CHAPTER 4

DISCUSSION

As AFB_1 poses a great health risk in many parts of world, a great deal of effort has been made to investigate the protective effect of various chemical substances against AFB_1 carcinogenicity (Guengerich *et al.*, 1998). Numerous studies in animal models have examined the relationships between administration of chemoprotective agents and carcinogenic exposure. Several biomarkers have been validated, which allows assessment of whether or not levels of these biomarkers can be modulated in experimental models of chemopreventive agents (Egner, *et.al.*, 1995; Kensler *et.al.*, 1997). Among the various possible biomarkers of aflatoxin B_1 exposure, the measurement of carcinogen -DNA and -protein adducts is of major interest because they are direct products of damage to a critical cellular macromolecular target (Essigmann *et.al.*, 1977). Aflatoxin (AF) had been shown to bind to plasma proteins, primarily albumin, in rats after a single dose of AFB_1 (Skipper *et. al.*, 1985; Wong *et.al.*, 1981). The serum albumin adduct has been examined as a biomarker of exposure and because of the longer *in vivo* half-life of albumin, compared to the urinary DNA adduct, the serum albumin adduct can integrate exposure over longer time periods. Experimental data in rats demonstrated a dose-related increase in binding of AFB_1 to peripheral blood albumin and liver DNA in a fairly constant relationship between the two parameters after single dose or multiple doses (Wild *et.al.*, 1986). The present study was designed to investigate the effects of water extracts of *Centella asiatica* on aflatoxin B_1 (AFB_1) metabolism, using rats that received either a single dose of 40 μg/kg bodyweight of AFB_1 or twenty four doses (multiple doses) of 400 μg/kg bodyweight of AFB_1.

Primary study, the time course of disappearance of AFB_1-albumin adducts following a single dose of AFB_1 exposure was determined. The modulation effect of *C. asiatica* extract on AFB_1-albumin adduct formation was also assessed after rats had received a single dose of AFB_1 administered either before and after or after the AFB_1. The clearance of albumin-bound AFB_1 was measured over a period of 120 hours from the administered dose (40 μg/kg body weight). The maximum level 1.06 ± 0.05 ng/mg serum albumin of AFB_1-albumin adduct was able to be

detected at 4 hours after the dose of AFB_1. By 120 hours this level had declined to 0.12 ± 0.06 ng/mg serum albumin with half-life of the adducts of about 46 hours. For comparison, after a single dose of AFB_1 orally to rats, the plasma half-life of AFB_1 was reported at 55 hours by Wild and his colleagues (Wild *et.al.*, 1986). The half-life of the adduct in this study was therefore shorter than in the previous report. These values are more rapid than the half-life of non-adducted plasma albumin in rats; the half-life of non-adducted albumin was 2.66 days (~ 63 hours) for normal rats (Schreiber *et al.*, 1971). It has been reported that 4-aminobiphenyl- adducted hemoglobin *in vivo* in rats was cleared at a faster rate than unmodified hemoglobin (Green *et. al.*, 1984). From the data obtained, it could be stated conclusively that AF-albumin adduct was cleared more rapidly than the undamaged albumin.

To assess the modulating effect of *C. asiatica* extract on AFB_1 bound to albumin in serum, the time course of AFB_1-albumin adduct formation in rats that received the extract was determined. It was shown that administration of low dose (10 mg/kg bodyweight) of *C. asiatica* extract for 5 days before and after exposure to AFB_1 (group 2) resulted in more accelerated binding of AFB_1 to albumin as demonstrated by the maximum levels of AFB_1-albumin adduct at 2 hours compared with 4 hours in the rats that received AFB_1 only (group 1), while a peak of adduct in serum rats that received high dose (100 mg/kg bodyweight) of *C. asiatica* extract (group 3) was detectable after 4 hours of AFB_1 exposure similar as AFB_1 control group (group 1). In addition, the maximum of adduct level was also observed at 4 hours of AFB_1 dosage in the rats that received the *C. asiatica* extract after AFB_1 exposure (group 4 and 5). It was found the adduct level was cleared from serum by 50% within 24 hours in the AFB_1 control group. While the clearance of adduct in rat serum was delayed by administration of the *C. asiatica* extract prior to AFB_1 exposure (group 2 and 3), a rapid decline of adduct level was observed within 8 hours in the rat that received *C. asiatica* extract after AFB_1 (group 4 and 5). The results showed that *C. asiatica* extract may alter AFB_1-metabolism by not only acceleration of adduct formation but also elimination of adduct from serum.

