Modulation of Cytochrome P450 1 Activity and DMBA-DNA Adduct Formation by Baicalein, Isoflavones and Theaflavins

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ABSTRACT

CYP1 enzymes (CYP1A1, 1A2, 1B1) are ubiquitous members of cytochrome P450 superfamily. They play a key role in the metabolism of procarcinogens, such as polycyclic aromatic hydrocarbons (PAHs), to their ultimate carcinogenic forms. Regulation of *CYP1* gene expression can be mediated through aryl hydrocarbon receptor (AhR). PAHs bind and activate AHR; this increases the transcription of *CYP1* gene and results in CYP1 enzyme induction. The CYP1 enzymes bioactivate PAHs and generate genotoxic metabolites. These metabolites attack DNA and form PAH-DNA adducts which may lead to subsequent mutation. Recently, certain phytochemicals such as resveratrol are reported to inhibit CYP1 activity. They reduce the risk of chemical carcinogenesis by either interfering the AHR-mediated transcriptional pathway or direct inhibiting CYP1 enzymes. Their biochemical actions are believed to be an important part for chemoprevention of cancer.

In this project, the effects of baicalein, (-)-epigallocatechin (EGCG), four theaflavins (TF1, TF2A, TF2B and TF3) and 3 isoflavones (biochanin A, genistein and daidzein) on human CYP1A1/1B1 activities and DMBA-induced DNA damage in the human breast cancer cell line MCF-7 were examined. Preliminary screening showed that all of the above phytochemicals inhibited EROD activity in recombinant human CYP1A1 and CYP1B1. Subsequent analysis indicated that only baicalein, biochanin A, EGCG and genistein did significantly inhibit DMBA-induced EROD activities in MCF-7 cells. Baicalein, EGCG, genistein and biochanin A treatments also reduced DMBA-induced *CYP1A1* and *CYP1B1* gene expressions as measured by semi-quantitative RT-PCR, while theaflavins and daidzein had no effect at this level. Transient transfection studies showed that baicalein, biochanin A, EGCG and genistein suppressed the XRE-dependent gene transcription. Furthermore,

DMBA-DNA adduct formation was reduced after the addition of the above phytochemicals. Interestingly, theaflavins did inhibit DMBA-induced DNA damage without affecting CYP1 enzymes activities and expression. This indicated that theaflavins might exert their anti-tumorigenic ability through other mechanisms. In conclusion, baicalein, biochanin A, EGCG and genistein likely inhibited DMBA-induced carcinogenesis via the intervention of AhR-mediated transcriptional pathway and CYP1A1/CYP1B1 activities. Although theaflavins also diminished the damage of DMBA in other studies, they did not alter DMBA-induced CYP1A1 and CYP1B1 activities at the concentration tested in the present study.

摘要

細胞色素(Cytochrome P450)是生物體內最大一群酵素的總稱，其中的酵素CYP1，是負責對體內和環境中的毒物如多環碳氫化合物(PAH)進行解毒或代謝的功用。CYP1 基因的表現是經由多環芳香烃類受器控制的，多環碳氫化合物在細胞膜外與多環碳氫化合物結合，引發受體反應，被活化的多環芳香烃類受器因此轉移到細胞核，誘導 CYP1 基因的轉錄及表達。之後，CYP1 酵素便爲多環碳氫化合物進行解毒，過程中有可能會釋放出致癌代謝物。這些代謝物一般具有搶奪其他物質電子的特性，成爲缺氧核醣酸 DNA 添加物，損害細胞的 DNA 而引致癌症。 研究發現，有些植物性化合物可以抑制 CYP1 酵素的表達，及干涉多環芳香烃類受器的受體反應，從而減少致癌物對基因的損害，預防癌症。

本篇我們將探討黃芩素 baicalein，異黃酮 isoflavones (genistein、daidzein and biochanin A) ，茶黃素 theaflavins 與綠茶兒茶素 EGCG 抑制 CYP1A1 及 CYP1B1 酵素於人類乳癌細胞系 MCF-7 的作用。我們發現上述的植物性化合物全部都可以減少活性氚化多環碳氫化合物 DMBA 與 DNA 所合成的 DNA 添加物的形成。雖然它們在試管中的實驗可以抑制重組的 CYP1A1 及 CYP1B1 酵素的活性，但是只有 baicalein、genistein、biochanin A and EGCG 能有效修飾 MCF-7 因 DMBA 誘導的 CYP1 酵素的活性。反轉錄聚合酶鏈式反應(RT-PCR)實驗證明 baicalein、genistein、biochanin A 與 EGCG 制止 DMBA 誘導的 CYP1 信使核醣核酸(mRNA)的表達。之後我們再以 XRE-Luc 微量質體做過渡性基因轉(transient transfection)的實驗, 結果亦顯示 baicalein、genistein、biochanin A 和 EGCG 能抑制 CYP1 轉錄依賴性螢光酵素的表現。本篇的結果，告訴我們 baicalein、genistein、biochanin A 和 EGCG 抑制 DMBA 誘導的 CYP1 酵素及基因毒性的現象，可能是因阻礙受質多環碳氫化合物與其受體的結合，影響 CYP1 酵素的表達及活性。以上結果顯示，植物中的化合物防止多環碳氫化合物誘發的基因突變，對於防癌是非常重要的。

