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Modulation of Rat Hepatic S9-Dependent Mutagenesis, DNA Binding, and Metabolism of Aflatoxin B1 and Benzo[a]pyrene by Four Chinese Medicinal Herbs

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MODULATION OF RAT HEPATIC S9-DEPENDENT MUTAGENESIS, DNA BINDING, AND METABOLISM OF AFLATOXIN B_1 AND BENZO[A]PYRENE BY FOUR CHINESE MEDICINAL HERBS

by

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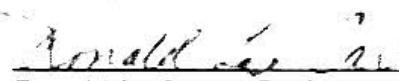

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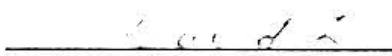

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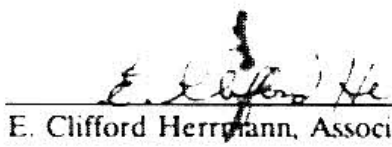

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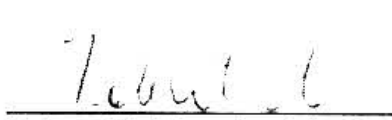

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CHAPTER 1

INTRODUCTION

Since the beginning of civilization, man has been using natural medicinal herbs. The Babylonians began using herbal therapy circa 2600 B.C. and at about the same time the Chinese were practicing herbal medicine. Chinese literature describes a number of herbs used in the treatment of cancer. *Oldenlandia diffusa* (OD) (common name Spreading Hedyotis of the Rubiaceae family) and *Scutellaria barbata* (SB) (Chinese name Pan-chih-lien of the Labiatae family) are two examples consistently found in various herbal formulas for treating liver, lung and rectal cancers. *Astragalus membranaceus* (AM) (Chinese name Huang-chi of the Leguminosae family) and *Ligustrum lucidum* (LL) (common name Privet of the Oleaceae family) are often used as adjuncts in cancer therapy (Hsu et al., 1986).

AM and LL are reported to have important immune enhancing properties. AM has been shown to prevent upper respiratory tract infections caused by the common cold and influenza viruses (Hou et al., 1981). Zhao et al. (1990) found that the injection of extracts of AM into normal and immunodeficient mice enhanced antibody production associated with elevated T helper cell activity. Lau et al. (1989) reported augmentation of macrophage oxidative burst by either

AM or LL, and a synergistic effect was observed with the combination of the two. It has been suggested that these two herbs contain potent immune stimulants which provide a rational basis for their therapeutic use as biological response modifiers in immune restoration (Sun, 1986; Sun et al., 1983a,b). Chu et al. (1988) reported that a fraction from the extract of AM potentiated interleukin-2 in the generation of lymphokine-activated killer cell activity *in vitro*. Chinese botanical literature describes OD and SB as having anti-neoplastic, anti-toxic, and anti-inflammatory properties (Hsu et al., 1986). Crude aqueous extracts of OD and *S. baicalensis*, a species related to SB, are reported to augment phagocytic activity in animals (Dharmananda, 1988). LL inhibited benzo[a]pyrene (BaP)-induced mutagenesis in *S. typhimurium* TA98 by more than 95%, while *A. mongholicus*, a species related to AM, showed no anti-mutagenicity (Meng et al., 1988).

Aflatoxin B_1 (AFB_1), a contaminant of animal and human food in various regions of the world, is a potent toxin produced by certain strains of the fungus *Aspergillus flavus*. It causes mutations in some microbes (Stark, 1980) and in mammalian cells (Ong, 1975; Promchaiyant et al., 1972). It is a potent hepatotoxin and hepatocarcinogen for some animal species and is implicated in the etiology of human hepatic disease in Asia and Africa (Stoloff, 1976; Parkin et al., 1988). AFB_1 is a potent procarcinogen requiring hepatic microsome-mediated metabolic activation to exert its carcinogenic and mutagenic effects. Certain microsomal cytochrome P-450 enzymes catalyze the epoxidation of AFB_1 to form

AFB_1-8,9 oxide which can covalently bind at the N^7 atom of guanine residues of cellular DNA (Essigmann et al., 1977; Garner, 1973; Gurtoo and Dave, 1975; Swenson et al., 1977; Croy et al., 1978; Singer and Grunberger, 1983). It has been demonstrated that the amount of AFB_1 metabolite binding to DNA correlates with increased hepatic carcinogenesis and mutagenesis. AFB_1-DNA binding is a measure of hepatocellular carcinoma initiation (Stark, 1980; Swenson et al., 1977; Whitham et al., 1982; Lotlikar et al., 1984), and is modulated by both phase I and phase II cellular enzymes (Degen and Neumann, 1981). Figure 1 illustrates the metabolic activation and DNA binding of AFB_1 (Beland and Poirier, 1989).

Benzo[a]pyrene (BaP), one of the most common polycyclic aromatic hydrocarbons (PAHs), is a major pollutant of air, water, soil and the food chain (Singer and Grunberger, 1983). BaP and several of its metabolites produce tumors in newborn mice (Buening et al., 1978). Significant correlation between the incidence of human cancer and exposure to PAHs is suggested by epidemiologic studies (Singer and Grunberger, 1983). BaP is a procarcinogen which requires cytochrome P-450 enzymes for activation to BaP 7,8-dihydrodiol-9,10-epoxide (BPDE) that is believed to be essential for the expression of its mutagenicity and carcinogenicity (Gurtoo and Bejba, 1974; Gurtoo and Dave, 1975). BPDE is the major metabolite that binds to DNA *in vitro* and *in vivo*. This epoxide reacts at the C-10 position with the exocyclic amino group at the N^2 position of guanine residues in DNA (Jeffrey et al., 1977; Koreeda et al., 1978). Both phase I and phase II enzymes influence binding of metabolites of BaP to

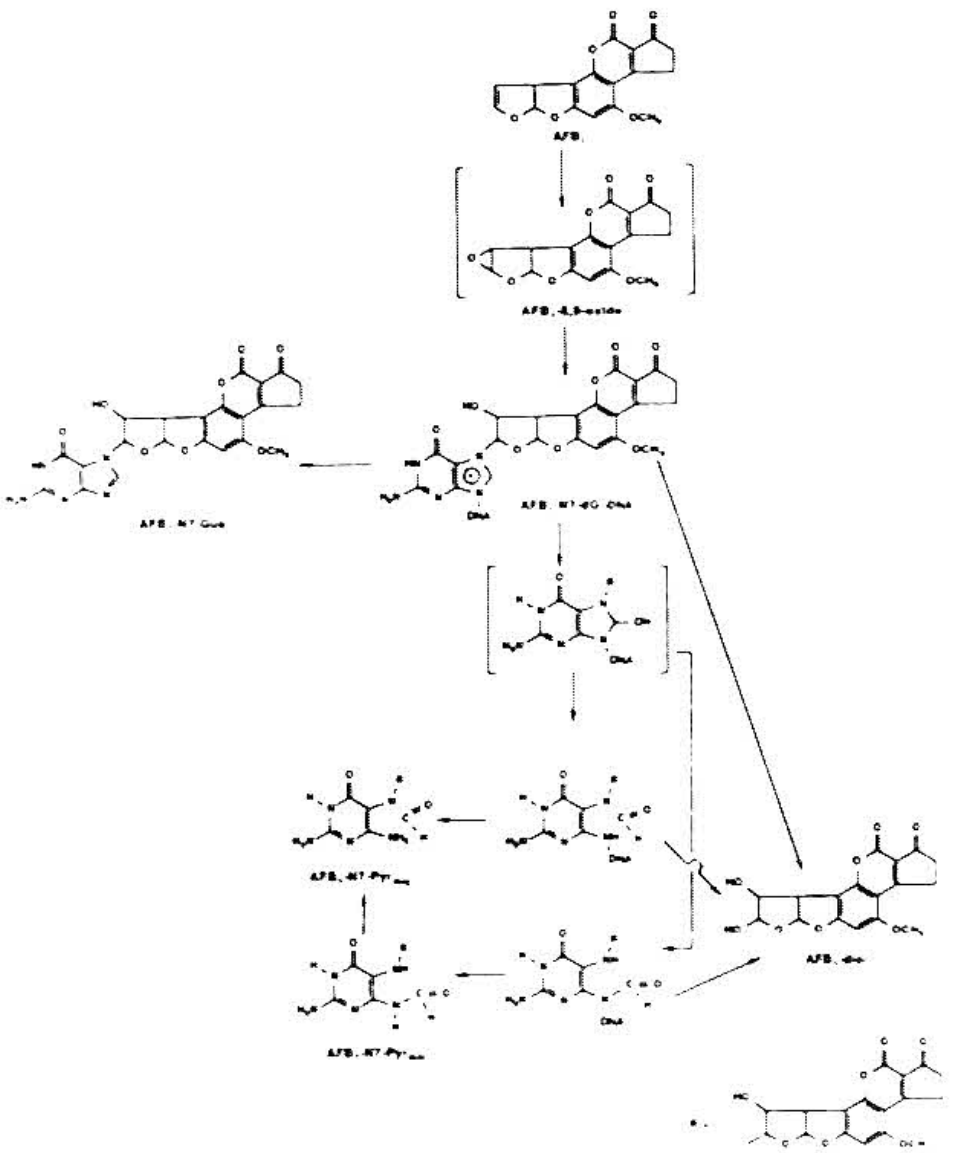

Figure 1 Metabolic activation and DNA binding of AFB_1 (Beland and Poirier, 1989).

DNA (Singer and Grunberger, 1983). Figure 2 shows the metabolism and activation of BaP (Gelboin, 1980) while Figure 3 illustrates the stereospecific metabolism and DNA binding of BaP (Beland and Poirier, 1989).

Cytochrome P450 enzymes play an important role in both carcinogen activation and detoxification. They are members of a group of hemoproteins that catalyze mixed function oxidase reactions. They all exhibit a characteristic ferrous : carbon monoxide complex with an absorbance peak near 450 nm. They are found in most tissues and are highly concentrated in liver endoplasmic reticulum (Guengerich, 1988). Multiple forms of cytochrome P450 enzymes with different catalytic specificity yield diverse products. Oxidation of some xenobiotics renders them more electrophilic with potential for binding covalently to electron-rich nucleic acids and proteins, while other oxidative processes make them more polar and thus more readily excretable (Guengerich, 1988, 1991; Forrester et al., 1990; Paolini et al., 1991). P450 enzymes found in the liver and other organs may enable the organism to inactivate and excrete diverse chemicals encoutered in the environment. A primary route of exposure of animals and humans to environmental chemicals is dietary. These include chemicals synthesized by plants or formed during food storage and preparation. Both P450 (phase I) and conjugating enzymes (phase II) are inducible by many chemicals and drugs, some of which are also substrates for these enzymes. Barbiturates, PAHs found in cigarette smoke, and some compounds present in foods are well known examples of inducers (Okey, 1990; Anderson and Kappas, 1991)

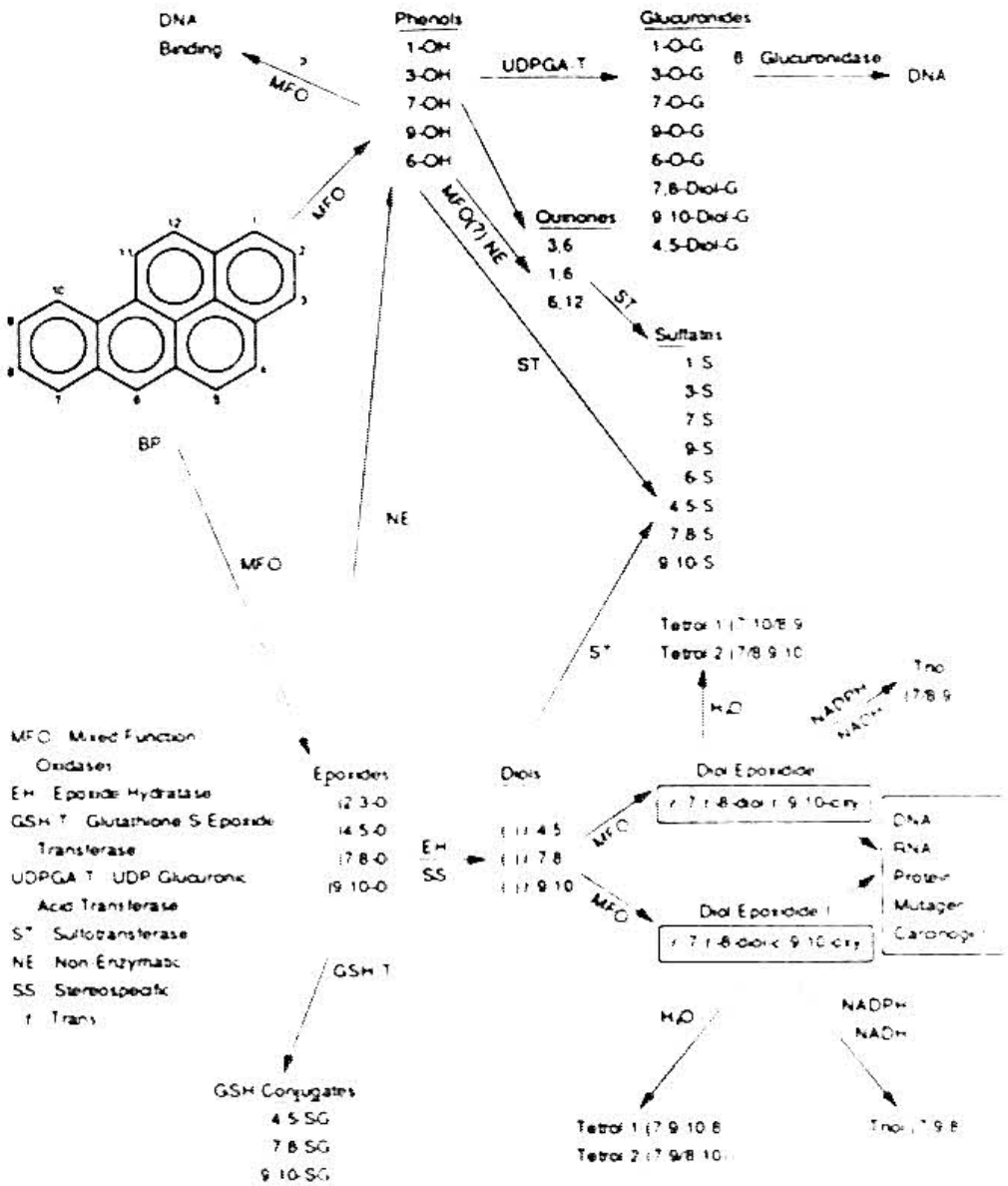

Figure 2. Metabolism and activation of BaP (Gelboin, 1980).