Many mechanisms of action of chemopreventive agents involve alteration of the metabolic fate of carcinogens by modulating the activities of either, or both the phase I and/or phase II drug metabolizing enzymes. Thus possible mechanisms to explain the observed protective effects might include (1) induction of phase I enzymes (i.e., cytochrome P450) to enhance carcinogen

detoxification: (2) inhibition of phase I enzymes to retard metabolic activation; (3) induction of phase II xenobiotic-metabolizing enzymes (e.g., UDP-glucuronosyl transferase, glutathione *S*-transferase) to enhance carcinogen detoxification and elimination; and (4) nucleophilic trapping of reactive intermediates. As shown in Figure 28, induction of phase II detoxification enzymes enhance AFB_1 inactivation by facilitating the clearance of activated metabolites through conjugation with glutathione (Coles *et al.*, 1985; Kensler *et al.*, 1986). The detoxification pathway of AFB_1 is mainly conjugated by glutathione depended on the glutathione *S*-transferase (GST) activity (Johnson *et al.*, 1997). In addition to Phase II mechanism, the hydrolysis of the epoxide constitutes another pathway against AFB_1 and is mainly due to spontaneous reaction rather than catalyzed by epoxide hydrolase. Rat and human epoxide hydrolases show very little rate acceleration of hydrolysis of AFB_1 epoxide (Guengerich *et al.*, 1996; Johnson *et al.*, 1997). The other pathway in order to eliminate AFB_1 from the body is inhibition of CYP450 enzymes and/or activation of the demethylation of AFB_1 biotransformation (Sukbunteung, 1996). No direct evidence is available from the present study. It was postulated that the effect of *C. asiatica* extract on the faster rate of AFB_1 bound to albumin in serum in the present study may be caused by either induction of phase I enzymes (i.e., cytochrome P450) or catalysis by epoxide hydrolase since the induction by these enzymes was believed to increase the risk of AFB_1 bound to albumin (Figure 27). In addition, it may be suggested that *C. asiatica* extract administration might also affect the AFB_1 detoxification pathway via GSTs. *C. asiatica* administration could increase glutathione content as reported by Veerendra and Gupta (Veerendra and Gupta, 2003). However, further study is needed to clarify these possible mechanisms responsible for the modulation effect of *C. asiatica* extract against albumin adducts formation.

The effectiveness of chemopreventive agent modulation of biological potency may reside in the balance between these activation and inactivation pathways. Since these pathways may be modified during multiple carcinogen exposures, examination of albumin adduct formation and removal during multiple dose protocol may be more relevant than examination after a single dose for studying initiation processes by chemical carcinogens. It is well established that single dose administration of AFB_1 alone is not sufficient to induce liver tumor formation, where daily administration over 2-8 weeks period induces 20-100% tumor incidence (Kensler *et al.*, 1986). Therefore, the effect of *C. asiatica* extract on the formation and removal of AFB_1-albumin adduct

in rats treated with multiple-dosing protocol of 24 doses of AFB_1 was studied, according to the study of Yang and coworker (Yang *et al.*, 2000)