ABBREVIATIONS

AHR	Aryl hydrocarbon receptor
APS	Ammonium persulfate
ARNT	Aryl hydrocarbon receptor nuclear translocator
B[a]P	Benzo[a]pyrene
CYP or P450	Cytochrome P450
DMBA	7, 12-dimethylbenz[a]anthracene
DMSO	Dimethyl sulfoxide
EGCG	(-)-epigallocatechin gallate
EROD	Ethoxyresorufin-O-deethylase
GST	Glutathione-S-transferase
Hsp90	Heat shock protein 90
MROD	methoxyresorufin-O-demethylase
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
OME	Omeprazole
PAH	Polycyclic aromatic hydrocarbons
PAS	Per-Arnt-Sim
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RT-PCR	Reversed transcription - polymerase chain reaction
SDS	Sodium codicil sulphate
TCDD	2,3,7,8 tetrachlorodibenzo-*p*-dioxin
TF1	Theaflavin
TF2A	Theaflavin-3-gallate
TF2B	Theaflavin-3'-gallate
TF3	Theaflavin-3,3'-digallate
XRE	Xenobiotics responsive element
β-NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form

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CHAPTER 1 GENERAL INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous toxicants that are commonly found in our environment, such as diesel exhaust, char-broiled meat, tobacco smoke, over-heated cooking oil, etc (IARC, 1983; EPC, 1990). Studies have showed that PAHs elicit a board spectrum of adverse biological responses including tumor promotion, immunosuppression, cardiovascular diseases and hormonal dysregulation (Birbnaum, 1994; Couture et al, 1994; Nebert et al, 1993). Apart from this, PAHs are reported to be genotoxic and capable of forming carcinogen-DNA adducts in human or animal tissues (Dipple et al, 1984; Kriek et al, 1998). Upon exposure, PAHs are metabolized to reactive PAHs intermediates mediated by cytochrome P450s, drug-metabolizing enzymes that play a major role in the activation of hydrophobic xenobiotics. These activated epoxide diol metabolites bind to mutational hotspots of critical genes including p53 tumor suppressor gene, may initiate tumorigenesis (Smith et al, 2000). Moreover, a higher amount of DNA-adduct is observed in various tumor tissues including breast, lung and stomach (Li et al, 1996; Lee et al, 1998; Li et al, 2001).

Xenobiotic-metabolizing enzymes

The metabolism of xenobiotics is catalyzed by a number of enzymes which are involved in phase I and phase II reactions (Figure 1.1). Phase I reactions including oxidation, hydroxylation and reduction, result in reactive metabolites that can be further metabolized by phase II enzymes and excreted. Cytochrome P450s are the most extensively studied phase I enzyme systems that are responsible for the oxidation of a large number of xenobiotics such as PAHs (Gonzalez et al, 1994). The

products of phase I reactions are usually substrates for the phase II enzymes, but some xenobiotics can be directly conjugated to awter-soluble moieties bypassing phase I enzymes. Phase II detoxification enzymes such as glutathione-S-transferase are involved primarily in conjugation reactions. The conjugated chemicals can then be excreted. Although the phase I and phase II enzymes are believed to be evolved for the detoxification of xenobiotics, they are also involved in the generation of reactive intermediates. These active electrophiles may attack cellular macromolecules, leading to toxicity or carcinogenicity. Cytochrome P450s are reported to play an essential role in the activation of a variety of toxicants and carcinogens.