This study was initiated to determine effects of four Chinese medicinal herbs (AM, LL, OD, and SB) on the mutagenesis, DNA binding and metabolism of AFB_1 and BaP.

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CHAPTER 2

CHINESE MEDICINAL HERBS MODULATE MUTAGENESIS, DNA BINDING AND METABOLISM OF AFLATOXIN B_1

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[2]Student's research committee chairman who supervised the research and manuscript preparation

[3]Graduate student who provided technical advice in the early phase of the research

[4]Student's research committee member who supervised the research and manuscript preparation

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CHINESE MEDICINAL HERBS MODULATE MUTAGENESIS, DNA BINDING AND METABOLISM OF AFLATOXIN B_1

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Key words Aflatoxin B_1; Chinese Medicinal Herbs; Mutagenesis; DNA binding

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SUMMARY

Oldenlandia diffusa (OD) and *Scutellaria barbata* (SB) have been used in traditional Chinese medicine for treating liver, lung and rectal tumors while *Astragalus membranaceus* (AM) and *Ligustrum lucidum* (LL) are often used as an adjunct in cancer therapy. In this study, we determined the effects of aqueous extracts of these four herbs on aflatoxin B_1 (AFB_1)-induced mutagenesis using *Salmonella typhimurium* TA100 as the bacterial tester strain and rat liver 9000 x g supernatant as the activation system. The effects of these herbs on [^{3}H]AFB_1 binding to calf-thymus DNA were assessed. Organosoluble and water-soluble metabolites of AFB_1 were extracted and analyzed by high performance liquid chromatography (HPLC). Mutagenesis assays revealed that all of these herbs produced a concentration-dependent inhibition of histidine-independent revertant (His^+) colonies induced by AFB_1. At a concentration of 1.5 mg/plate, SB and OD in combination exhibited an additive effect. The trend of inhibition of these four herbs on AFB_1-induced mutagenesis was: SB > LL > OD > AM. LL, OD and SB significantly inhibited AFB_1 binding to DNA, reduced AFB_1-DNA adduct formation, and also significantly decreased the formation of organosoluble metabolites of AFB_1. Our data suggest that these Chinese medicinal herbs possess cancer chemopreventive properties.

INTRODUCTION

Since the beginning of civilization, man has been using natural medicinal herbs. The Babylonians began using herbal therapy circa 2600 B.C. At about the same time, the Chinese were practicing herbal medicine. Chinese literature describes a number of herbs used in cancer treatment. Among them *Oldenlandia diffusa* (OD) (common name Spreading Hedyotis of the Rubiaceae family) and *Scutellaria barbata* (SB) (Chinese name Pan-chih-lien of the Labiatae family) are consistently found in various herbal formulas, while *Astragalus membranaceus* (AM) (Chinese name Huang-chi of the Leguminosae family) and *Ligustrum lucidum* (LL) (common name Privet of the Oleaceae family) are often used as an adjunct in cancer therapy (Hsu et al., 1986).

AM and LL are important immune enhancing herbs. AM has been demonstrated to prevent upper respiratory tract infections due to the common cold and influenza viruses (Hou et al., 1981). Zhao et al. (1990) found that the injection of AM extracts into normal and immunodeficient mice enhanced antibody production associated with elevated T helper cell activity. Lau et al (1989) reported augmentation of macrophage oxidative burst by either AM or LL, and a synergistic effect was observed with the combination. It was suggested that these two herbs contain potent immune stimulants which may provide the rational basis for their therapeutic use as biological response modifiers in immune restoration (Sun, 1986, Sun et al., 1983a,b). Chu et al. (1988) reported that a

fraction of AM extract potentiated interleukin-2 in the generation of lymphokine-activated killer cell activity *in vitro*. Chinese botanical literature describes OD and SB as having anti-neoplastic, anti-toxic, and anti-inflammatory properties (Hsu et al., 1986). The crude extracts of OD and *S. baicalensis*, a related species of SB, have been reported to augment phagocytic activity in animals (Dharmananda, 1988).

LL inhibited benzo(a)pyrene (BaP)-induced mutagenesis in *S. typhimurium* TA98 by more than 95%; while *A. mongholicus*, a related species of AM, showed no anti-mutagenicity (Meng et al., 1988). The anti-mutagenic and anti-carcinogenic properties of OD and SB have not been studied.

Aflatoxin B_1 (AFB_1) is a potent toxin produced by certain strains of the fungus *Aspergillus flavus*. It contaminates animal and human food in different parts of the world and causes mutations in different microbes (Stark, 1980), and in mammalian cells (Ong, 1975; Promchainant et al., 1972). AFB_1 is a potent hepatotoxic and hepatocarcinogenic agent in some animal species and is suspect in the etiology of human hepatic disease in Asia and Africa (Stoloff, 1976; Parkin et al., 1988). This potent procarcinogen requires hepatic microsome-mediated metabolic activation to exert its carcinogenic and mutagenic effects. Microsomal cytochrome P-450 activates the epoxidation process to form AFB_1-8,9-epoxide which binds covalently with the N^7 atom of guanine of cellular DNA (Essigmann et al., 1977; Garner, 1973; Gurtoo and Dave, 1975; Swenson et al., 1977, Singer and Grunberger, 1983). It has been demonstrated that the level of DNA binding

correlates with hepatic carcinogenesis and mutagenesis. AFB_1-DNA binding is regarded as a measure of hepatocellular carcinoma initiation (Stark, 1980; Swenson et al., 1977; Whitham et al., 1982; Lotlikar et al., 1984), and is modulated by both phase I and phase II cellular enzymes (Degen and Neumann, 1981).

This study determined the effects of four Chinese medicinal herbs on the mutagenesis, DNA binding and metabolism of AFB_1. We now report that three of these herbs (LL, OD and SB) significantly inhibited these processes.

MATERIALS AND METHODS

Chemicals

Nutrient Oxoid #2 broth was purchased from Unipath, Oxoid Division, Columbia, MD. Bactoagar was obtained from Difco Laboratories, Detroit, MI. Aroclor 1254 (polychorinated biphenyl) was obtained from Foxboro, North Haven, CT. Unlabeled AFB_1, authentic metabolites of AFB_1 and calf thymus DNA were purchased from Sigma Chemical Co., St. Louis, MO. [^{3}H]AFB_1 (15 Ci/mmol) was obtained from Moravek Biochemicals, Inc., Brea, CA. Liquid scintillation cocktail was purchased from Allied-Fisher Scientific, Pittsburg, PA. All solvents and chemicals for chromatography were purchased from J.T. Baker Chemical Co., Phillipsburg, NJ. All other chemicals were acquired from Sigma Chemical, St. Louis, MO.

Preparation of herbal extracts

The dried root of AM was a gift from Dr. Yan Sun of the Cancer Institute of the Chinese Academy of Medical Sciences in Beijing, China. The dried seed of LL was a gift from Dr. Robert Lin of Irvine, CA. The crude herbs OD and SB were purchased from Nuherbs Co., Oakland, CA. Each of these herbs was ground to fine powder in a coffee mill (Waring Products, New Hartford, CT) Aqueous extracts were prepared as previously described (Lau et al., 1989). Ten grams of the dried powder of each herb were suspended in 100 ml of distilled water and heated at 56° C for 1 h. The suspension was centrifuged at 2000 rpm for 10 min and then filtered. The filtrate was centrifuged again at 3000 rpm for 10 min and the supernatant was filter-sterilized. The sterile extract was used in all the experiments. The dry weight of each extract was determined. The concentration used in the experiments was based on the dry weight of the extract.

Preparation of hepatic S9

Liver S9 was prepared as previously described (Tadi et al., 1991). Briefly, male Sprague-Dawley rats, each weighing 150-160 g, were given a single injection of Aroclor 1254 (500 mg/kg) five days before time of sacrifice. They were given drinking water and Purina laboratory chow ad libitum until 12 h before sacrificing when the chow was removed. Liver homogenates were prepared aseptically (Maron and Ames, 1983). After determining the protein concentration (Lowry et

al., 1951; Hartree, 1972), one ml aliquots of S9 (31 mg protein/ml) were frozen at -80° C and used as needed.

Mutagenesis assay

The plate incorporation assay of Maron and Ames (1983) was carried out using *Salmonella typhimurium* TA100 (gift from Dr. Bruce N. Ames, University of California, Berkeley, CA) as the tester strain. The following materials were added to sterile disposable test tubes in triplicate in sequence: 100 μl overnight nutrient broth culture of TA100, 500 μl of cofactor mix, various concentrations of herbal extract (0, 1.5, 3 and 6 mg/plate), 50 μl (1.55 mg) of S9 protein, and 10 μl (0.1 μg) of AFB_1 (dissolved in dimethylsulfoxide). The mixture was vortexed with 2 ml of molten top agar containing histidine-biotin and poured onto minimum glucose agar plates. After 48 h incubation at 37° C , His^+ revertant colonies were scored. Spontaneous revertant colonies were subtracted. The experiments were repeated twice. Data were expressed as mean number of His^+ revertants per plate and % inhibition in the presence of various concentrations (mg/plate) of herbal extract. The non-toxic, optimal inhibitory concentrations of these herbs were used for subsequent experiments.

[^{3}H]AFB_1-DNA binding and adduct identification

Liver S9 protein (1.55 mg in 50 μl), herbal extract (AM, 3 mg/ml; LL, 3 mg/ml; OD, 3 mg/ml; SB, 1.5 mg/ml), and 10 μl of [^{3}H]AFB_1 (500 pmol) were

incubated in 1 ml cofactor buffer containing 0.5 mg purified calf thymus DNA for 1 h at 37° C in a shaking waterbath. After incubation, DNA was extracted and processed (Tadi et al., 1991). Radioactivity in the samples was determined by liquid scintillation spectroscopy (Model LS 5801 counter, Beckman Instrument Division, Berkeley, CA). DNA was quantitated by the diphenylamine assay (Burton, 1956). Data were expressed as picomoles per milligram (pmol/mg) DNA. DNA samples were hydrolyzed and extracted in water-saturated *n*-butanol. The butanol layer was dried under N_2 and the residue was redissolved and adducts were separated by HPLC. The radioactivity in collected fractions was determined by liquid scintillation spectroscopy. AFB_1-DNA adducts from hydrolyzed DNA were identified by comparing experimental chromatograms with profiles of authentic adduct standards published by Groopman et al. (1981). Data were expressed as pmol/mg DNA.

Analysis of [^{3}H]AFB_1 metabolites

Liver S9 protein (1.55 mg in 50 µl), herbal extract (AM, 3 mg/ml; LL, 3 mg/ml; OD, 3 mg/ml; SB, 1.5 mg/ml), and 10 µl of [^{3}H]AFB_1 (66.7 pmol) were incubated in 1 ml cofactor buffer in a shaking waterbath at 37° C for 30 min. Samples were extracted with chloroform : ethyl acetate (1:1). The organosoluble fractions were dried under N_2 and redissolved in methanol. Aliquots were separated isocratically by HPLC in 5% tetrahydrofuran, 20% methanol, 20% acetonitrile and 55% water. One ml fractions were collected over 30 min.

Aqueous fractions remaining after chloroform : ethyl acetate extraction were lyophilized, redissolved in 1 ml 50% methanol, passed through a C_{18}-Sep-pak cartridge (Water Associates, Milford, MA.) and analyzed isocratically by HPLC in 25% ethanol and 20 mM triethylammonium formate, pH 3 (Lotlikar et al., 1989) Radioactivity in eluted organosoluble and water-soluble fractions was determined by liquid scintillation spectroscopy and analyzed with a Beckman Datagraph Program in the DU Data Leader software package on an IBM PS/2 model 50 computer. Organosoluble metabolites and water-soluble glutathione conjugates of AFB_1 were identified by comparing profiles with authentic standards. Data were expressed as pmol/mg S9 protein.

Calculation of pmol and statistical analysis of data

The radioactivity (DPM), determined by liquid scintillation spectroscopy, was converted to pmol based on the specific activity of [^{3}H]AFB_1 used. The data from DNA binding and metabolism and those from mutagenesis assays were analyzed with one way analysis of variance (ANOVA) followed by Tukey's multiple range test for honestly significant difference (HSD). Statistical significance was defined as $p < 0.05$. Statistical procedures were performed with Stratgraphics software version 3.1 (STSC, Inc., Rockville, MD)

RESULTS

Mutagenesis assay

Table 1 compares the numbers of His^+ revertants per plate and the percent inhibition of the four herbs on AFB_1-induced mutagenesis from two experiments. All herbs exhibited a concentration-dependent inhibitory effect. SB was more inhibitory than the other herbs at concentrations of 1.5 and 3 mg/plate. LL exhibited slightly greater inhibitory effect than OD. AM exhibited modest inhibition at all concentrations.

When the combined effect of OD and SB on mutagenesis to that of the individual herbs was compared, an additive effect was observed at a concentration of 1.5 mg/plate. The combination of AM and LL at a concentration of 1.5 mg/ml had an effect approaching additive. At 3 and 6 mg/ml the combined effect was less than LL alone. All the dosages used were nontoxic to the bacteria.