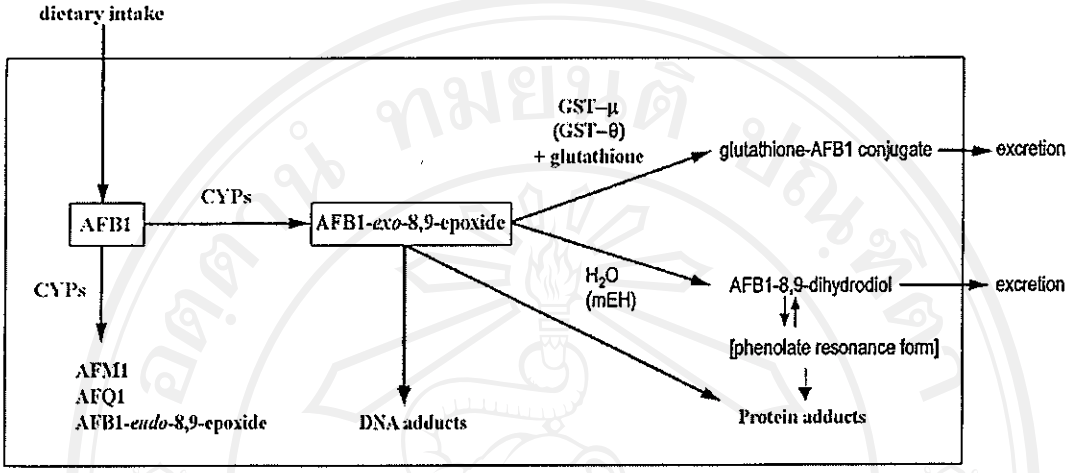

Figure 27 Proposed metabolism of AFB_1. Adapted from Guengerich *et al.* and Eaton *et al.* AFM1, aflatoxin M1; AFQ1, aflatoxin Q1; CYP, cytochrome P450; mEH, microsomal epoxide hydrolase.

It was found that the multiple protocol of AFB_1 administration in this study could not induce rat liver tumors after 24 doses of 400 μg/kg bw of AFB_1. This might be due to rat strains used in the present study which were Wistar instead of F344 as in Yang's report. The Fischer rat was shown to be more susceptible (5 times) to induced liver cancer occurrence than the Wistar rat (Hayes *et al.*, 1991a). It is believed that the toxicity of AFB_1 was selective towards certain species. In contrast with mouse and hamster, rat, guinea pig and man are more susceptible to the hepatotoxic effects of AFB_1 (O'Brien *et al.*, 1983; Hayes *et al.*, 1991b). The toxicity of the mycotoxin is based on a balance between the rate of primary activation of AFB_1 and the rate of detoxification of metabolites or repair of cellular damage. The differential toxicity of AFB_1 between species is though to be due mainly to different levels of activity of xenobiotic-metabolizing enzymes. The livers of mice which are resistant to the hepatotoxic effects of AFB_1 contain high concentrations of an Yc-type GST subunit (McLellan *et al.*, 1991) that has considerable GSH-conjugating activity towards AFB_1-epoxide (Quinn *et al.*, 1990; Ramsdell and Eaton, 1990; Hayes *et al.*, 1991b; Hayes *et al.* 1992). Although this multiple protocol could not induce liver cancer in Wistar rats, the significance elevation of AFB_1-albumin adducts and 8-

OHdG as well as GGT activity was observed in the rats that treated with AFB_1 alone (group 5) after 24 doses of 400 μg/kg bw of AFB_1. This was indicated that this was sufficient model for study of AFB_1 metabolism.

In the present study, accumulation of AFB_1-albumin adduct occurred in rat serum after 4 doses of 400 μg/kg bw of AFB_1 administration. The temporary change of accumulation at 16 doses of AFB_1 exposure was unexpected and required further investigation, however the adduct level reached to the steady stead within 20 doses of AFB_1 exposure (Figure 20). Treatment of low dose (10 mg/kg bw) of *C. asiatica* extract led to significant overaccumulation of the AFB_1-albumin adduct after 4 doses of AFB_1. The mildest accumulation of AFB_1-albumin adduct was observed with administration of high dose (100 mg/kg bw) of *C. asiatica* extract. As shown in the single dose protocol, the pre-administration of *C. asiatica* extract resulted in the lower rate of clearance of AFB_1-albumin adducts in the serum (Figure 17). Therefore, the higher accumulation that occurred in the first period of multiple dose of AFB_1 treatment might result from the lower rate of clearance. Despite the fact that the AFB_1-albumin adduct were gradually cleared from serum as compared to AFB_1 administration only, the reduction of adducts was observed in rats receiving the *C. asiatica* extract. However, the significant diminution of the adduct levels occurred only by administration of the low dose of extract. That accumulation can be diminished with several chemopreventive agents has been reported to be inversely correlated with hepatic glutathione level and glutathione *S*-transferase activity across species (Gorelick, 1990). *C. asiatica* administration could increase glutathione content as reported by Veerendra and Gupta (Veerendra and Gupta, 2003), thus it was postulated that the reduction of AFB_1-albumin adduct as modulated by *C. asiatica* extract might involve glutathione. The pattern of increasing and decreasing of AFB_1 binding in the present study suggested that *C. asiatica* may be involved in the induction and interplay with activating and detoxifying enzymes, including the cytochrome P450 monooxygenase system (CYP450) and glutathione *S*-transferase as well as DNA repair systems. However, more study is needed to clarify which mechanism is responsible for the modulating effect of *C. asiatica* extract.

