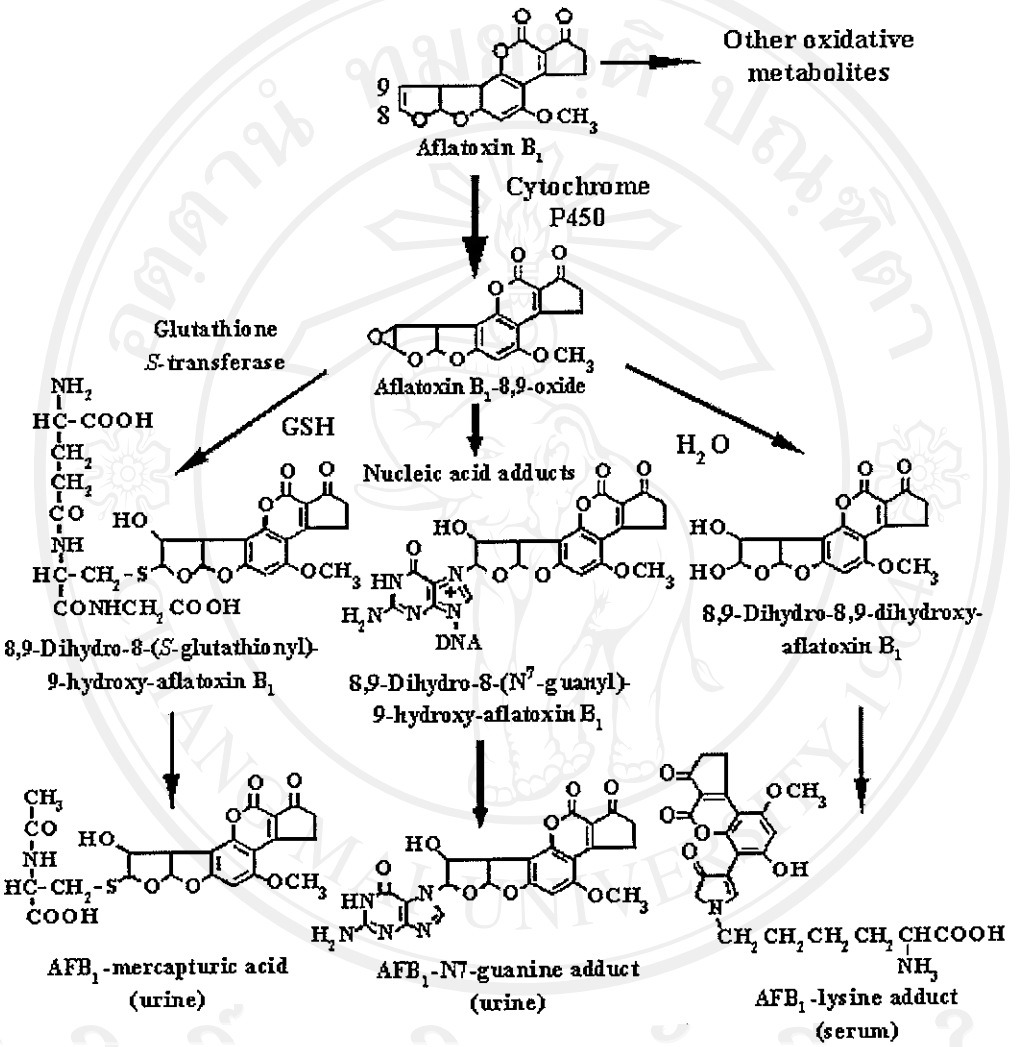

Figure 3 Aflatoxin B_1 exposure and biomarker (Derived from Groopmann, 1994)