[^{3}H]AFB_1-DNA binding and adduct formation

Table 2 illustrates the inhibition of the binding of [^{3}H]AFB_1 metabolites to calf thymus DNA by Chinese herbs. SB at a concentration of 1.5 mg/ml inhibited AFB_1-DNA binding by 90%. OD, LL and AM at 3 mg/ml inhibited the binding by 72%, 74% and 18% respectively. The effects of the four herbs on [^{3}H]AFB_1-DNA adduct formation are shown in Table 3. Except for AM, the herbs

TABLE 1

EFFECTS OF *Astragalus membranaceus* (AM), *Ligustrum lucidium* (LL), *Oldenlandia diffusa* (OD), AND *Scutellaria barbata* (SB) ON AFB_1 - INDUCED MUTAGENESIS IN *S typhimurium* TA100

Treatments		Revertants/plate[a]	% inhibition
Herbal Extracts	Concentration[b] (mg/plate)		
SB	0	1103	–
	1.5	416	63
	3.0	139	87
	6.0	127	89
OD	0	1103	--
	1.5	798	27
	3.0	500	55
	6.0	140	87
OD + SB[c]	0	1103	--
	1.5	83	92
	3.0	63	94
	6.0	76	93
LL	0	1103	--
	1.5	597	46
	3.0	259	76
	6.0	102	91
AM	0	1103	--
	1.5	982	11
	3.0	823	25
	6.0	686	38
AM + LL[c]	0	1103	--
	1.5	496	55
	3.0	273	75
	6.0	275	75

[a]Values are average numbers of histidine-independent revertants of triplicate plates after subtraction of spontaneous revertants (169). [b]Zero concentration indicates AFB_1 alone without any herbal treatment. [c]Equal amounts of OD and SB or AM and LL

TABLE 2

EFFECTS OF CHINESE MEDICINAL HERBS ON [^{3}H]AFB$_1$ METABOLITE BINDING TO DNA*

Herbal Extract	[^{3}H]AFB$_1$ - Bound to DNA (pmol/mg)	% Inhibition
Control	39 ± 2.0	--
SB (1.5 mg/ml)	4 ± 0.8*	90
OD (3 mg/ml)	11 ± 0.5*	72
LL (3 mg/ml)	10 ± 0.3*	74
AM (3 mg/ml)	32 ± 4.0	18

*Calf thymus DNA, rat liver S9, [^{3}H]AFB$_1$, and herbal extract were incubated as described in Materials and Methods. DNA was extracted and radioactivity determined by liquid scintillation spectroscopy. Values are means ± SE of duplicates. * indicates significantly different from control, $p < 0.01$

TABLE 3

EFFECTS OF CHINESE MEDICINAL HERBS ON THE FORMATION OF [^{3}H] AFB_1 DNA ADDUCTS

Herbal Extracts	[^{3}H]AFB_1 Metabolite - DNA Adducts (pmol/mg DNA)				Total AFB_1-DNA Adducts
	AFB_1-N^7-FAPyr (minor)	AFB_1-N^7-FAPyr (major)	AFB_1-diol	AFB_1-N^7-Gua	
Control	0.94 ± 0.03	0.33 ± 0.01	0.71 ± 0.05	26.58 ± 3.86	28.58 ± 2.12
SB (1.5 mg/ml)	0.09 ± 0.05* (90%)	0.05 ± 0.01* (85%)	0.08 ± 0.02* (89%)	2.26 ± 1.04* (93%)	2.48 ± 1.23* (91%)
OD (3 mg/ml)	0.02 ± 0.01* (99%)	0.17 ± 0.13 (49%)	0.05 ± 0.01* (93%)	7.07 ± 0.56* (73%)	7.31 ± 0.61* (74%)
LL (3 mg/ml)	0.04 ± 0.02* (96%)	0.09 ± 0.08 (73%)	0.04 ± 0.03* (94%)	6.14 ± 0.14* (77%)	6.31 ± 0.13* (77%)
AM (3 mg/ml)	0.71 ± 0.23 (25%)	0.18 ± 0.07 (46%)	0.58 ± 0.11 (18%)	22.18 ± 6.19 (17%)	23.65 ± 7.80 (18%)

Calf thymus DNA, rat liver S9, [^{3}H]AFB_1, and each herb extract was incubated as described in Materials and Methods. DNA was extracted, hydrolyzed, and [^{3}H]AFB_1-DNA adducts were analyzed by HPLC. Radioactivity was determined by liquid scintillation spectroscopy. Values are means ± SE. Numbers in parenthesis indicate percent inhibition. *indicates significantly different from control ($p < 0.05$)

significantly inhibited the formation of 8,9-dihydro-8-(2-amino-6-formamido-4-oxo-3,4-dihydropyrimid-5-yl amino)-9-hydroxyaflatoxin B_1 (AFB_1-N^7-FAPyr minor), 8,9-dihydro-8,9-dihydroxyaflatoxin B_1 (AFB_1-diol) and *trans*-8,9-dihydro-8-(guan-7-yl)-9-aflatoxin B_1 (AFB_1-N^7-Gua) at the concentrations tested.

Analysis of [^{3}H]*AFB$_1$ metabolites*

Fig. 1 shows the effects of the four herbs on the formation of organosoluble and water-soluble metabolites of [^{3}H]AFB_1. Except for AM, the herbs significantly inhibited the formation of organosoluble metabolites and therefore yielded higher amounts of unmetabolized AFB_1. The metabolites consisted of aflatoxin M_1 (AFM_1), aflatoxin P_1 (AFP_1), and aflatoxin Q_1 (AFQ_1). Significant inhibition of these metabolites was observed with LL, OD and SB. The inhibition of AFB_1 metabolism is also reflected in the significantly smaller amount of AFB_1-glutathione conjugates formed in the presence of the herbs.

DISCUSSION

We studied four Chinese medicinal herbs which have long been used in cancer therapy in China. Immune modulating effects of AM and LL have been reported (Lau et al., 1989; Chu et al., 1988; Sun, 1986; Sun et al., 1983a,b; Zhao, 1990). OD and SB are often used in the treatment of liver, lung, and rectal cancers in traditional Chinese medicine (Hsu et al., 1986; Chong and Lee, 1988). A related

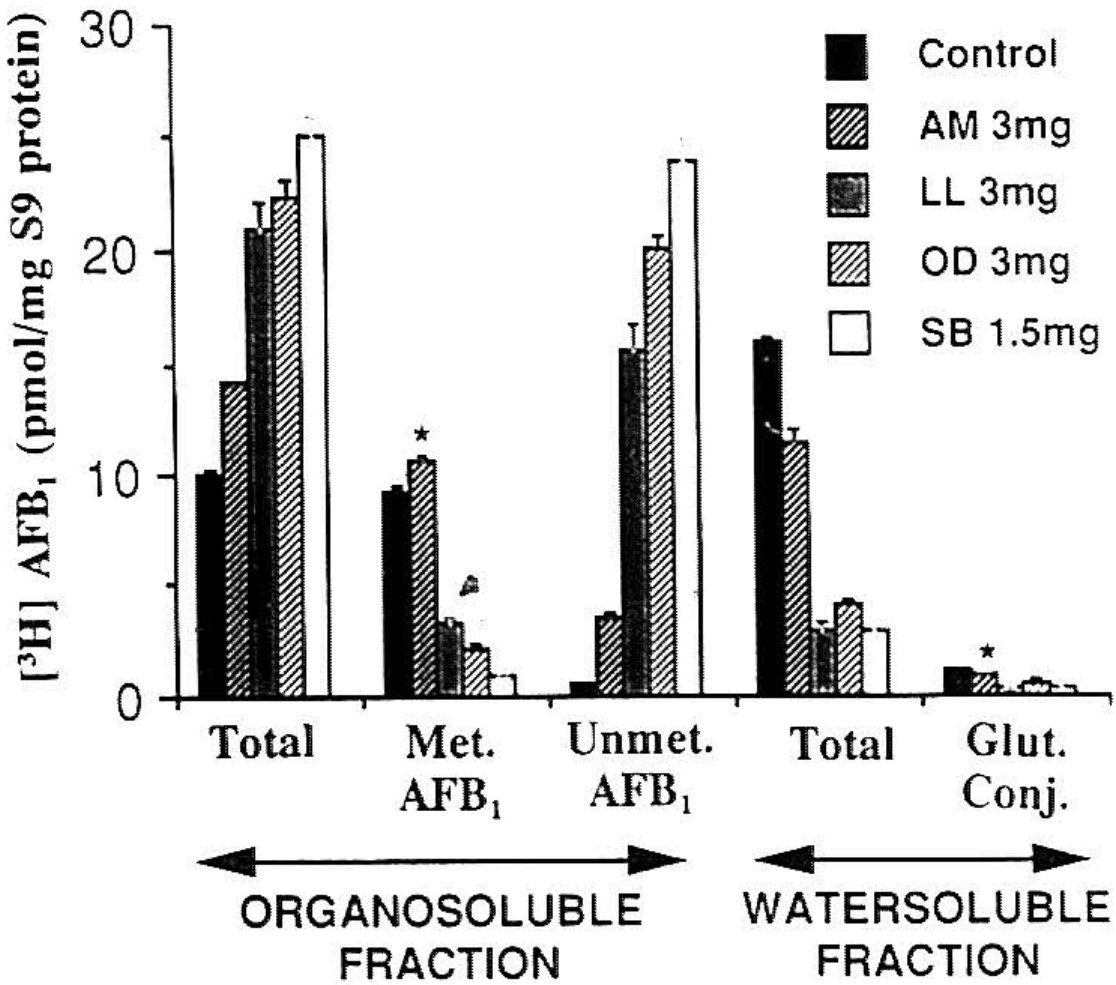

Fig. 1. Effects of Chinese medicinal herbs on the formation of organosoluble and water-soluble metabolites of [^{3}H]AFB$_1$. Metabolites were extracted and analyzed by HPLC as described in Materials and Methods. * indicates not significant from control. All other values were significantly different from controls ($p < 0.05$).

species of AM was reported to have no anti-mutagenic effects while LL showed significant inhibition of BaP-induced mutagenesis in *S. typhimurium* TA98 (Meng et al., 1988).

Wattenberg (1985) has classified chemopreventive agents into three classes based on the mode of action. (1) Metabolic inhibitors prevent the formation of carcinogens from precursor compounds, (2) blocking agents inhibit carcinogenic compounds from reacting with cellular targets, and (3) suppressing agents inhibit the process of neoplastic manifestation. For example, phenols are chemopreventive at all levels, flavones are blocking agents, and plant sterols such as β-sitosterol are suppressing agents. Plant phenols also act as nucleophilic traps (Newmark, 1987). Among the four Chinese medicinal herbs we studied, SB and LL contain phenols and flavones while OD and AM contain β-sitosterol (Hsu et al., 1986).

In this study, we assessed the anti-mutagenic properties of these four herbs in the mutagenesis assay of Maron and Ames (1983) using *S. typhimurium* TA100 as the tester strain. All the herbs exhibited a concentration-dependent inhibition of AFB_1-induced mutagenesis. SB was the most inhibitory and AM was the least. When used in combination, SB + OD had a synergistic or additive effect (Table 1).

The Ames assay has been shown to be useful in correlating *in vitro* bacterial mutagenesis with *in vivo* carcinogenicity in animals (Rinkus and Legator, 1979). In the case of aflatoxins, the relative mutagenic *in vitro* potency has been shown to

correlate with *in vivo* carcinogenic data (Wong and Hsieh, 1976). Metabolic activation of AFB_1 to the 8,9-epoxide is believed to be essential to the expression of its mutagenicity and carcinogenicity (Gurtoo and Bejba, 1974). This epoxide reacts with the N^7 atom of guanine to form AFB_1-N^7-Gua (Croy et al., 1978). Our results suggest that the effects of the Chinese herbs on AFB_1-induced mutagenesis in *S. typhimurium* TA100 (Table 1), on [^{3}H]AFB_1 binding to calf thymus DNA (Table 2), and on AFB_1-N^7-Gua adduct formation (Table 3) were mediated through the inhibition of the metabolism of AFB_1. HPLC analysis of the organosoluble (phase I-mediated) fractions showed a significant decrease in the metabolism of AFB_1 in the presence of LL, OD, and SB (Fig. 1). The greatest effect was with SB. The decreased formation of water-soluble conjugates, specifically glutathione conjugates, in the presence of LL, OD and SB reflects the effect of these herbs on phase I metabolism of AFB_1. An inhibition of the phase I metabolism of AFB_1 would necessarily result in reduced amounts of AFB_1-8,9-epoxide available for conjugation with glutathione. A specific effect of these herbs on phase II enzyme activity cannot be conclusively supported by our data. Aqueous extracts of all four Chinese herbs we tested inhibited AFB_1-induced mutagenesis although variably. Three of them (LL, OD, and SB) significantly inhibited DNA binding and metabolism of AFB_1. Our results therefore suggest that the Chinese medicinal herbs LL, OD and SB may possess cancer chemopreventive properties.