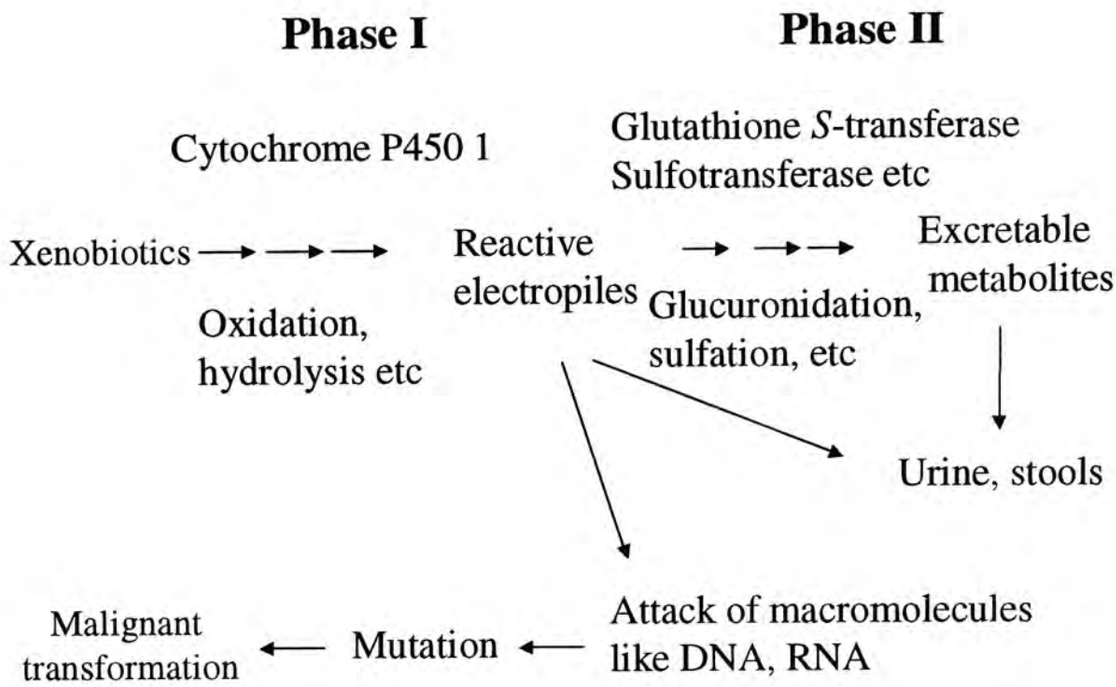


Figure 1.1 Phase I and phase II reactions involved in biotransformation.

Cytochrome P450 1 family

Cytochrome P450 (CYP) monooxygenases are a superfamily of microsomal hemeproteins. They metabolize a number of endogenous compounds such as estradiol and fatty acids and an infinite number of exogenous substances (xenobiotics). CYP1 subfamily containing CYP1A1, CYP1A2 and CYP1B1 is the most ubiquitous of all cytochrome P450 families, and is inducible by PAHs via the aryl hydrocarbon receptor (AhR) transactivation pathway. The family is featured by its specificity of substrates in a planer molecular configuration, such as PAHs, TCDD. Thus, PAHs are not only the substrates but they also are inducers of CYP1 enzymes. A major characteristic, which distinguishes CYP1 from other cytochrome P450 families, is their propensity to metabolize compounds at site and generate reactive electrophiles (Guengerich et al., 1998). In the presence of epoxide hydroxylase, CYP1 enzymes catalyze the conversion of benzo[a]pyrene into mutagenic diol epoxides (Shimada et al., 1999; Kim et al., 1998). Biotransformation of DMBA is found to be dependent on CYP1A1/1B1 (Conney et al., 1994; Larsen et al., 1998). The metabolites from the above reactions intercalate with DNA and may lead to cancer (Figure 1.2). This explains the significance of CYP1 enzymes in the biotransformation of chemical carcinogens into genotoxic moieties. Furthermore CYP1A1 induction has long been considered as a biomarker of identifying individuals with high cancer risk. (Karki et al., 1987; Guengerich, 1991; Kawajiri et al., 1993) A more detail information on the characteristic of individual CYP1 enzymes is given below.

CYP1A1

CYP1A1 is the most studied enzyme in the CYP1 family. It is mainly extrahepatic and is responsible for the metabolism of PAHs. Constitutive expression of CYP1A1 in human and rat liver is hardly detectable but it is highly inducible in liver and other tissues by PAHs. It is one of the AhR battery genes and is also possible to be activated by other ligand independent pathways induced by omeprazole (OME) (Backlund et al., 1997). CYP1A1 polymorphism affects its enzyme level toward PAHs, i.e., more readily inducible variant can produce greater carcinogenic activation (Schwarz et al., 2001). Woman with CYP1A1 polymorphism who also smoked cigarettes has a higher risk of lung cancer.

CYP1A2

Human CYP1A2 is the most abundantly expressed CYP1 enzyme in the liver (Landi, 1999). Substrates for metabolic activation by CYP1A2 include aminoflurenes, nitrosamines and a number of PAHs. Unlike CYP1A1, the molecular mechanism underlying the induction of CYP1A2 is obscure. It is because the constitutive or inducible expression of CYP1A2 is exceptionally low (Roberts et al., 1994) or even absence in human or other mammalian cell lines even though it is one of the most abundant P450s in human liver.

CYP1B1

CYP1B1 is expressed in a tissue-specific manner and can be induced by AhR ligands. Not only does it bioactivate PAHs and aryl amines, but it is also a

catalytically efficient estradiol 4 hydroxylase that is likely to participate in endocrine regulation and the toxicity of estrogen. CYP1B1 appears to be more active than CYP1A1 in the conversion of a number of PAHs to their genotoxic intermediates (Shimada et al., 1996). Low levels of CYP1B1 mRNA have been observed in normal human tissue (Sutter et al., 1994; Willey et al., 1997; Vadlamuri et al., 1998), while CYP1B1 protein has only been detected in cancer cells, e.g. mammary fibroblast (McKay et al., 1995; Murray et al., 1997). Elevated CYP1B1 expression and putative CYP1B1 catalyzed E2 4'-hydroxylation have been suggested to be probable biomarkers of carcinogenesis in several tissues including breast and uterus myometrium (Spink et al., 1998).