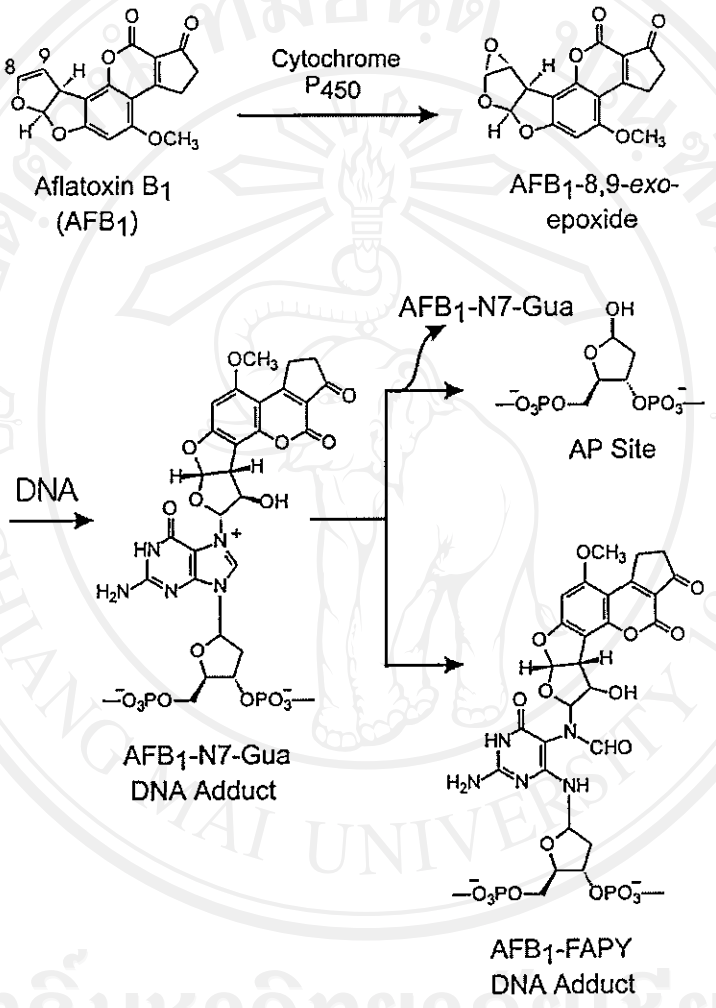

Figure 4 Pathway of metabolic activation leading to DNA adduct formation by AFB_1 (Derived from Smela, 2001)

1.2.4.2 AFB_1-DNA adduct

AFB_1-DNA adduct formation in rat liver is also dose dependent, and DNA adducts are considered to be the promutagenic lesions which give rise to initiate cells, required for transformation and carcinogenesis. As shown in Figure 4, AFB_1-8,9-epoxide reacts covalently with DNA to form adducts that presumably account for biological effects of the toxin. Of the various adducts formed, the most abundant both *in vivo* and *in vitro* is 8,9-dihydro-8-(N_7-Guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N7-Gua). The positively charged imidazole ring of the principle adduct promotes depurination, giving rise to an apurinic site (AP). Alternatively, the imidazole ring of AFB_1-N7-Gua opens to form the more chemically and biologically stable AFB_1-formamidopyrimidine (AFB_1-FAPY) (Busby *et al.*, 1984). While this reaction occurs favorably under basic conditions, the AFB_1-FAPY adduct is a significant product *in vivo* (Croy and Wong, 1981). It is likely that the initial AFB_1-N7-Gua adduct, the AFB_1-FAPY adduct, and the AP site, individually or collectively, represent the chemical precursors to the genetic effects of AFB_1. The AFB_1-N7-Gua adduct has mutagenic properties that correlate with those of the biologically relevant DNA lesion of AFB_1 (Bailey *et al.*, 1996).

1.2.4.3 AFB_1-albumin adduct

The major adduct in rats has been characterized as an aflatoxin-lysine residue (Sabbioni *et al.*, 1987). In addition, 1-3% of the administered aflatoxin dose was bound to circulating albumin after a single dose exposure (Pereira and Chang, 1980). The half-life of albumin in humans is about 20 days. The half-life of albumin was 2.66 days for normal rats (Schreiber *et al.*, 1971). The serum albumin adducts have also been examined as a biomarker of exposure and, because of the longer *in vivo* half-life of albumin, compared to urinary DNA adduct. The serum albumin adduct can integrate exposures over longer time periods. Furthermore, data from human exposure studies have shown that the excretion of the urinary aflatoxin-nucleic acid adducts and formation of the serum albumin adducts are highly correlated (Gan *et al.*, 1988; Groopman *et al.*, 1992).

The measurement of aflatoxin-serum albumin adducts offers a convenient diagnostic test that can be used to screen very large numbers of samples. This method has been extensively validated in experimental trials, and the technique is described in detail by *Wild et al.* (Wild *et al.*, 1990a). The measurement of aflatoxin bound to albumin is valuable for number of reasons:

1. A significant percentage of ingested aflatoxin is covalently bound to albumin, in rat and human 1-3% of single dose (Wild *et al.*, 1986; Sabbioni *et al.*, 1987; Gan *et al.*, 1988).

2. The half life of albumin (approximately 20 days in human and 3 days in rat) allows accumulation of aflatoxin adducts following repeated exposure and thus the adduct level gives an integration of recent past exposure (1-3 month) (Skipper and Tannenbaum, 1990).

3. In rats the level of binding to serum albumin reflects the level of aflatoxin bound to liver DNA (Wild *et al.*, 1986). In humans a similar relationship is suggested by data demonstrating a correlation between aflatoxin nucleic adducts in the urine and aflatoxin bound to albumin (Groopman *et al.*, 1989). These observations are probably a result of the activation of aflatoxin in the hepatocytes and the subsequent binding to the DNA or albumin in the same cells.