The formation of 8-OHdG is a result of direct interaction between reactive oxygen species (ROS) and DNA bases (Floyd *et al.*, 1986). Any factors interfering with the formation of ROS will also affect 8-OHdG formation. It has been shown that AFB_1 enhanced ROS formation in

hepatocytes in a dose- and time-dependent manner, leading to oxidative damage (Shen *et al.*, 1995; Yarborough, *et al.*, 1996). Moreover, the metabolic processing of AFB_1 by CYP 450, which accounted for the activation of AFB_1 to the reactive intermediate, AFB_1-8,9-epoxide, is postulated to be the source of the increased levels of ROS in AFB_1-treated hepatocytes (Shen *et al.*, 1995). CYP450 may form ROS by releasing iron from the P450 heme (Baliga *et al.*, 1996). GSH plays a critical role in important cellular functions, which include the maintenance of status of proteins, the destruction of H_2O_2, lipid peroxides, free radicals, translocation of amino acids across cell membrane, the detoxification of foreign compounds and the biotransformation of drugs (James and Hrabison, 1982). The present study showed an increase in the level of 8-OHdG formation by the multiple dose of AFB_1 administration. Administration of *C. asiatica* extract alone could not induce the 8-OHdG formation in liver (Figure 24). The significant higher formation of 8-OHdG was found after 4 doses of AFB_1 by the administration of low dose (10 mg/kg bw) of *C. asiatica* extract. It appeared that *C. asiatica* extract in either low dose of high dose tended to decrease 8-OHdG formation after 8 doses of AFB_1 exposure compare to treatment with AFB_1 alone (Figure 25). Although the 8-OHdG level was not significantly decreased by this protocol, the results showed that 8-OHdG tended to decrease more with a longer treatment of *C. asiatica* extract (Figure 25). By pretreatment with *C. asiatica* extract, it was postulated that raising of 8-OHdG in the first period of the study might be induced by CYP450, as mentioned above, whereas the reduction of 8-OHdG after 8 doses of AFB_1 exposure corresponding AFB_1-albumin adduct might result in increasing of glutathione content by *C. asiatica*. Together with the lower level of AFB_1-albumin adduct formation by *C. asiatica* extract at low dose, it may be concluded that *C. asiatica* extract exerts chemopreventive activity.

γ-glutamyl transpeptidase (GGT) is present in the endoplasmic reticulum of hepatocytes, where its activity is increased in situations leading to microsomal enzyme induction. This is partly explainable in terms of hepatic microsomal enzyme induction, which occurs after exposure to alcohol or to certain drugs. GGT activity is an indicator of the carcinogenicity of AFB_1 (Shen *et al.*, 1995). In the present study the significant increase of the GGT activity was observed in the 24 doses of AFB_1 over control range (3.00-5.00 IU/L). The significant decrease of this enzyme was observed in rats that received the high dose (100 mg/kg bw) of *C .asiatica* extract. This indicated that high dose of the extract might protect against AFB_1-induced cytotoxicity in primary

rat hepatocytes. Taken together with the lower level of 8-OHdG formation by *C. asiatica* extract, it may be concluded that *C. asiatica* extract administration may prevent AFB_1-induced liver damage.

Due to the fact that the modulatory effects of *C. asiatica* on AFB_1-metabolism, as demonstrated by the reduction of AFB_1-albumin adduct level and reduction of oxidative liver DNA damage, were a dose independent manner, thus administration of the *C. asiatica* extract at the low concentration (10 mg/ kg bodyweight) was able to prevent liver damage resulting from AFB_1-metabolism.