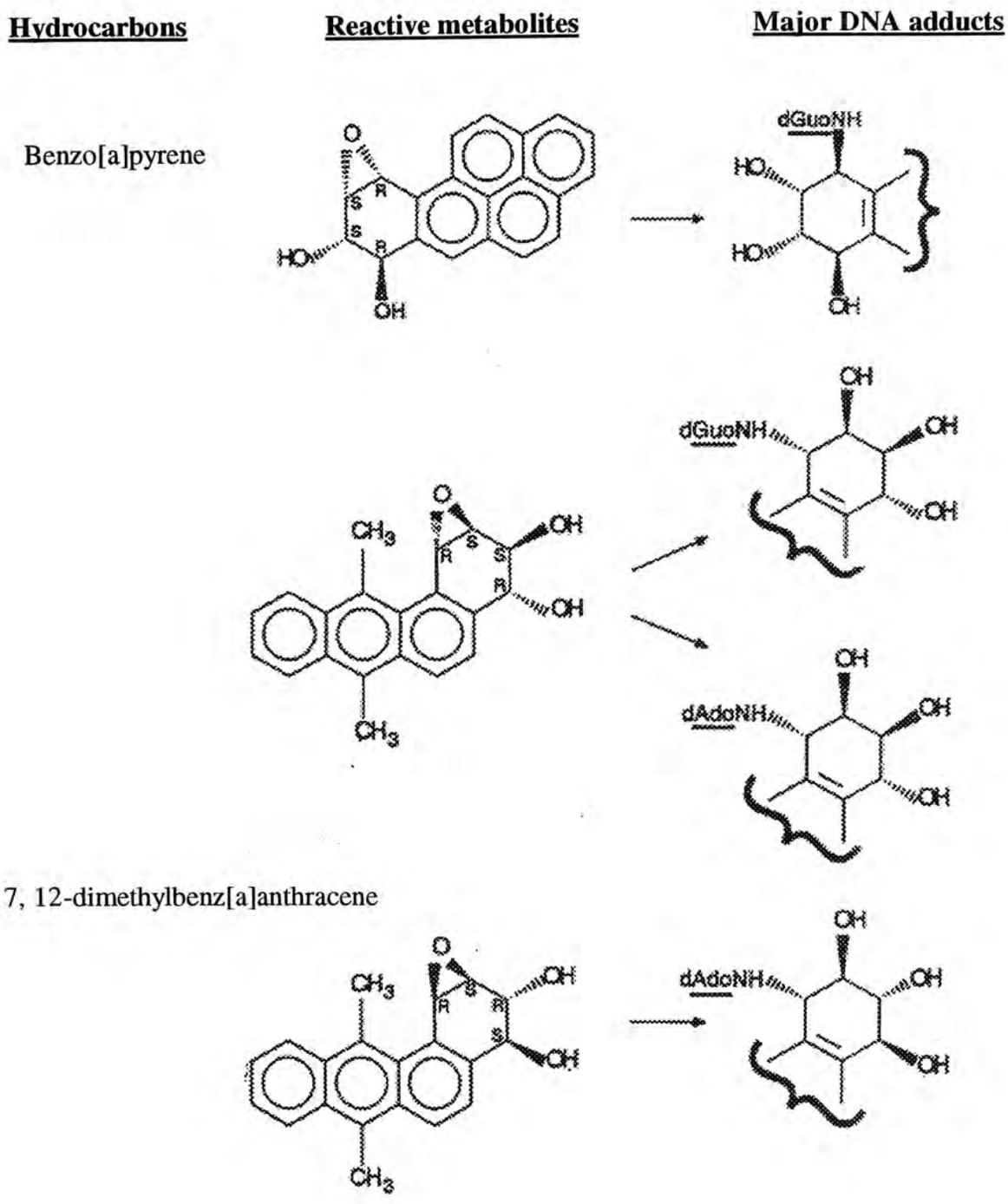


Figure 1.2 Reactive metabolites of B[a]P and DMBA and the principle adducts that they form with DNA (Adopted from Dipple et al., 1994).

Transactivation of CYP1 enzymes by Aryl hydrocarbon receptor (AhR)

What are the mechanisms governing CYP1 induction and how are they regulated? Studies have implicated that Ah receptor (AhR) plays a pivotal role in regulating the transcription of CYP1 enzymes. Ah receptor is a ligand-activated basic helix-loop-helix (bHLH) transcriptional factor, which belongs to the PAS (Per-Arnt-Sim) homology domain family. Unliganded AhR in cytoplasm is associated with two 90kDa heat shock proteins and immunophilin chaperon, AhR-interacting protein (AIP). Binding to ligands like PAHs triggers the conformational change of AhR, which results in the dissociation of AhR with the complex. The activated AhR translocates into the nucleus and subsequently dimerizes with aryl hydrocarbon receptor nucleus translocator (ARNT). The ligand-bound heterodimer binds xenobiotic responsive elements (XRE) and can initiate gene transcriptions with the recruitment of coactivators and other general transcription factors (Figure 1.3). XRE with the core sequence "TNGCGTG" is present in the promoter regions of several genes involved in the metabolism of xenobiotics, including cytochrome P450 1 family (Chang et al., 1998; Tian et al., 1999; Kronenberg et al., 2000).