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CHAPTER 3

CHINESE MEDICINAL HERBS MODULATE MUTAGENESIS, DNA BINDING AND METABOLISM OF BENZO[A]PYRENE

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CHINESE MEDICINAL HERBS MODULATE MUTAGENESIS, DNA BINDING AND METABOLISM OF BENZO[A]PYRENE

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ABSTRACT

Oldenlandia diffusa (OD) and *Scutellaria barbata* (SB) have been used in traditional Chinese medicine for treating liver, lung and rectal tumors while *Astragalus membranaceus* (AM) and *Ligustrum lucidum* (LL) are often used as an adjunct in cancer therapy. In this study, we determined the effects of aqueous extracts of these four herbs on benzo[a]pyrene (BaP)-induced mutagenesis using *Salmonella typhimurium* TA100 as the bacterial tester strain and rat liver 9000 x g supernatant as the metabolic activation system. The effects of these herbs on [^{3}H]BaP binding to calf thymus DNA and formation of BaP-DNA adducts were assessed. Organosoluble and water-soluble metabolites of BaP were extracted and analyzed by high performance liquid chromatography (HPLC) and alumina column liquid chromatography. Mutagenesis assays revealed that all four herbs produced a significant concentration-dependent inhibition of His$^+$ revertants induced by BaP. At a concentration of 1.5 mg/plate, SB and OD in combination exhibited an additive effect whereas AM and LL in combination demonstrated an antagonistic effect. The trend of inhibition of these four herbs on BaP-induced mutagenesis was: SB > LL > OD > AM. LL, OD and SB significantly inhibited BaP binding to DNA. LL and SB significantly reduced BaP-DNA adduct formation and SB significantly decreased the formation of organosoluble metabolites of BaP. Our data suggest that these Chinese medicinal herbs possess cancer chemopreventive properties.

INTRODUCTION

The Chinese have practiced herbal medicine for thousands of years. Chinese literature describes a number of herbs used in cancer treatment. Among them *Oldenlandia diffusa* (OD) and *Scutellaria barbata* (SB) are consistently found in various herbal formulas, while *Astragalus membranaceus* (AM) and *Ligustrum lucidum* (LL) are often used as an adjunct in cancer therapy (Hsu et al., 1986).

AM and LL are important immune enhancing herbs. AM has been demonstrated to prevent upper respiratory tract infections due to the common cold and influenza viruses (Hou et al., 1981). The injection of AM extracts into normal and immunodeficient mice enhanced antibody production associated with elevated T helper cell activity (Zhao et al., 1990). Lau et al. (1989) reported augmentation of macrophage oxidative burst by either AM or LL. A synergistic effect was observed with AM and LL in combination. It was suggested that these two herbs contain potent immune stimulants which may provide the rational basis for their therapeutic use as biological response modifiers in immune restoration (Sun, 1986; Sun et al., 1983a,b). Chu et al. (1988) reported that a fraction of AM extract potentiated interleukin-2 in the generation of lymphokine-activated killer cell activity *in vitro*. Chinese botanical literature describes OD and SB as having anti-neoplastic, anti-toxic, and anti-inflammatory properties (Hsu et al., 1986). Crude extracts of OD and *S. baicalensis*, a species related to SB, have been reported to augment phagocytic activity in animals (Dharmananda, 1988).

LL inhibited benzo[a]pyrene (BaP)-induced mutagenesis in *S. typhimurium* TA98 by more than 95%; while *A. mongholicus*, a species related to AM, showed no anti-mutagenicity (Meng et al., 1988). The anti-mutagenic and anti-carcinogenic properties of OD and SB have not been studied.

BaP, one of the most common polycyclic aromatic hydrocarbons (PAHs), is a major pollutant of air, water, soil and the food chain (Singer and Grunberger, 1983). BaP and several of its metabolites produce tumors in newborn mice (Buening et al., 1978) and significant correlation between the incidence of human cancer and PAHs is suggested by epidemiologic studies (Singer and Grunberger, 1983). BaP is a procarcinogen which requires cytochrome P-450 enzymes for activation (Guntoo and Dave 1975). BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) is the major metabolite that binds to DNA *in vitro* and *in vivo*. It reacts at the C-10 position with the exocyclic amino group at the N^2 position of guanine (Jeffrey et al., 1977; Koreeda et al., 1981). Both phase I and phase II enzymes are involved in BaP-DNA binding (Singer and Grunberger, 1983). This study examined the effects of four Chinese medicinal herbs on the mutagenesis, DNA binding and metabolism of BaP.

MATERIALS AND METHODS

Chemicals. Nutrient broth (Oxoid #2) was purchased from Unipath, Oxoid Division (Columbia, MD, USA). Bactoagar was obtained from Difco Laboratories (Detroit, MI, USA). Aroclor 1254 (polychorinated biphenyl) was obtained from Foxboro (North Haven, CT, USA). Unlabeled BaP and calf thymus DNA were purchased from Sigma (St. Louis, MO, USA). Authentic BaP metabolites were obtained from National Cancer Institute Repository (Kansas City, MO, USA). [^{3}H]BaP (66.7 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA). Aluminum oxide for column chromatography was purchased from Bio-Rad (Richmond, CA, USA). Liquid scintillation cocktail was purchased from Allied-Fisher Scientific (Pittsburg, PA, USA). Solvents and chemicals for chromatography were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). All other chemicals were acquired from Sigma (St. Louis, MO, USA).

Preparation of herbal extracts. The dried root of AM was a gift from Dr. Yan Sun of the Cancer Institute of the Chinese Academy of Medical Sciences in Beijing, China. The dried seed of LL was a gift from Dr. Robert Lin of Irvine, CA, USA. The crude herbs OD and SB were purchased from Nuherbs Co., Oakland, CA, USA. Each of these herbs was ground to fine powder in a coffee mill (Waring Products, New Hartford, CT, USA). Aqueous extracts were

prepared by suspending 10 g of the dried powder of each herb in 100 ml of distilled water and heating at 56° C for 1 h. The suspension was centrifuged at 2000 rpm for 10 min and then filtered. The filtrate was centrifuged again at 3000 rpm for 10 min and the supernatant was filter-sterilized (Lau et al., 1989). The dry weight of each extract was determined.

Preparation of hepatic S9. Male Sprague-Dawley rats, each weighing 150-160 g, were given a single injection of Aroclor 1254 (500 mg/kg) five days before time of sacrifice. Drinking water and Purina laboratory chow was provided ad libitum until 12 h before sacrificing when the chow was removed. Liver S9 was prepared aseptically (Maron and Ames, 1983). After determining the protein concentration (Lowry et al., 1951; Hartree, 1972), one ml aliquots of S9 (31 mg protein/ml) were frozen at -80° C and used as needed.

Mutagenesis assays. The plate incorporation assay of Maron and Ames (1983) was employed using *Salmonella typhimurium* TA100 (a gift from Dr. Bruce N. Ames, University of California, Berkeley, CA, USA) as the tester strain. The following materials were added to sterile disposable test tubes in triplicate in sequence: 100 μl overnight nutrient broth culture of TA100, 500 μl of cofactor buffer, different concentrations of herbal extract (0, 1.5, 3 and 6 mg/plate), 1.55 mg of S9 protein, and 5 μg of BaP. The mixture was vortexed with 2 ml of molten top agar containing histidine-biotin and poured onto minimum glucose

agar plates. After 48 h incubation at 37° C , histidine-independent revertant (His$^+$) colonies were scored. Spontaneous revertant colonies were subtracted. Data were expressed as % inhibition in the presence of various concentrations (mg/plate) of herbal extract. The optimal inhibitory concentrations of these herbs were used for subsequent experiments.

[^{3}H]BaP-DNA binding and adduct formation. Liver S9 protein (1.55 mg), herbal extract (AM, LL, OD, 3 mg/ml; SB, 1.5 mg/ml), and [^{3}H]BaP (150 pmol) were incubated in 1 ml of cofactor buffer containing 0.5 mg purified calf thymus DNA for 1 h in 37° C in a shaking waterbath. After incubation, DNA was extracted and processed (Rubano et al., 1990). Radioactivity in the samples was determined by liquid scintillation spectroscopy (Model LS 5801 counter, Beckman Instrument Division, Berkeley, CA, USA). DNA was quantitated by the diphenylamine assay (Burton, 1956). Data were expressed as picomoles per milligram (pmol/mg) DNA. DNA samples were hydrolyzed and extracted in water-saturated *n*-butanol. The butanol layer was dried under N_2 and the residue was redissolved. Adducts were separated by HPLC. The radioactivity in collected fractions was determined by liquid scintillation spectroscopy. BaP-DNA adducts were determined by comparing profiles with authentic adduct standards. Data were expressed as pmol/mg DNA.

Analysis of [^{3}H]BaP metabolites. Liver S9 protein (1.55 mg), herbal extract (AM, LL, OD, 3 mg/ml; SB, 1.5 mg/ml), and [^{3}H]BaP (18.74 pmol) were incubated in 1 ml of cofactor buffer in a shaking waterbath at 37° C for 30 min. Samples were extracted with ethyl acetate : acetone (2:1). The organosoluble fractions were dried under N_2 and redissolved in 1 ml of 60% methanol. Aliquots were analyzed by HPLC, separated in a linear methanol and water gradient of 60%-100% over 50 min at a flow rate of 1 ml/min (Rubano et al., 1990). Aqueous fractions remaining after chloroform : ethyl acetate extraction were lyophilized, redissolved in 2.5 ml of 70% ethanol, and chromatographed on an alumina column (Autrup, 1979). By alumina column chromatography unmetabolized and unconjugated [^{3}H]BaP were eluted with 100 ml of absolute ethanol. Sulfate ester conjugates were eluted with 60 ml of 2X distilled water and glucuronide conjugates were eluted with 80 ml of 0.05 M ammonium phosphate buffer (pH 3). Glutathione conjugates were eluted with 60 ml of 25% formic acid. Samples were collected at a flow rate of 2 ml/min. Radioactivity in eluted organosoluble and water-soluble fractions was determined by liquid scintillation spectroscopy and analyzed with a Beckman Datagraph Program in the DU Data Leader software package on an IBM PS/2 model 50 computer. Organosoluble metabolites and water-soluble conjugates of BaP were identified by comparing profiles with authentic standards. Data were expressed as pmol/mg S9 protein.

Calculation of pmol and statistical analysis of data. The radioactivity (DPM), determined by liquid scintillation spectroscopy, was converted to pmol based on the specific activity of [^{3}H]BaP. The data from DNA binding and metabolism and those from mutagenesis assays were analyzed with one way analysis of variance (ANOVA) followed by Tukey's multiple range test for honestly significant difference (HSD). Statistical significance was defined as $P < 0.05$. Statistical procedures were performed with Stratgraphics software version 3.1 (STSC, Inc., Rockville, MD, USA).

RESULTS

Mutagenesis assays. Figure 1 compares the percent inhibition of the four herbs on BaP-induced mutagenesis. All these herbs exhibited a concentration-dependent effect. SB was significantly more inhibitory than the other herbs at all concentrations. OD, LL and AM had similar inhibitory effects at all concentrations except at 6 mg/plate for AM.

Figure 2A compares the combined effect of OD and SB on mutagenesis to that of the individual herbs. An additive effect was observed at a concentration of 1.5 mg/plate but not at 3 and 6 mg/plate . In Figure 2B, the combination of AM and LL at a concentration of 1.5 and 3 mg/ml was less inhibitory than AM or LL alone and at 6 mg/ml the combined effect was less than LL alone.

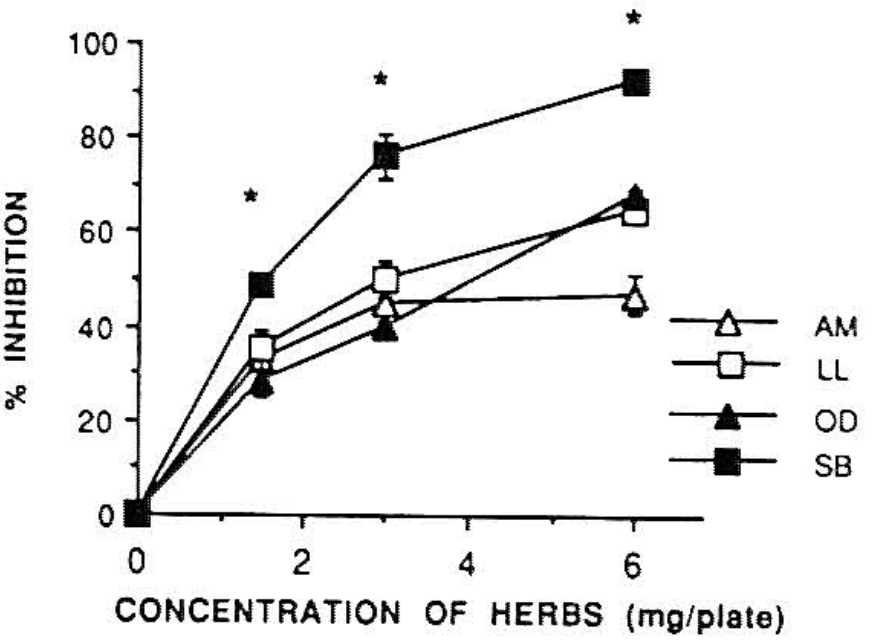

Figure 1. Effects of *Astragalus membranaceus* (AM), *Ligustrum lucidum* (LL), *Oldenlandia diffusa* (OD), and *Scutellaria barbata* (SB) on BaP-induced mutagenesis in *S. typhimurium* TA100. With the exception of LL and OD at the concentration of 1.5 mg/plate, these herbs at all concentrations significantly inhibited mutagenesis, $p < 0.05$ compared with control. * indicates SB was statistically different from the other herbs by Tukey's multiple range test.