4. The structural identification of major albumin adduct formed by AFB_1 (Sabbioni *et al.*, 1987) and AFG_1 (Sabbioni and Wild, 1991) has permitted the development of radioimmunoassays (Gan *et al.*, 1988), enzyme-linked immunosorbent assays (ELISA) (Wild *et al.*, 1990a) and HPLC-fluorescence techniques (Wild *et al.*, 1990a) to measure this adduct.

5. The ELISA has provided a simple, inexpensive assay which is sensitive, specific and applicable to field studies (Wild *et al.*, 1990b). The technique has been designed to be applicable with a minimum of expensive equipment and experience of workers.

1.2.5 DNA damage by Aflatoxin

Enhanced production of reactive oxygen species (ROS), such as superoxide anion, peroxyl, hydrogen peroxide and hydroxyl radicals leads to so-called oxidative stress. Oxidative stress appears to be an important factor in a number of human diseases, including the induction of cancer. Several chemical carcinogens seem to induce oxidative stress either indirectly through modification of cellular antioxidant defense mechanisms or directly. Tumor progression resulted in the development of malignant growth from benign lesions. In this stage oxidative stress may play a direct role in the development of cancer characteristics such as uncontrolled growth, genomic instability, chemotherapy resistance, invasion and metastasis. Tumor cells continually underwent high and persistent oxidative stress, as was shown by the measurement of higher 8-OHdG levels in human carcinoma cells than in surrounding normal cells (Toyokuni *et al.*, 1995). This persistent oxidative stress does not appear large enough to induce cell death, because tumor

cells have decreased cell sensitivity to oxidative stress (Palozza *et al.*, 1994). Cancer cells emerging from the multistep carcinogenic process with inactivated or deleted tumor-suppressor genes and/or activated oncogenes are much less dependent than normal cells on external growth factors because they can manufacture their own factors. High levels of antioxidants induced by persistent oxidative stress in cancer cells increase the chemotherapy resistance of the cells. Increased protein oxidative damage on certain protease inhibitors facilitates tumor invasion. It is postulated that the metabolic process of AFB_1 by cytochrome P450 might be the possible source of the elevated ROS level in AFB_1-treated hepatocytes. The enhanced level of ROS may be responsible for the oxidative damage caused by AFB_1, which may ultimately contribute to the cytotoxic and carcinogenic effect (Shen *et al.*, 1996). Common oxidative DNA damage, including formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), was observed in rat hepatic DNA following exposure to AFB_1. A time- and dose-dependent increase in hepatic levels of 8-OHdG residues in liver DNA treated with AFB_1 has been recently reported (Shen *et al.*, 1995; Yaborough *et al.*, 1996). As a biomarker for oxidative DNA damage, 8-hydroxy-deoxyguanosine (8-OHdG) in tissue or body fluid is known as a sensitive indicator (Shigenaga *et al.*, 1989). 8-OHdG, a major product of oxidative DNA damage, is produced by enzymatic cleavage after 8-hydroxylation of the guanine base of DNA (Richter *et al.*, 1988). Because 8-OHdG is an unstable molecule, it is further hydrolyzed to 8-hydroxyguanine (8-OHG), and both 8-OHdG and 8-OHG are released into systemic circulation and secreted in urine. However, measurement of 8-OHdG has been hampered by the risk of auto-oxidation of deoxyguanosine (dG) during the preparation of samples, resulting in false-high background and low sensitivity (Moller *et al.*, 1997). Measurement of plasma 8-OHdG may provide a more accurate and stable assay for oxidative DNA damage because 8-OHdG is not affected by the work-up procedure. (Shigenaga *et al*, 1994).

1.3 Chemoprevention for AFB_1-induced carcinogenesis

Natural products have long been a fertile source of cures for cancer, which is projected to become the major causes of death in this century. Until recently, cancer prevention has involved mainly attempts to eliminate carcinogenic agents and to detect and remove precancerous lesions. Cancer chemoprevention, defined as the incidence of cancer prevention by administration of one

or more compounds, is becoming one of the most important subjects in cancer research at the present. Chemoprevention is the process of inhibiting, delaying, or reversing carcinogenesis in the premalignant phase and aims to halt or reverse the development and progression of precancerous cells through use of non-cytotoxic nutrients or pharmacological agents during the lengthy time period between tumor initiation and progression (Sporn *et al.*, 1976). Potential chemopreventive agents are structurally heterogeneous and mechanistically diverse. Activities include anti-proliferative, anti-inflammatory, anti-mutagenic and anti-oxidative mechanisms as well as inducing effects on drug detoxication, apoptosis and cell differentiation. There is evidence from epidemiological observations that nutrition influences cancer incidence and offers a variety of potential preventive dietary factors, e.g. vitamins, micronutrients and non-nutritive plant metabolites, including carotenoids, phytosterines, terpenoids, phenolic antioxidants, coumarins, isothiocyanates, tannin, fiber, saponines, etc.