To determine whether AhR plays a role in modulating carcinogenesis through the induction of xenobiotic-metabolizing enzymes, Shimizu et al. (2000) expose AhR-deficient mice to benzo[a]pyrene (B[a]P). They find that the carcinogenicity of this agent is lost in AhR-deficient mice, while cytochrome p450 1A1 has been implicated to be responsible for the toxicity of B[a]P. In another CYP1B1 knockout mouse model, fewer DMBA-induced lymphoma incidences have been reported in the null mice (Buters et al., 1999). From these data, the positive roles of AHR, CYP1A1, and CYP1B1 in PAH-induced carcinogenesis are substantiated.

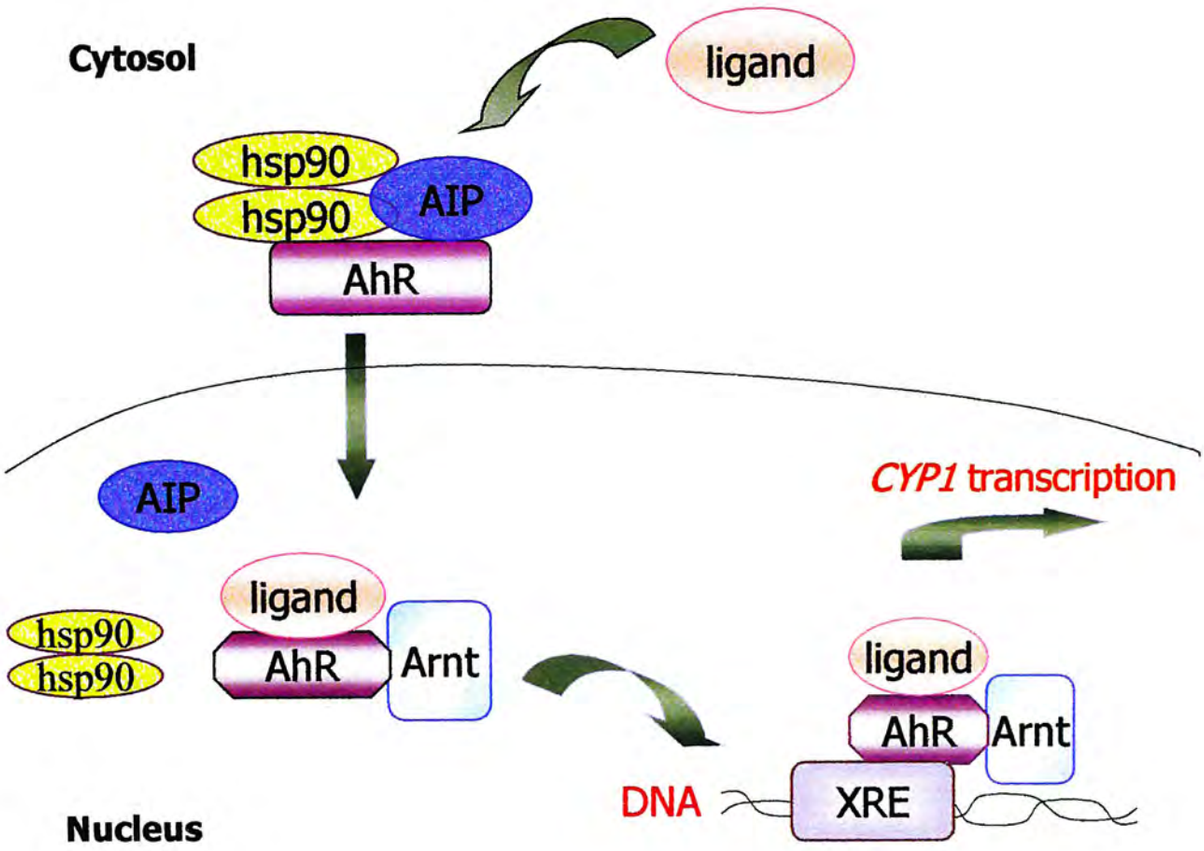


Figure 1.3 Transactivation of *CYP1* gene by AhR.