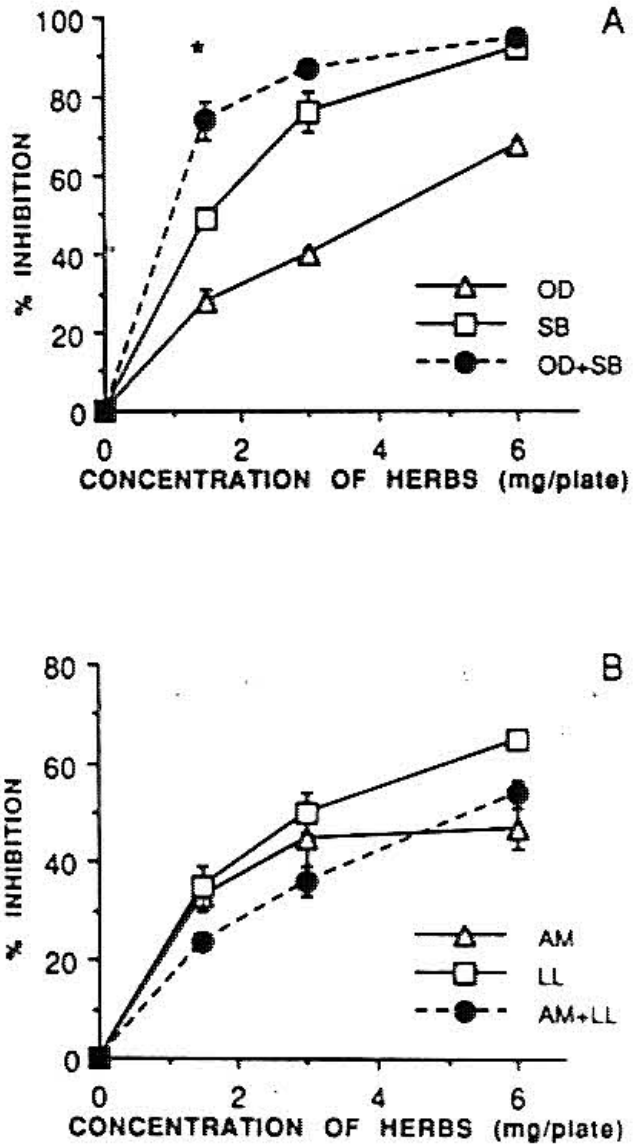

Figure 2. Combination effects of Chinese medicinal herbs on BaP-induced mutagenesis in *S. typhimurium* TA100. (A) OD + SB compared to OD and SB alone. (B) AM + LL compared to AM and LL alone.

[^{3}H]BaP-DNA binding and adduct formation. Table 1 illustrates the inhibition of the binding of [^{3}H]BaP metabolites to calf thymus DNA and the formation of [^{3}H]BaP-DNA adducts by these herbs. SB at a concentration of 1.5 mg/ml inhibited BaP-DNA binding by 75%. OD, LL and AM at 3 mg/ml inhibited the binding by 35%, 36% and 13% respectively. SB and LL significantly inhibited the formation of total [^{3}H]BaP-DNA adducts (7-S BPDE-1, 7-R BPDE-1, BPDE-2, and unknown) at the concentrations tested.

Analysis of [^{3}H]BaP metabolites. Figure 3 shows the effects of the four herbs on the formation of organosoluble metabolites of [^{3}H]BaP. SB significantly inhibited the formation of organosoluble metabolites. In the presence of LL and OD, there were also less formation of organosoluble metabolites but statistically not significant. LL, OD, and SB had significantly higher amounts of unmetabolized BaP. The inhibition of BaP metabolism by the herbs is also reflected in the significantly smaller amount of water-soluble metabolites bound as sulfate ester, glucuronide and glutathione conjugates (Figure 4)

DISCUSSION

We studied four Chinese medicinal herbs which have long been used in cancer therapy in China. Immune modulating effects of AM and LL have been reported (Lau et al., 1989, Chu et al., 1988; Sun, 1986; Sun et al., 1983a,b; Zhao, 1990).

Table 1. Effects of Chinese medicinal herbs on [^{3}H]BaP metabolite binding to DNA[a] and the formation of [^{3}H]BaP-DNA adducts[b]

Herbal Extracts	[^{3}H]BaP Bound to DNA (pmol/mg)	% Inhibition	Total [^{3}H]BaP-DNA Adducts (pmol/mg)	% Inhibition
Control	17.3 ± 2.5	-	4.475 ± 0.92	-
SB (1.5 mg/ml)	4.3 ± 0.4*	75	1.228 ± 0.15*	72.6
OD (3 mg/ml)	11.3 ± 0.1*	35	2.688 ± 0.31	39.9
LL (3 mg/ml)	11.2 ± 0.2*	36	1.928 ± 0.18*	56.9
AM (3 mg/ml)	15.1 ± 0.2	13	3.866 ± 0.36	13.6

[a]Calf thymus DNA, rat liver S9, [^{3}H]BaP, and each herbal extract was incubated as described in Materials and Methods. DNA was extracted and radioactivity determined by liquid scintillation spectroscopy. [b]DNA was hydrolyzed and [^{3}H]BaP-DNA adducts were analyzed by HPLC. Radioactivity of adducts were determined by liquid scintillation spectroscopy. Values are means ± SE of duplicates. *indicates difference from control ($p < 0.05$)

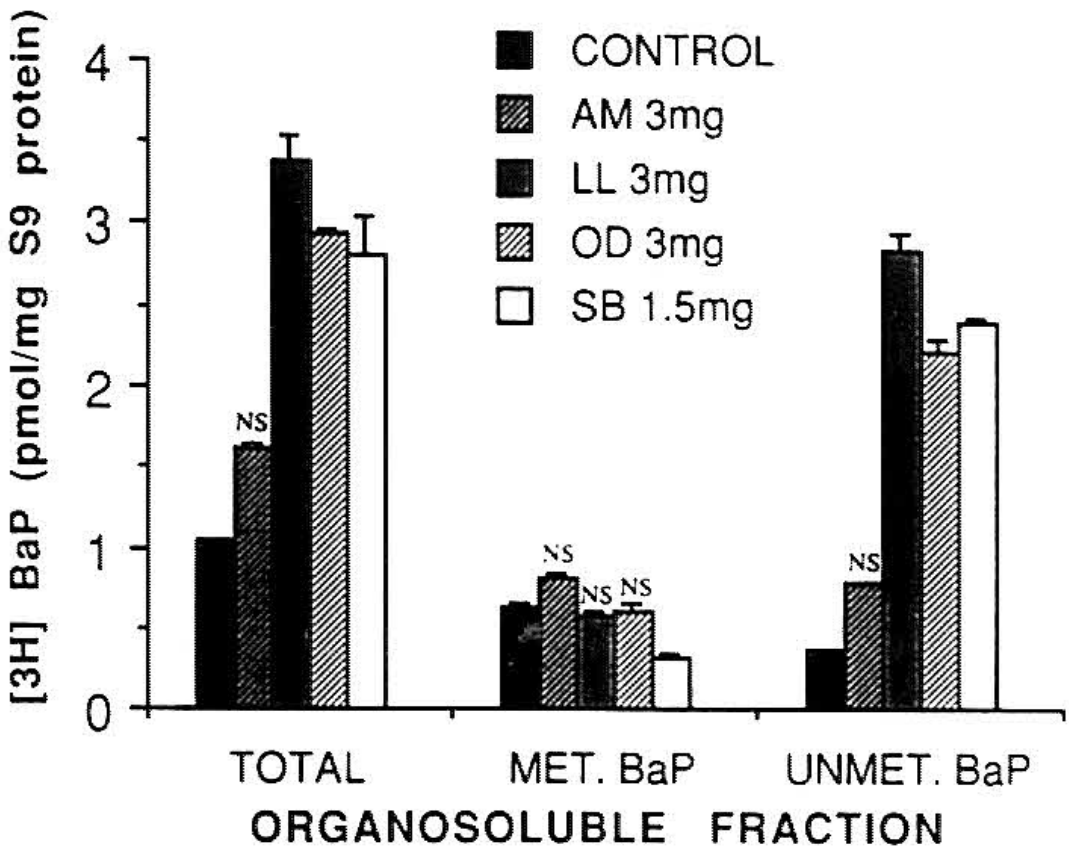

Figure 3. Effects of Chinese medicinal herbs on the formation of organosoluble metabolites of [^{3}H]BaP. Metabolites were extracted and analyzed by HPLC as described in Materials and Methods. NS indicates not significant from control All other values were significantly different from controls ($p < 0.05$).

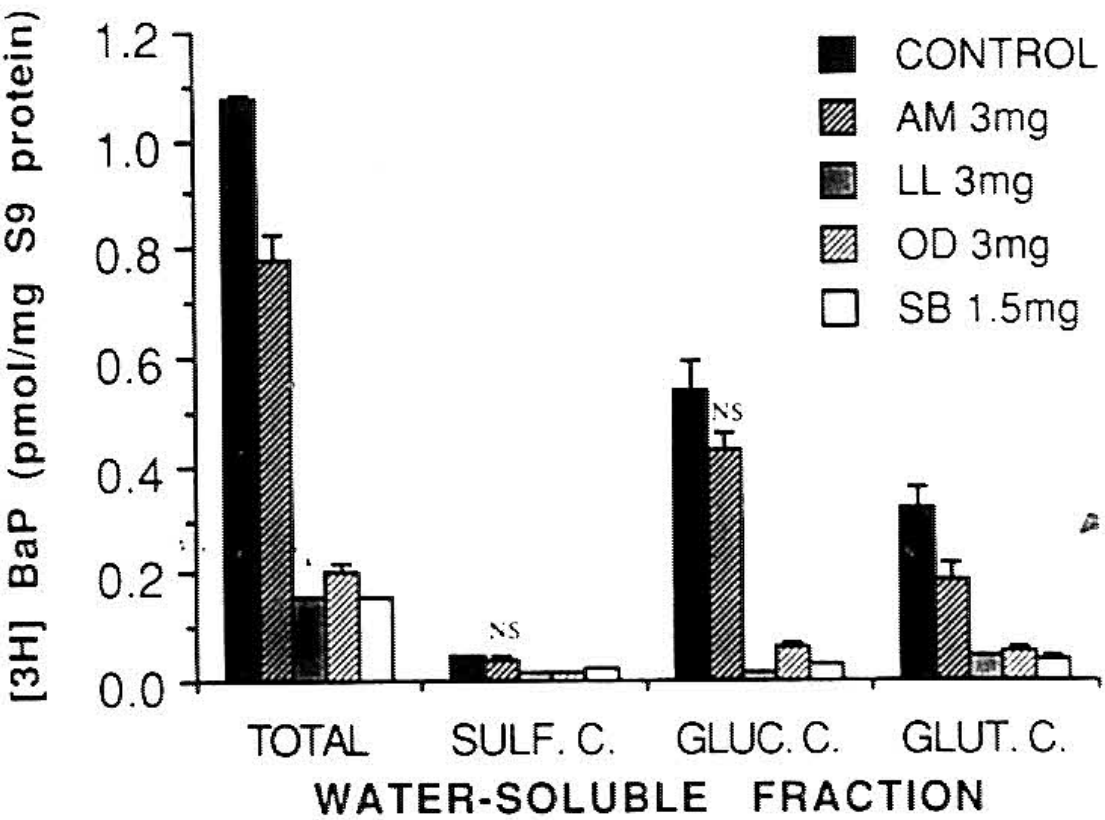

Figure 4. Effects of Chinese medicinal herbs on the formation of water-soluble conjugates of [^{3}H]BaP (Sulf. C. = Sulfate ester Conjugates, Gluc. C. = Glucuronide Conjugates, Glut. C. = Glutathione Conjugates). Conjugates were extracted and analyzed by alumina column chromatography as described in Materials and Methods. NS indicates not significant from control. All other values were significantly different from controls ($p < 0.05$).

OD and SB are often used in the treatment of liver, lung, and rectal cancers in traditional Chinese medicine (Hsu et al., 1986; Chong and Lee, 1988). A related species of AM was reported to have no antimutagenic effects while LL showed significant inhibition of BaP-induced mutagenesis in *S. typhimurium* TA98 (Meng et al., 1988).

Wattenberg (1985) has classified chemopreventive agents into three classes based on the mode of action. (1) Metabolic inhibitors prevent the formation of carcinogens from precursor compounds, (2) blocking agents inhibit carcinogenic compounds from reacting with cellular targets, and (3) suppressing agents inhibit the process of neoplastic manifestation. For example, phenols are chemopreventive at all levels, flavones are blocking agents, and plant sterols such as β-sitosterol are suppressing agents. Plant phenols also act as nucleophilic traps (Newmark, 1987). Among the four Chinese medicinal herbs we studied, SB and LL contain phenols and flavones while OD and AM contain β-sitosterol (Hsu et al., 1986).

In this study, we assessed the antimutagenic properties of these four herbs in the mutagenesis assay of Maron and Ames (1983) using *S. typhimurium* TA100 as the tester strain. All the herbs significantly inhibited BaP-induced mutagenesis to varying degrees. SB was the most inhibitory and AM was the least. When used in combination, only SB and OD had an additive effect (Figure 2A,B).

The Ames assay has been shown to be useful in correlating *in vitro* bacterial mutagenesis with *in vivo* carcinogenicity in animals (Rinkus and Legator, 1979).

Metabolic activation of BaP to the 7,8-dihydrodiol-9,10-epoxide is believed to be essential to the expression of its mutagenicity and carcinogenicity (Gurtoo and Bejba, 1974). This epoxide reacts with the N^2 atom of guanine (Jeffery et al., 1977; Koreeda et al., 1981). Our results suggest that the effects of the Chinese herbs on BaP-induced mutagenesis in *S. typhimurium* TA100 (Figures 1, 2), on [^{3}H]BaP binding to calf thymus DNA and adduct formation (Table 1) were mediated through the inhibition of the metabolism of BaP (Figures 3, 4). HPLC analysis of the organosoluble (phase I-mediated) fractions showed a significant decrease in the metabolism of BaP in the presence of SB and a significantly higher amount of unmetabolized BaP in the presence of LL, OD, and SB (Figure 3). The decreased formation of water-soluble conjugates (sulfate ester, glucuronide, and glutathione conjugates) in the presence of LL, OD and SB reflects the effect of these herbs on phase I metabolism of BaP. An inhibition of the phase I metabolism would necessarily result in reduced amounts of BaP 7,8-dihydrodiol-9,10 epoxide available for conjugation. A specific effect of these herbs on phase II enzyme activity cannot be conclusively determined by our assays.

In conclusion, aqueous extracts of all four Chinese herbs we tested inhibited BaP-induced mutagenesis. Three of them (LL, OD, and SB) significantly inhibited DNA binding. Two of them (LL and SB) significantly reduced BaP-DNA adduct formation and SB significantly inhibited metabolism of BaP. Our results therefore suggest that the Chinese medicinal herbs LL, OD and SB possess cancer chemopreventive properties.