Metabolism of AFB_1 in humans has been well characterized, with activation to AFB_1 8,9-*exo*-epoxide resulting in DNA adduct formation. CYP1A2, 3A4, 3A5, 3A7 and GSTM1 enzymes among others mediate metabolism in humans. The expression of these enzymes can be modulated with chemopreventive agents, resulting in inhibition of DNA-adduct formation and hepatocarcinogenesis in rats. Oltipraz is a chemopreventive agent that increases glutathione conjugation and inhibits some cytochrome P450 enzymes. Results from clinical trials in China using oltipraz are consistent with experimental data in showing that, following dietary exposure to aflatoxins, modulation of the metabolism of aflatoxins can lead to reduced levels of DNA adducts. Experimentally, aflatoxin-induced hepatocarcinogenesis can be inhibited by more than a score of different chemopreventive agents with multiple mechanisms of action (Kensler *et al.*, 1994). Recently, effective inhibition of experimental AFB_1-induced hepatocarcinogenesis by oltipraz, produced protective alterations in the excretion of metabolites of AFB_1 in exposed individuals (Wang *et al.*, 1999).

1.4 Possible useful of *Centella asiatica* as cancer chemopreventive agent

Centella asiatica (L.) Urban, has been used as a traditional herbal medicine in Asiatic countries for hundreds of years. It is a perennial, herbaceous creeper growing to 50 cm with fan shaped leaves. The whole plant is collected and dried for use. It contains a variety of compounds,

but the active ingredients are triterpene saponins, mainly asiaticoside, sapogenin asiatic acid, (Singh and Rastogi, 1969), madecassoside madecassic acid, thiamin, riboflavin, pyridoxine, vitamin K, aspartate, glutamate, serine, threonine, alanine, lysine, histidine, magnesium, calcium and sodium. The plant is indigenous to the warmer regions of both hemispheres, including Africa, Australia, Cambodia, Central America, China, Indonesia, the Lao People's Democratic Republic, Madagascar, the Pacific Islands, South America, Thailand, southern United States of America, and Vietnam. It is especially abundant in the swampy areas of India, the Islamic Republic of Iran, Pakistan, and Sri Lanka up to an altitude of approximately 700 m (Iwu, 1993; Tyler *et al.*, 1988.). *C. asiatica* was found to contain high phenolic contents which exhibit strong association with antioxidative activities (Zainol *et al.*, 2003). The major principles in *C. asiatica* are the triterpenes asiatic acid and madecassic acid, and their derived triterpene ester glycosides, asiaticoside and madecassoside (Kartnig, 1988; Farnsworth and Bunyapraphatsara, 1992). *C. asiatica* is reported to be used in the treatment of leprous ulcers and venous disorders (Iwu, 1993; Kartnig, 1988; Chaudhuri *et al.*, 1978,). Studies suggest that extracts of *C. asiatica* cause regression of inflammatory infiltration of the liver in cirrhosis patients (Darnis *et al.*, 1979). A water-soluble fraction of *C. asiatica* was reported to inhibit hepatic enzymes responsible for barbiturate metabolism (Leung and Stevey, 1996). The antioxidant activity of *C. asiatica* was evaluated by Shuka *et al.* They found that administration of asiaticoside significantly increased the levels of superoxide dismutase, catalase, glutathione peroxidase, vitamin E and ascorbic acid in excision-type cutaneous wounds in rats. The level of antioxidant activity was highest during the initial stages of treatment (Shukla *et al.*, 1999) Researchers at the Amala Cancer Research Centre in Kerala, India, tested both a crude extract of *C. asiatica* and its partially purified fractions (AF) for their anti-tumor activity. AF significantly inhibited the proliferation of the transformed cell lines in Ehrlich ascites tumor cells and Dalton's lymphoma ascites tumor cells with no toxic effects on normal human lymphocytes. AF was also found to inhibit the development of mouse lung fibroblast. Oral administration of both *C. asiatica* and AF retarded the development of solid and ascites tumors, and increased the life span of tumor bearing mice. Tritiated thymidine, uridine and leucine incorporation assays suggested that the fraction acts directly on DNA synthesis (Babu *et al.*, 1995). Fresh juice is reported to have moderate cytotoxic action in human ascites tumor cells (Lin *et al.*, 1972). Reseachers found improvement in 5 of 12

clients with chronic hepatic disorders, treated with a titrated extract of *C. asiatica* (Darnis *et al.*, 1979).

1.5 Objectives of the study

1. To study modulation effects of AFB_1-albumin adduct formation in a single dose of AFB_1-treated rats, using water extracts of *Centella asiatica*.
2. To study the effects of water extracts of *Centella asiatica* on AFB_1-metabolism after multiple doses of AFB_1-exposure in Wistar rats.