Implication of PAHs and CYP1 family in breast cancer

Some mammary carcinogens can be transformed by phase I enzymes and attack DNA, and constant exposure over time may result in an accumulation of gene mutation in certain critical genes like p53. Carcinoma development only occurs when those mutations impair normal cell cycle regulation. To date, the significance of PAHs and CYP1 family expression in breast cancer still remain unclear, as no conclusive effects can be drawn. While PAH-DNA adduct may also be formed and accumulate in breast tumor, breast cancer risk cannot be predicted from the level of PAH-DNA adduct in normal breast tissue (Williams et al., 2000; Rundle et al., 2000). Nevertheless, as researches have indicated that mammary gland is susceptible to tumor initiation by DNA-damaging agents like PAHs, it would therefore be surprising if exposing xenobiotic genotoxins do not have a consequence for tumor initiation. Studies have shown that a single injection of DMBA, a synthetic PAH, induce mammary tumor in rat. Acute exposure to benzo[*a*]pyrene inhibits the expression of BRCA-1, a major tumor suppressor gene, in breast cancer cells (Jeffy et al., 1999). Furthermore, it is conceivable that people with certain alleles of polymorphic genes encoding higher expressions of metabolizing enzymes would lead to increased rates of breast cancer. Polymorphisms of CYP1A1 and CYP1B1 have been related to a high risk of breast cancer (Hanna et al., 2000). Benzo[*a*]anthracene-mediated induction of CYP1A1 expression is paralleled by increases in the formation of IQ-DNA adducts (Williams, 1998). Studies indicate that CYP1B1 mediated E2 4 hydroxylation is higher in human breast tumor compared with normal breast cells. (Trombino et al., 2000) Moreover, the dysregulation of AhR, CYP1A1 and CYP1B1 have been suggested to be the molecular biomarkers for PAH-induced mammary cell transformation (Dialyna et al., 2001).

Potential role of phytochemicals on cancer prevention

Experimental and epidemiological data have revealed that certain phytochemicals are effective in preventing neoplastic development and carcinogen-induced DNA damages. They exert a multiplicity of biochemical actions that is believed to be responsible for their chemopreventive actions on cancer. Antioxidants like polyphenols from green tea function as nucleophile-interacting electrophiles prevent carcinogen bioactivation to form PAH-DNA adduct. Other phytochemicals such as curcumin suppress tumor progression by inhibiting cell proliferation enzymes and induce apoptosis of tumor cells. One of the most significant mechanisms by which these compounds may perform their putative anticancer effects is through the modulation of phase I activating enzymes and phase II detoxifying enzymes. Table 1.1 shows the effects of certain phytochemicals on xenobiotic metabolism and the related enzymes. Phytochemicals may affect gene transcription and translation of specific xenobiotic metabolizing enzymes as well as the degradation of their mRNA and protein.

Compounds	Effects	Xenobiotic metabolism and enzymes
Quercetin	↓↓↓	Ethoxyresorufin *O*-deethylase activities, aryl hydrocarbon hydroxylase
Naringenin	↓↓	Ethoxyresorufin *O*-deethylase activities, metabolism of benzo[a]pyrene
Indole-3-carbinol	↑	P450s 1A1, 2B1, UDP-glucuronyl transferase, pentoxyresorufin *O*-dealkylase activities,
Limonene	↑	P450, P450s 2B, 2C
	↑↑	DMBA metabolism
Flavones	↑	P450, glutathione *S*-transferase
	↓↓	Ethoxyresorufin *O*-deethylase activities

Table 1.1 Effects of food phytochemicals on xenobiotic metabolism.

(Modified from Smith et al., 1994).

Significance of this project

To find out the potential role of phytochemicals in drug-metabolizing enzyme modulation, extensive studies have been carried out to delineate the effects of phytochemicals on CYP1 activating enzymes. Researches indicate that inhibition of CYP1 enzymes is beneficial in the prevention of PAH-DNA adduct formation *in vivo* and *in vitro*, possibly by reducing the formation of activated intermediates. Galangin from India root suppresses DMBA-DNA adduct formation in a breast cancer cell line by acting as an inhibitor of CYP1A1 activity and an agonist of AhR (Ciolino et al., 1999); whereas furanocoumarins prevents DMBA diol-epoxide formation catalyzed by CYP1 enzymes in female SENCAR mice (Kleiner et al., 2002).

In light of this, we would like to address the effects of certain phytochemicals including baicalein, isoflavones and theaflavins on CYP1 enzyme regulation and the DNA-adduct formation in the breast tumor cell line MCF-7 in response to DMBA. Studies have demonstrated that MCF-7 cells expressing CYP1A1 and CYP1B1 are similar to normal human mammary epithelial cells with regard to AhR expression and CYP1A1 activity (Larson et al., 1998). A synthetic PAH, DMBA, is chosen because it is a mammary carcinogen that has been used extensively as a prototypical agent in cancer research. Not only does it act as an inducer for CYP1 transcription but is also a substrate of CYP1 enzymes. The activated DMBA intermediates can covalently bind to DNA that may trigger DNA damage. Since certain phytochemicals have been shown to be AhR antagonists that render the induction of CYP1 enzymes, the present study will investigate the effects of phytochemicals on DMBA-induced XRE transactivation.