ACKNOWLEDGEMENTS

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CHAPTER 4

CHINESE MEDICINAL HERBS MODULATE MUTAGENESIS, DNA BINDING AND METABOLISM OF BENZO[A]PYRENE 7,8-DIHYDRODIOL AND BENZO[A]PYRENE 7,8-DIHYDRODIOL-9,10-EPOXIDE

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Cancer Letters, 62, 123-131

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Key words: Chinese medicinal herbs; *Oldenlandia diffusa*, *Scutellaria barbata*; BaP 7,8-DHD and BPDE metabolism; DNA binding; mutagenesis; scavenging

SUMMARY

Oldenlandia diffusa (OD) and *Scutellaria barbata* (SB) have been used in traditional Chinese medicine for treating liver, lung and rectal tumors. In this study, the effects of aqueous extracts of these two herbs on benzo[a]pyrene 7,8-dihydrodiol (BaP 7,8-DHD) and benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide (BPDE)-induced mutagenesis using *Salmonella typhimurium* TA100 as the bacterial tester strain and rat liver 9000 X g supernatant (S9) as the metabolic activation system were assessed. We also determined the effects of these two herbs on BaP 7,8-DHD and BPDE binding to calf thymus DNA. Organosoluble metabolites of BaP 7,8-DHD and water-soluble conjugates of BaP 7,8-DHD and BPDE were analyzed by high-performance liquid chromatography (HPLC) and alumina column liquid chromatography. Mutagenesis assays revealed that these two herbs produced a significant concentration-dependent inhibition of histidine-independent (His^+) revertants induced by BaP 7,8-DHD and BPDE. OD and SB also inhibited BPDE-induced mutagenesis in a concentration-dependent manner in the absence of S9. SB had a greater inhibitory effect than OD. SB significantly inhibited BaP 7,8-DHD and BPDE binding to DNA while OD significantly enhanced DNA binding of both compounds. OD and SB inhibited the formation of organosoluble metabolites of BaP 7,8-DHD and decreased the formation of water-soluble conjugates of BaP 7,8-DHD and BPDE. However, the fraction of the total radioactivity in the water-soluble conjugates present as sulfate

and glutathione was increased by OD and SB. Glucuronide fraction was decreased. The results of this study affirm our previous work suggesting that these two Chinese medicinal herbs possess anti-mutagenic properties and further suggest that they act as blocking agents through a scavenging mechanism.

INTRODUCTION

The Chinese have practiced herbal medicine for thousands of years. Chinese literature describes a number of herbs used in cancer treatment. Among them *Oldenlandia diffusa* (OD) (common name Spreading Hedyotis of the Rubiaceae family) and *Scutellaria barbata* (SB) (Chinese name Pan-chih-lien of the Labiatae family) are consistently found in various herbal formulas. They are described as having anti-neoplastic, anti-toxic, and anti-inflammatory properties [11]. The crude extracts of OD and *S. baicalensis*, a species related to SB, is reported to augment phagocytic activity in animals [5]. In a previous study we show that OD and SB produce a significant concentration-dependent inhibition of His^+ revertants induced by BaP in in *Salmonella typhimurium* TA100 and significantly inhibit BaP binding to calf thymus DNA. SB significantly inhibits BaP-DNA adduct formation that correlates with an inhibition of the metabolism of BaP [24].

Benzo[a]pyrene (BaP), one of the most common polycyclic aromatic hydrocarbons (PAHs), is a major pollutant of air, water, soil and the food chain [20]. BaP and several of its metabolites produce tumors in newborn mice and a

significant correlation between the incidence of human cancer and PAHs in the environment is suggested by epidemiologic studies [2,20]. BaP is a procarcinogen requiring cytochrome P-450 enzymes for activation [8]. BaP 7,8-DHD, a major metabolic intermediate, can be converted to BPDE by mixed-function oxidases [6]. BPDE is the metabolite that binds to DNA *in vitro* and *in vivo* [2,21]. It reacts at the C-10 position with the exocyclic amino group at the N^2 position of guanine and both Phase I and Phase II enzymes are involved in the metabolism and DNA binding of BaP [13,14,20]. In the present report we have expanded our study of the effects of two Chinese medicinal herbs (OD and SB) by investigating their effects on the mutagenesis, DNA binding and metabolism of BaP 7,8-DHD and BPDE.

MATERIALS AND METHODS

Chemicals

Bactoagar was purchased from Difco Laboratories, Detroit, MI. Nutrient broth (Oxoid #2) was obtained from Unipath, Oxoid Division, Columbia, MD. Aroclor 1254 (polychorinated biphenyl) came from Foxboro, North Haven, CT. Unlabeled BaP 7,8-DHD, BPDE, authentic BaP metabolites and [^{3}H]BaP 7,8-DHD (767 mCi/mmol) were obtained from the National Cancer Institute Repository, Kansas City, MO. [^{3}H]BPDE (439 mCi/mmol) was purchased from Chemsyn Science Lab, Lenexa, KS. Liquid scintillation cocktail was obtained

from Allied-Fisher Scientific, Pittsburg, PA. Aluminum oxide for column chromatography, and solvents and chemicals for chromatography were purchased from J.T. Baker Chemical, Phillipsburg, NJ. Calf thymus DNA and all other chemicals were acquired from Sigma Chemical Co, St. Louis, MO.

Preparation of herbal extracts

The crude herbs OD and SB were purchased from Nuherbs Co., Oakland, CA. Each of these herbs was ground to fine powder in a coffee mill (Waring Products, New Hartford, CT). Aqueous extracts were prepared by suspending 10 g of the dried powder of each herb in 100 ml of distilled water and heating at 56° C for 1 h. The suspension was centrifuged at 2000 rpm for 10 min and then filtered. The filtrate was centrifuged again at 3000 rpm for 10 min and the supernatant was filter-sterilized [15]. The extract was lyophized and the dry weight determined. The concentrations used in the experiments were based on the dry weight of the extract.

Preparation of hepatic S9

Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN), each weighing 150-160 g, were given a single injection of Aroclor 1254 (500 mg/kg) five days before time of sacrifice. Drinking water and Purina laboratory chow was provided ad libitum until 12 h before sacrificing when the chow was removed. Liver S9 was prepared aseptically (17). After determining the protein

concentration [9,16], one ml aliquots of S9 (62.7 mg protein/ml) were frozen at -80° C and used as needed.

Mutagenesis assays

The plate incorporation assay of Maron and Ames [17] was employed using *Salmonella typhimurium* TA100 (a gift from Dr. Bruce N. Ames, University of California, Berkeley, CA) as the tester strain. The following materials were added to sterile disposable test tubes in triplicate in sequence: 100 μl overnight nutrient broth culture of TA100, 500 μl of cofactor buffer, different concentrations of herbal extract (0, 1.5, 3 and 6 mg/plate), 25 μl of S9 protein (1.57 mg) , and 10 μl of BaP 7,8-DHD (0.5 μg) or BPDE (1 μg) (in dimethylsulfoxide). The mixture was vortexed with 2 ml of molten top agar containing histidine-biotin and poured onto minimum glucose agar plates. After 48 h incubation at 37° C , His^+ colonies were scored. Spontaneous revertant colonies were subtracted. The experiments were repeated at least twice. Data were expressed as number of His^+ revertants (mean ± SE) per plate in the presence of various concentrations (mg/plate) of herbal extract. The non-toxic, optimal inhibitory concentrations of these herbs were used for subsequent experiments.

[^{3}H]BaP 7,8-DHD and [^{3}H]BPDE-DNA binding and adduct formation

Liver S9 protein (3.14 mg in 50 μl), herbal extract (OD or SB, 3 mg/ml), and 10 μl of [^{3}H]BaP 7,8-DHD (1.88 μmol, 1.44 μCi) or [^{3}H]BPDE (4.9 μmol,

2.15 μCi) were incubated in 1 ml of cofactor buffer containing 0.5 mg purified calf thymus DNA for 1 h at 37° C in a shaking waterbath. After incubation, DNA was extracted and processed [19]. Radioactivity in the samples was determined by liquid scintillation spectroscopy (Model LS 5801 counter, Beckman Instrument Division, Berkeley, CA). DNA was quantitated by the method of Burton [3]. Data were expressed as picomoles per milligram (pmol/mg) DNA. For the isolation of adducts, the DNA samples were hydrolyzed and extracted in water-saturated *n*-butanol. The butanol layer was dried under N_2 and the residue was redissolved. Adducts were separated by high-performance liquid chromatography (HPLC). The radioactivity in collected fractions was determined by liquid scintillation spectroscopy. Adducts were identified by comparing profiles with authentic adduct standards of BaP-DNA adducts prepared by the reaction of BPDE [12]. Data were expressed as pmol/mg DNA.

Analysis of [^{3}H]BaP 7,8-DHD and [^{3}H] BPDE metabolites

Liver S9 protein (3.14 mg in 50 μl), herbal extract (OD or SB, 3 mg/ml), and 10 μl of [^{3}H]BaP 7,8-DHD (2,347 pmol, 1.8 μCi) or [^{3}H]BPDE (2,449 pmol, 1.075 μCi) were incubated in 1 ml of cofactor buffer in a shaking waterbath at 37° C for 30 min. Samples were extracted with ethyl acetate : acetone (2:1). The organosoluble fractions were dried under N_2 and redissolved in 1 ml of 60% methanol. Aliquots were analyzed by HPLC in a linear methanol and water gradient of 60%-100% over 50 min at a flow rate of 1 ml/min [19]. Aqueous

fractions remaining after ethyl acetate : acetone extraction were lyophilized, redissolved in 2.5 ml of 70% ethanol, and separated on an alumina column [1]. Samples were collected at a flow rate of 2 ml/min. Radioactivity in eluted organosoluble and water-soluble fractions was determined by liquid scintillation spectroscopy and analyzed with a Beckman Datagraph Program in the DU Data Leader software package on an IBM PS/2 model 50 computer. Organosoluble metabolites and water-soluble conjugates of [^{3}H]BaP 7,8-DHD and water-soluble conjugates of [^{3}H]BPDE were identified by comparing profiles with authentic standards [19,1]. Data were expressed as pmol/mg S9 protein and as % total radioactivity.

Calculation of pmol and statistical analysis of data

The radioactivity (DPM), determined by liquid scintillation spectroscopy, was converted to pmol based on the specific activity of [^{3}H]BaP-7,8-DHD or [^{3}H]BPDE. All experimental data were analyzed with one way analysis of variance (ANOVA) followed by Tukey's multiple range test for honestly significant difference (HSD). Statistical significance was defined as $p < 0.05$. Statistical procedures were performed with Stratgraphics software version 3.1 (STSC, Inc., Rockville, MD).

RESULTS

Mutagenesis assays

Table 1 compares the number of His$^+$ revertants (means ± SE, n=6) per plate and the percent inhibition of OD and SB on BaP 7,8-DHD or BPDE-induced mutagenesis in *S. typhimurium* strain TA100 from two experiments. Except for the effect of 1.5 mg SB on BaP 7,8-DHD-induced mutagenesis, the two herbs exhibited a concentration-dependent inhibitory effect. SB exhibited more inhibition than OD at concentrations of 3 and 6 mg/plate in each group. Both herbs exhibited a concentration-dependent inhibitory effect on BPDE-induced mutagenesis in TA100 whether liver S9 was present or absent. The generally higher number of His$^+$ revertants in the absence of S9 were not significantly different statistically from those with S9.

[^{3}H]BaP 7,8-DHD and [^{3}H]BPDE-DNA binding and adduct formation

Table 2 illustrates that SB significantly inhibited the binding of [^{3}H]BaP 7,8-DHD and [^{3}H]BPDE to calf thymus DNA and the formation of DNA adducts (7R- and 7S-BPDE-I: deoxy-guanosine adducts, BPDE-II: deoxy-guanosine adducts, as well as unknown adducts) at the concentration tested. OD significantly enhanced the binding of [^{3}H]BaP 7,8-DHD and [^{3}H]BPDE to DNA and the formation of DNA adducts at the concentration tested. In the absence of S9 more BPDE-DNA adducts were formed in all the samples (controls included).

Table 1. Effects of *Oldenlandia diffusa* (OD) and *Scutellaria barbata* (SB) on BaP 7,8-DHD or BPDE-induced mutagenesis in *S. typhimurium* TA100.

Treatments		Revertants/plate[a]					
Herbal Extracts	Concentration (mg/plate)	BaP 7,8-DHD with S9		BPDE with S9		BPDE without S9	
OD	0[b]	551 ± 5	----	508 ± 33	----	607 ± 41	----
	1.5	445 ± 27	(19)[c]	450 ± 47	(11)	422 ± 52	(30)
	3.0	408 ± 7*	(26)	257 ± 26*	(49)	316 ± 30*	(48)
	6.0	373 ± 4*	(32)	167 ± 11*	(67)	215 ± 35*	(65)
SB	0[b]	551 ± 5	----	508 ± 33	----	607 ± 41	----
	1.5	744 ± 52*	(0)	322 ± 21*	(37)	366 ± 25*	(40)
	3.0	370 ± 29*	(33)	243 ± 18*	(52)	263 ± 19*	(57)
	6.0	111 ± 7*	(80)	139 ± 13*	(73)	246 ± 17*	(60)

[a]Values are means ± SE of histidine-independent revertants from triplicate plates from two separate experiments. Spontaneous revertants (113 in the BaP 7,8-DHD group and 134 in the BPDE group) were subtracted.

[b]Indicates BaP 7,8-DHD or BPDE in the absence of herbs.

[c]Percent inhibition is given in parenthesis.

*Significantly different from BaP 7,8-DHD or BPDE alone ($p < 0.05$).

Table 2. Effects of Chinese medicinal herbs on [^{3}H] BaP 7,8-DHD and [^{3}H]BPDE metabolite binding to DNA[a] and the formation of DNA adducts

Herbal Extracts	[^{3}H]BaP 7,8-DHD Bound to DNA	Total [^{3}H]BaP 7,8-DHD-DNA Adducts	[^{3}H]BPDE Bound to DNA		Total [^{3}H]BPDE-DNA Adducts	
			with S9	without S9	with S9	without S9
Control	81.8 ± 5.8	15.5 ± 1.7	309.5 ± 2.2	337.3 ± 37.6	166.0 ± 6.5	292.1 ± 4.8[c]
OD (3mg/ml)	311.1 ± 4.6[b]	41.5 ± 0.3[b]	693.5 ± 77.7[b]	676.0 ± 20.1[b]	287.6 ± 0.5[b]	378.0 ± 14.3[b,c]
SB (3mg/ml)	49.3 ± 1.0[b]	8.4 ± 0.4[b]	148.7 ± 2.1[b]	201.8 ± 10.0[b,c]	89.8 ± 2.4[b]	120.9 ± 2.5[b,c]

[a] Calf thymus DNA, with or without rat liver S9, [^{3}H]BaP 7,8-DHD or [^{3}H]BPDE, and each herbal extract were incubated as described in Materials and Methods. DNA was extracted and radioactivity determined by liquid scintillation spectroscopy. Adducts from hydrolyzed DNA were separated by HPLC. Values (pmol/mg) are means ± SE of duplicate samples.

[b] Indicates significant difference from control ($p < 0.05$).

[c] Significantly different from [^{3}H]BPDE-DNA binding or adducts in the presence of rat liver S9 ($p < 0.05$).

Analysis of [^{3}H]BaP 7,8-DHD and [^{3}H] BPDE metabolites

Figure 1 shows the effects of the two herbs on the formation of organosoluble metabolites of [^{3}H]BaP 7,8-DHD. Both OD and SB significantly inhibited the formation of organosoluble metabolites and therefore yielded significantly higher amounts of unmetabolized [^{3}H]BaP 7,8-DHD (Fig. 1A). The amount of metabolites (tetrols: 7,10/8,9- and 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; and triols: 7,8,9-trihydrooxy-7,8,9,10-tetrahydrobenzo[a]pyrene) expressed as % total radioactivity in each sample indicated significant inhibition of [^{3}H]BaP 7,8-DHD metabolism by these herbal extracts (Fig. 1B). The inhibition of BaP 7,8-DHD metabolism by the herbs was also reflected in the significantly smaller amount of water-soluble metabolites bound as glucuronide and glutathione conjugates (Fig. 2A). However, when compared with controls, the % total radioactivity present in each sample as sulfate and glutathione conjugates were increased by OD and SB (Fig. 2B). Glucuronide conjugates were decreased.

Figure 3 shows the effects of OD and SB on the formation of water-soluble conjugates of [^{3}H]BPDE. There was a significant inhibition of total conjugates formed that was primarily due to the effect of the herbs on glucuronide conjugate formation (Fig. 3A). As with BaP 7,8-DHD, the % total radioactivity in each sample present as BPDE- sulfate and glutathione conjugates was increased in the presence of OD and SB (Fig. 3B). Glucuronide conjugates were significantly less than control.

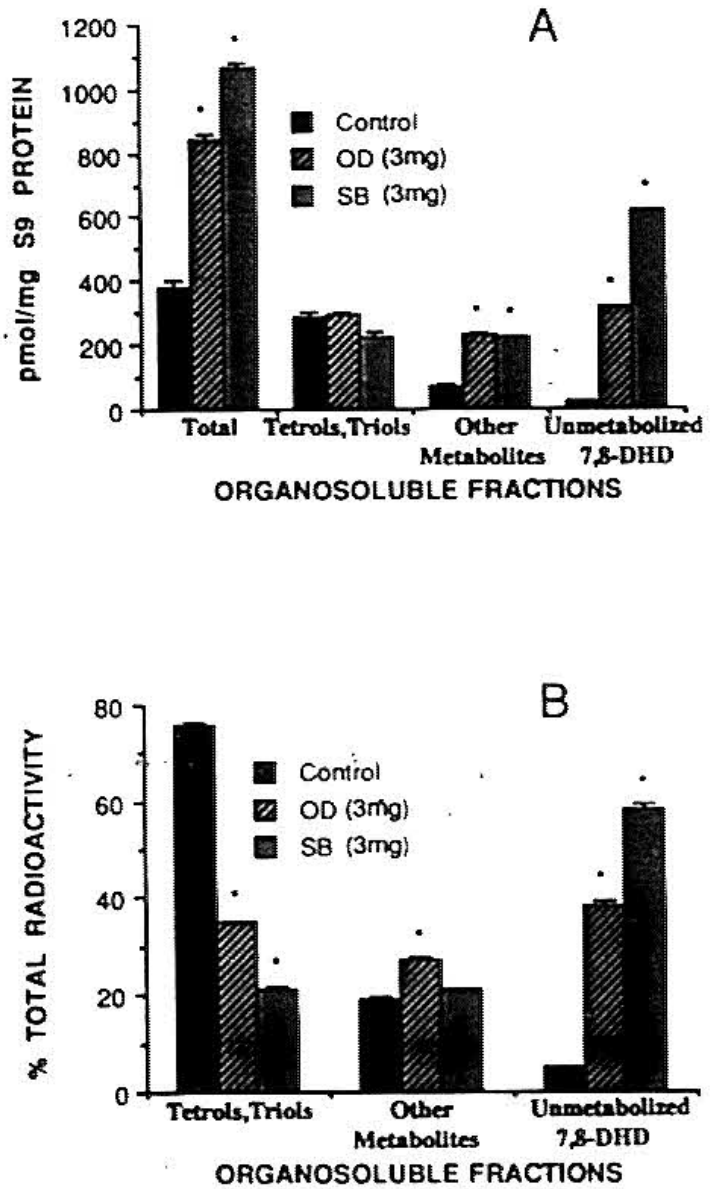

Figure 1. Effects of *Oldenlandia diffusa* (OD) and *Scutellaria barbata* (SB) on the formation of organosoluble metabolites of [^{3}H]BaP 7,8-DHD. Metabolites were extracted and analyzed by HPLC as described in Materials and Methods. (A) Data shown as pmol/mg S9 protein. (B) Data shown as % total radioactivity. * Significantly different from control ($p < 0.05$).

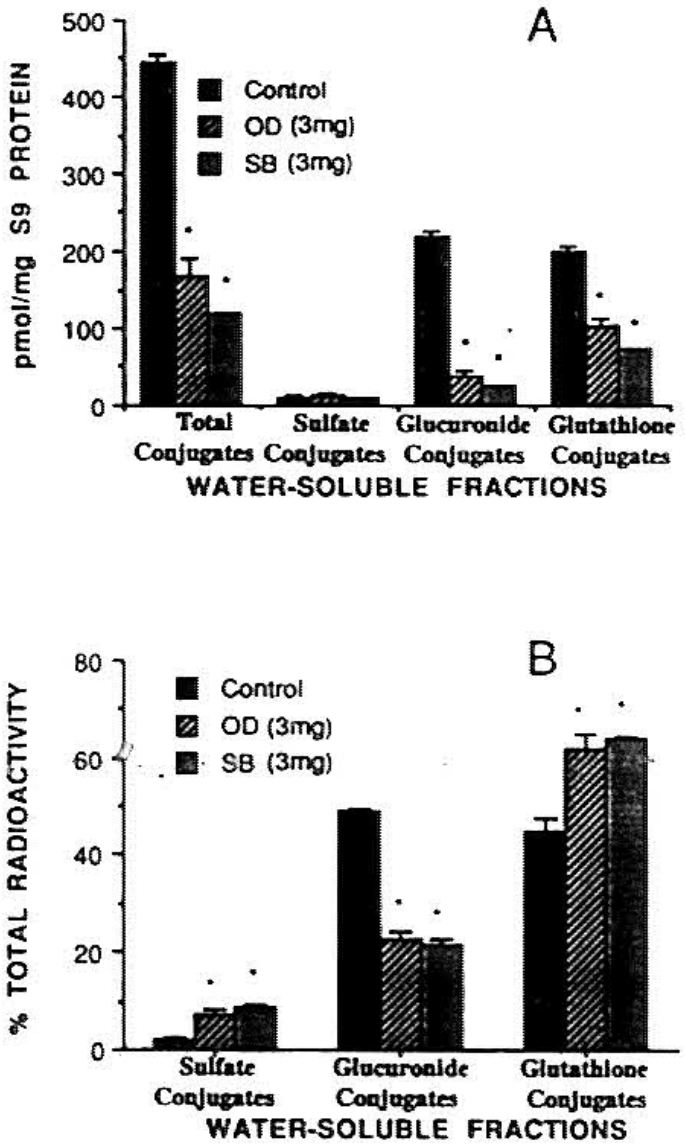

Figure 2. Effects of OD and SB on the formation of water-soluble conjugates of [^{3}H]BaP 7,8-DHD. Conjugates were extracted and analyzed by alumina column chromatography as described in Materials and Methods. (A) Data shown as pmol/mg S9 protein. (B) Data shown as % total radioactivity. * Significantly different from control ($p < 0.05$).

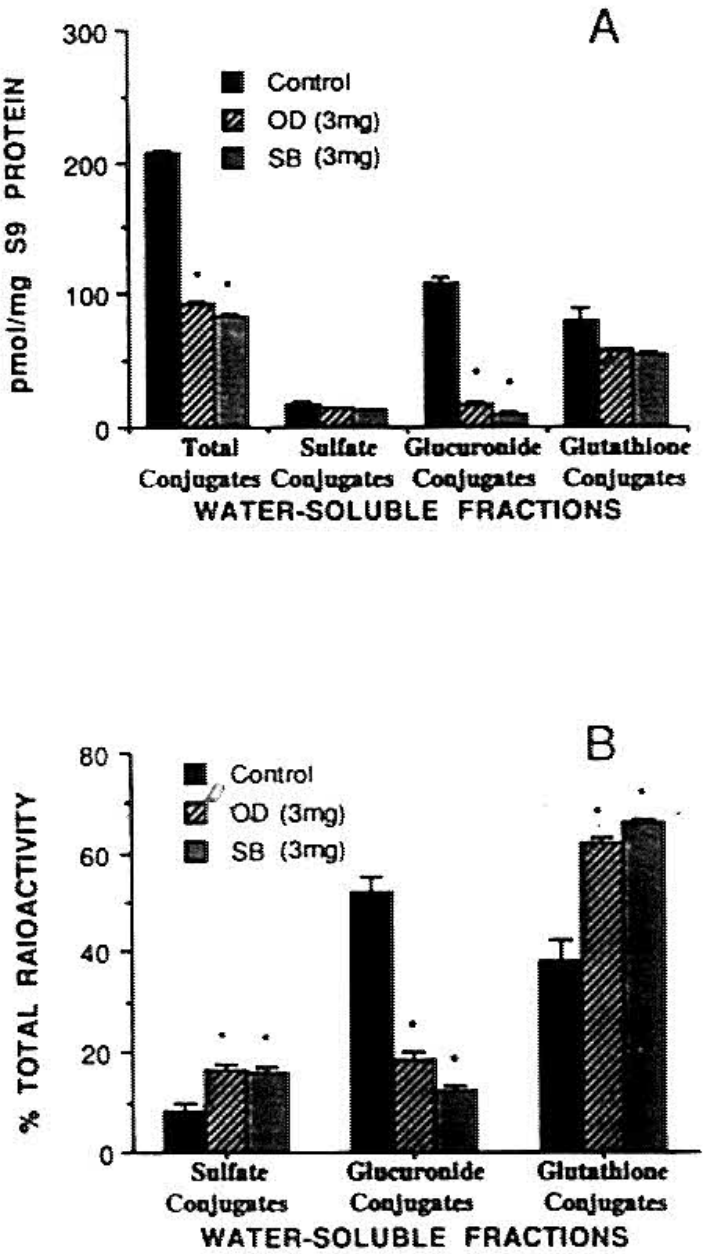

Figure 3. Effects of OD and SB on the formation of water-soluble conjugates of [^{3}H]BPDE. (A) Data shown as pmol/mg S9 protein. (B) Data shown as % total radioactivity. * Significantly different from control ($p < 0.05$).

DISCUSSION

Wattenberg [23] has classified chemopreventive agents into three groups based on their mode of action. (1) Metabolic inhibitors prevent the formation of carcinogens from precursor compounds, (2) blocking agents inhibit carcinogenic compounds from reacting with cellular targets, and (3) suppressing agents inhibit the process of neoplastic manifestation. For example, phenols are chemopreventive at all levels, flavones are blocking agents, and plant sterols such as β-sitosterol are suppressing agents. Involved in these actions are: (a) inhibition of metabolic activation pathways, (b) induction of detoxification pathways, and (c) scavenging of the reactive electrophilic radicals [23]. Hocman [10] has reviewed the reported inhibitory effects of plant phenols in PAH-induced mutagenesis and carcinogenesis. The phenolic hydroxyl groups were mainly responsible for their anti-mutagenic and chemopreventive properties. Flavones inhibit BaP-DNA adduct formation by the methylation or glycosylation of their particular hydroxyl groups and the saturation of the 2,3 double bonds [10]. Of the two Chinese medicinal herbs used in this study, SB contains phenols and flavones while OD contains β-sitosterol [11]. OD and SB have been used in the treatment of liver, lung and rectal cancers in traditional Chinese medicine [4,11].