CHAPTER 2 MATERIALS AND METHODS

Chemicals

Baicalein, EGCG, genistein, biochanin A and daidzein were obtained from Sigma-Aldrich, Milwaukee, WA, U.S.A. Ethoxyresorufin, and DMBA were purchased from Sigma Chemicals (St Louis, MO, USA). The carrier solvent DMSO was also obtained from Sigma Chemicals. All other chemicals, if not stated, were acquired from Sigma Chemicals.

Maintenance of cells

MCF-7 cells (gift from Dr. V.C. Jordan) were grown in RPMI – 1640 phenol red free media (Sigma Chemicals) supplemented with 50 units/ml Penicillin -Streptomycin (GibcoBRL, USA) and 10% v/v fetal bovine serum (Invitrogen Life Technology, Rockville, MD) in a humidified incubator at 37°C, in 5% CO_2. When cells reached about 80% confluence, the medium was removed and the cells were trypsinized with 3 ml trypsin-EDTA for 3 min. The cell suspension was transferred to a 15-ml centrifuge tube containing 7 ml PBS and centrifuged at 1,000 rpm at room temperature for 5 min. The cell pellet was resuspended with fresh medium and transferred to new flasks and incubated as described above.

Preparation of cell stock

Semi-confluent cells in 75 cm^2 flasks were washed with PBS and trypsinized as described above. After centrifugation, the cell pellet was suspended in 450 µl of RPMI supplemented with 10% v/v FBS and transferred into a 2-ml freezing tube containing 450 µl of FBS and 100 µl of DMSO. The tube was put in an isopropanol-filled freezing container (Nalgene, USA) and the container was put into

a –80°C freezer so that the cell stock was frozen at a constant rate of about 1°C /min. cell stocks were stored at -80°C or liquid nitrogen.

Cell recovery from liquid nitrogen stock

The cell stock was taken out from the liquid nitrogen tank and thawed immediately in a 37°C water bath. To remove the DMSO, the cell stock was diluted into 10 ml of RPMI with 10% v/v FBS. The cell suspension was centrifuged as described above and the supernatant was discarded. The cell pellet was then suspended in 10 ml of RPMI with 10% v/v FBS, plated on a 75 cm^2 culture flask and incubated as above. Medium was changed one day after recovery of the cells.

Measurement of cell viability

Cell viability was assessed by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) straining as described by Mosmann (1983). Briefly, MCF-7 cells were plated in 96-well plates at 10^4 cells per well, and 1 μM DMBA and various concentrations of phytochemicals were administered for 24 h. At the end of the treatment, 50 μl of 1 mg/ml MTT was added and the cells were incubated at 37°C for 4 h. Then 100 μl of DMSO was added and the plate was allowed to stand at room temperature for 30 min. Cell viability was determined by the absorbance at 600nm.

Preparation of cell lysates (NP-40 cell lysis buffer)

To harvest the cells, the medium was discarded and the cells were washed once with PBS. The cells were scraped off from the plate and resuspended in the residual PBS. The suspension was transferred into a microfuge tube. Another 1 ml of PBS was added to the plate and the residual cells were scraped off. The suspension was

again transferred to the same microfuge tube and centrifuged at 13,000 rpm in a desktop centrifuge for 30 s; the supernatant was discarded. The cell pellet was resuspended with three pellet-volumes of cell lysis buffer supplemented with protease inhibitors mixture and incubated on ice for 30 min. To get rid of the cell debris, the resuspension was centrifuged at 13,000 rpm in a desktop centrifuge for 30 min at 4°C. The supernatant was saved and transferred to a new microfuge tube. The protein concentration of the lysate was measured as described above. For confirmation of expression of expected proteins, the lysate was analyzed by SDS-PAGE followed by Western blot.

XRE-luciferase gene reporter assay

Transient transfection of cell using lipofectamine PLUS reagent

MCF-7 cells were seeded at 10^5 cells/well in 24-well plates. After 24 h, the cells were transiently transfected with 4.0 μg of the XRE reporter plasmid and 1.0 μg of renilla luciferase control vector pRL (Promega, Madison, WI, USA) in LipofectAmine PLUS (Invitrogen Life Technologies) as described in the manual. After 16 h, the transfection medium was removed and 1ml of fresh medium was added to each well. Cells were then treated with 1 μM DMBA and various concentrations of phytochemicals for 24 h. The amounts of these two luciferases were determined using Dual-Luciferase Assay Kit (Promega).

Dual Luciferase Assay

Dual Luciferase assays were preformed according to the manual (Promega). 20 μl cell lysate was mixed with 50 μl of reaction buffer supplemented with firefly luciferase substrate. The luciferase bioluminescence was measured by using a

FLUOstar Galaxy plate reader. The XRE transactivation activities represented by firefly luciferase light units were then normalized with that of renilla luciferase.