In this study, we assessed the anti-mutagenic properties of OD and SB in the mutagenesis assay of Maron and Ames [17] using *S. typhimurium* TA100 as the tester strain. Both herbs significantly inhibited BaP 7,8-DHD and BPDE-

induced mutagenesis (Table 1). The inhibitory effect of OD and SB on BPDE-induced mutagenesis occurred in both the presence and absence of liver S9. The Ames assay is useful in correlating *in vitro* bacterial mutagenesis with *in vivo* carcinogenicity in animals [18]. Because of their ability to trap reactive species of carcinogens, blocking agents can exhibit inhibition of mutagenesis [23]. OD and SB inhibited BPDE-induced mutagenesis in TA100 (Table 1) suggesting that these two herbs may work as blocking agents through a scavenging action.

Metabolic activation of BaP and BaP 7,8-DHD to BPDE is believed to be essential to the expression of its mutagenicity and carcinogenicity [2,7,21]. BPDE reacts with the N^2 atom of guanine [13,14]. In a previous report, we suggested that the inhibitory effects of the Chinese herbs OD and SB on BaP-induced mutagenesis in *S. typhimurium* TA100, on [^{3}H]BaP binding to calf thymus DNA and adduct formation were mediated through the inhibition of the Phase I-mediated metabolism of BaP [24]. In the present study, HPLC analysis of the organosoluble (Phase I-mediated) fraction showed a significant decrease in the metabolism of BaP 7,8-DHD in the presence of OD and SB. This decrease was indicated by the amount of tetrols and triols and unmetabolized BaP 7,8-DHD (Fig. 1A,B). An inhibition of the metabolism of BaP 7,8-DHD would result in reduced amounts of metabolites available for conjugation (Fig. 2A). The effect of OD and SB on the formation of BaP 7,8-DHD metabolite conjugates with sulfate, glucuronide and glutathione (Fig. 2A) correlates with their effect on BPDE conjugate formation (Fig. 3A). It is of interest that although the total amount of

water-soluble conjugates formed in the presence of OD and SB was less than those in controls, the distribution of conjugates was affected. Both OD and SB appeared to enhance the sulfate and glutathione fractional portion of total conjugates and decrease the glucuronide fraction (Fig. 2B,3B). Additional experiments are required to clarify this observation. The inhibitory effect of SB on BaP 7,8-DHD metabolite binding to DNA and adduct formation (Table 2) correlates with the metabolism data in Fig. 1 and the hypothesis of its phenolic properties [10,23]. Further support for a scavenging effect by SB is presented in Table 2 where SB inhibited BPDE binding to DNA and adduct formation in the absence of S9.

The significantly enhanced binding of BaP 7,8-DHD and BPDE to DNA and formation of adducts by OD (Table 2) appears to be at variance with its anti-mutagenic effects (Table 1). This enhanced effect also occurred at a concentration of 1.5 mg (data not shown). BPDE, a product from the metabolism of BaP and BaP 7,8-DHD, is further metabolized by microsomes via triol-epoxide intermediates which bind to microsomal protein and glutathione [6]. Less binding occurred in the presence of S9 in our study (Table 2). A possible explanation for the enhanced DNA binding of BaP 7,8-DHD and BPDE in the presence of OD is that OD in the scavenging mode binds metabolites (BPDE) and also binds to DNA. The nature of this interaction would somehow permit an anti-mutagenic effect (Table 1) possibly by preventing BPDE from directly interacting with the DNA. This mechanism has been proposed for ellagic acid [22].

In summary, aqueous extracts of two Chinese herbs (OD and SB) significantly inhibited BaP 7,8-DHD and BPDE-induced mutagenesis in *S. typhimurium* TA100. OD and SB significantly inhibited the metabolism of BaP 7,8-DHD and the formation of water-soluble conjugates of BaP 7,8-DHD and BPDE. Both OD and SB modified the distribution of conjugates within the water-soluble fraction. SB significantly inhibited BaP 7,8-DHD and BPDE binding to DNA and reduced the formation of adducts. Our data suggest that the Chinese medicinal herbs OD and SB possess anti-mutagenic properties and possibly anti-carcinogenic effects. Additional experiments are necessary to clarify the anti-carcinogenic properties, particularly for OD.

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CHAPTER 5

DISCUSSION

In the previous three chapters, I discussed experiments in which four Chinese medicinal herbs (OD, SB, LL, and AM) were used. These herbs have been used in cancer therapy in China for generations. OD and SB are often used in the treatment of liver, lung, and rectal cancers in traditional Chinese medicine (Hsu et al., 1986; Chong and Lee, 1988). Immune modulating effects of AM and LL have been reported (Lau et al., 1989; Chu et al., 1988; Sun, 1986; Sun et al., 1983a,b; Zhao, 1990). LL showed significant inhibition of BaP-induced mutagenesis in *S. typhimurium* TA98 while a species related to AM showed no effect (Meng et al., 1988).

Wattenberg (1985) has classified chemopreventive agents into three groups based on their mode of action: (1) Metabolic inhibitors prevent the formation of carcinogens from precursor compounds, (2) blocking agents inhibit carcinogenic compounds from reacting with cellular targets, and (3) suppressing agents inhibit the process of neoplastic manifestation. For example, phenols are chemo-preventive at all levels, flavones are blocking agents, and plant sterols such as β-sitosterol are suppressing agents. Involved in these actions are: (a) inhibition of metabolic activation pathways, (b) induction of detoxification pathways, and (c)

scavenging of the reactive electrophilic radicals (Wattenberg, 1985). Hocman (1989) has reviewed the reported inhibitory effects of plant phenols in PAH-induced mutagenesis and carcinogenesis. The phenolic hydroxyl groups were mainly responsible for their anti-mutagenic and chemopreventive properties. Flavones inhibit BaP-DNA adduct formation by the methylation or glycosylation of particular hydroxyl groups and the saturation of the 2,3 double bonds on the flavone molecules (Hocman, 1989). Among the four Chinese medicinal herbs studied, SB and LL contain phenols and flavones while OD and AM contain β-sitosterol (Hsu et al., 1986).

In this study, we assessed the anti-mutagenic properties of the above four herbs in the mutagenesis assay of Maron and Ames (1983) using *S. typhimurium* TA100. The Ames assay has been used to correlate *in vitro* bacterial mutagenesis with *in vivo* carcinogenicity in animals (Rinkus and Legator, 1979). All four herbs exhibited a concentration-dependent inhibition of AFB_1 and BaP-induced mutagenesis. SB was the most inhibitory and AM was the least. When used in combination, SB and OD had an additive or synergistic effect. As described in chapter 4, OD and SB significantly inhibited BaP 7,8-DHD and BPDE-induced mutagenesis. The inhibitory effect of OD and SB on BPDE-induced mutagenesis occurred in both the presence and absence of liver S9. Because of their ability to trap reactive species of carcinogens, blocking agents can inhibit mutagenesis (Wattenberg, 1985). OD and SB inhibited BPDE-induced mutagenesis in TA100 suggesting that these two herbs act as blocking agents through a scavenging action.

Metabolic modification of BaP to the 7,8-dihydrodiol-9,10-epoxide is reported to be essential to the expression of its mutagenicity and carcinogenicity (Gurtoo and Bejba, 1974). This epoxide reacts with the N^2 atom of guanine (Jeffery et al., 1977; Koreeda et al., 1978). The results described in chapter 3 suggest that the effects of the Chinese herbs on BaP-induced mutagenesis in *S. typhimurium* TA100, on [^{3}H]BaP binding to calf thymus DNA and on [^{3}H]BaP-DNA adduct formation were mediated through the inhibition of the metabolism of BaP. HPLC analysis of the organosoluble (phase I-mediated) fractions showed a significant decrease in the conversion of BaP to its metabolites by S9 when extracts of LL, OD and SB were present. This was indicated by a significantly higher amount of unmetabolized BaP. The decreased formation of water-soluble conjugates (sulfate ester, glucuronide, and glutathione conjugates) in the presence of LL, OD and SB is interpreted as an effect of these herbs on phase I metabolism of BaP because an inhibition of phase I metabolism would decrease the amounts of BaP 7,8-dihydrodiol-9,10 epoxide and other metabolites available for conjugation.

As described in chapter 4, HPLC analysis of the organosoluble (phase I-mediated) fraction showed a significant decrease in the metabolism of BaP 7,8-DHD in the presence of OD and SB. Evidence of this decrease was in the diminished amounts of tetrols, triols and while the remaining unmetabolized [^{3}H]BaP 7,8-DHD was in larger proportions. The effect of OD and SB on the formation of BaP 7,8-DHD metabolite conjugates with sulfate, glucuronide and

glutathione correlated with their effect on BPDE conjugate formation. It is of interest that although the total amount of water-soluble conjugates formed in the presence of OD and SB was less than those in controls, the proportional distribution of conjugates was altered. Both OD and SB appeared to enhance the sulfate and glutathione fractional portion of total conjugates and decrease the glucuronide fraction. The inhibitory effect of SB on [^{3}H]BaP 7,8-DHD metabolite binding to DNA and adduct formation correlates as would be expected from the metabolism data and the hypothesis of its phenolic properties (Hocman, 1989; Wattenberg, 1985). Support for a scavenging effect by SB correlated with the inhibition of [^{3}H]BPDE binding to DNA and adduct formation by SB in the absence of S9.

In the case of aflatoxins, the relative mutagenic *in vitro* potency has been shown to correlate with *in vivo* carcinogenic data (Wong and Hsieh, 1976). Metabolic activation of AFB_1 to the 8,9-epoxide is believed to be essential to the expression of its mutagenicity and carcinogenicity (Gurtoo and Bejba, 1974). This epoxide reacts with the N^7 atom of guanine to form AFB_1-N^7-Gua (Croy et al., 1978). The results presented in chapter 2 suggest that the effects of the Chinese herbs on AFB_1-induced mutagenesis in *S. typhimurium* TA100, on [^{3}H]AFB_1 binding to calf thymus DNA, and on AFB_1-N^7-Gua adduct formation were mediated through the inhibition of the metabolism of AFB_1. HPLC analysis of the organosoluble (phase I-mediated) fractions showed an inhibition of the metabolism of [^{3}H]AFB_1 in the presence of LL, OD, and SB.

In conclusion, aqueous extracts of all four Chinese herbs tested inhibited AFB_1, BaP, BaP 7,8-DHD, and BPDE-induced mutagenesis. Three of the herbs (LL, OD, and SB) significantly inhibited [^{3}H]AFB_1 metabolite binding to calf thymus DNA, [^{3}H]AFB_1-DNA adduct formation and metabolism of [^{3}H]AFB_1. These three herbs also significantly inhibited the binding of [^{3}H]BaP to calf thymus DNA. Two of them (LL and SB) diminished [^{3}H]BaP-DNA adduct formation and SB significantly inhibited the metabolism of [^{3}H]BaP. OD and SB inhibited the metabolism of [^{3}H]BaP 7,8-DHD and the formation of water-soluble conjugates of [^{3}H]BaP 7,8-DHD and [^{3}H]BPDE. Both OD and SB modified the proportional distribution of conjugates within the water-soluble fraction. SB significantly inhibited [^{3}H]BaP 7,8-DHD and [^{3}H]BPDE binding to DNA and decreased the formation of adducts. These results therefore suggest that the Chinese medicinal herbs LL, OD and SB may possess anti-mutagenic and anti-carcinogenic properties. OD and SB may possess these properties in part by acting as blocking agents through a scavenging mechanism. Additional experiments are necessary to clarify the anti-mutagenic and anti-carcinogenic properties of these herbs, particularly those of OD.

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ABSTRACT

MODULATION OF RAT HEPATIC S9-DEPENDENT MUTAGENESIS, DNA BINDING, AND METABOLISM OF AFLATOXIN B_1 AND BENZO[A]PYRENE BY FOUR CHINESE MEDICINAL HERBS

by

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Extracts of the herbs *Oldenlandia diffusa* (OD) and *Scutellaria barbata* (SB) have been used in traditional Chinese medicine for treating liver, lung and rectal tumors while extracts of *Astragalus membranaceus* (AM) and *Ligustrum lucidum* (LL) are often used as adjuncts in cancer therapy. In this study, we determined the effects of aqueous extracts of these four herbs on aflatoxin B_1 (AFB_1), benzo[a]pyrene (BaP), benzo[a]pyrene 7,8-dihydrodiol (BaP 7,8-DHD) and benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide (BPDE)-induced mutagenesis, mediated by enzymes in rat hepatic 9000 x g supernatant (S9), in *Salmonella typhimurium* TA100. We also studied the effects of these four herbs on AFB_1 and BaP binding to calf thymus DNA and the effects of OD and SB on BaP 7,8-DHD and BPDE binding to calf thymus DNA. After incubation with rat hepatic S9, organosoluble metabolites of AFB_1, BaP, and BaP 7,8-DHD were analyzed by reverse phase high performance liquid chromatography (HPLC), and

water-soluble conjugates of BaP, BaP 7,8-DHD and BPDE were analyzed by alumina column liquid chromatography. Water-soluble conjugates of AFB_1 were determined by passing through C_{18}-Sep-pak cartridge and analyzed by HPLC. Mutagenesis assays showed that aqueous extracts of the herbs we tested produced a concentration-dependent inhibition of histidine-independent revertant (His^+) colonies induced by AFB_1, BaP, BaP 7,8-DHD, and BPDE. LL, OD, and SB inhibited AFB_1 metabolite binding to calf thymus DNA, formation of AFB_1-DNA adducts and metabolism of AFB_1. These three herbs also inhibited the binding of BaP metabolites to calf thymus DNA. LL and SB decreased BaP-DNA adduct formation and SB inhibited the metabolism of BaP. OD and SB significantly inhibited the metabolism of BaP 7,8-DHD and the formation of water-soluble conjugates of BaP 7,8-DHD and BPDE. Both OD and SB modified the distribution of conjugates within the water-soluble fraction. SB inhibited BaP 7,8-DHD and BPDE binding to DNA and decreased the formation of adducts. Our results therefore suggest that the Chinese medicinal herbs LL, OD and SB possess antimutagenic and anticarcinogenic properties. OD and SB may possess these properties because they are able to scavenge the reactive metabolites and thus prevent their interaction with DNA.