Manipulation of DNA and RNA

Separation and purification of DNA from agarose gel

Different percentages of agarose gels (typically 1.0% or 2.0%, w/v) were prepared with TAE buffer containing 0.4 μg/ml of ethidium bromide. DNA samples were loaded on the gel immersed in TAE buffer and separated at 70V for 45 min; 1 kb marker or 100 bp marker was used as size reference. The bands of interest were cut out from the gel and purified with the CONCERT™ Rapid Gel Extraction Systems (Gibco BRL). 30 μl of Gel Solubility Buffer (L1) was added for every 10 mg of gel in a 1.5-ml microtube at 50°C to allow complete dissolve. The mixture was then transfer to a cartridge on the top of a 2-ml wash tube and centrifuged at 13,000 rpm in a desktop centrifuge for 1 min. The solution in the collection tube was discarded; 0.7 ml Wash Buffer was added to the cartridge which was then incubated at room temperature for 3 min and centrifuged as above. The cartridge-collection tube assembly was centrifuged for further 1 min to remove the residual wash buffer. The DNA was eluted with 40 μl of MQ water and collected by centrifugation into a new clean microtube. The eluted DNA was stored at -20°C or used immediately.

Separation of DNA from acrylamide gel

The DNA samples in loading buffer were separated on 10% polyacrylamide gel. The apparatus (Mini-PROTEAN III, BioRad) and power supply (Power Pac 200/ 1000/ 3000, BioRad) used for running protein samples were products from BioRad. The 8% polyacrylamide gel mix (5 ml) was prepared by mixing 1.33 ml of 30%

acrylamide together with 2.7 ml MQ water, 1 ml 5X TBE 0.4 ml APS and 4 µl of TEMED. The DNA samples were then loaded on the wells and voltage (usually 200 V or lower) was applied to the buffer. The DNA bands were visualized by straining the gel with TBE containing 0.5 µg/ml of ethidium bromide for 15 min.

Restriction digestion

Restriction digest was used to prepare DNA inserts for subcloning or for confirmation of transformants. To prepare DNA inserts, 5 µg of plasmid DNA was used and the total reaction volume was 50 µl. To confirm transformants, 1 µg plasmid DNA was used and the total reaction volume was 15 µl. All restriction enzymes and the respective 10× buffers were products from New England BioLabs. Plasmid DNA was mixed with restriction enzymes (1 or 0.1 unit each) and 10X buffers (5 or 1.5 µl); then the volume was brought to 50 or 15 µl by MQ water. All the reactions were incubated in a 37°C water bath for 2 h unless instructed otherwise by the manufacturer.

Ligation of DNA fragments

The T4 ligase (400 unit/ µl) and 10× T4 ligase buffer used in all ligation reaction were products from NEB unless stated otherwise. The DNA insert and vector were mixed at a 3:1 concentration ratio (determined by resolving them on agarose gel), with 2 µl of 10× T4 ligase buffer and 400 units of T4 ligase (one unit being defined as the amount of enzyme required to give 50% ligation of Hind III fragments of Lambda DNA in 30 min at 16°C in 20 µl and a 5' DNA termini concentration of 0.12 µM). The volume was brought up to 20 µl with MQ water. The reaction was incubated at room temperature for 2 hrs or at 16°C for 16 h. Then the ligation product was then stored at -20°C or used immediately for transformation.

Transformation of DH5α

The whole ligation mix or 1 μg of plasmid DNA was transferred into a 1.5-ml microtube containing 200 μl of competent DH5α or BL21(DE3) cells and left on ice for 30 min. The cells were placed into 42°C water bath for 1.5 min for heat shock and chilled on ice for 3 min for cold shock. The transformation mix was mixed with 1 ml of LB medium and incubated at 37°C in a shaker for 30 min. The mixture was then centrifuged at 1,000 rpm in a desktop centrifuge for 5 s, and 900 μl of the supernatant was discarded. The pellet was resuspended in the residual medium and plated on LB^{amp} plate and incubated at 37°C overnight. Transformants that grew on plate as single colonies were transferred into 2 ml of LB^{amp} medium with toothpicks and incubated at 37°C with shaking overnight. The cultures were screened by PCR with appropriate primers and analyzed with agarose gel electrophoresis. Transformants that scored positive with the PCR screening were further analyzed by restriction digest after mini-prep. One of the transformants was then inoculated into 200 or 500 ml of LB^{amp} medium and grew with shaking at 37°C overnight. Large-scale plasmid isolation (maxi- or giga-prep) was then performed.

Small scale plasmid purification from DH5α (mini prep)

A single colony of transformed DH5α on a LB^{amp} plate was inoculated into 2 ml of LB^{amp} medium at 37°C with shaking overnight. The overnight culture was transferred into a microtube and pelleted; the supernatant was discarded. Plasmids were purified by QIAGEN Plasmid-Prep Kit. 100 μl of P1 buffer was added into the microtube, followed by vortexing. The suspension was then mixed with 100 μl of P2 and incubated at room temperature for 10 min to lyse the cells. P3 (100 μl) was then added and the lysate was incubated at 4°C for 5 min to neutralize the lysates. The lysate mixture was centrifuged at 4°C and 13,000 rpm in a desktop centrifuge for

10 min. The supernatant was then transferred to a new microtube and the plasmid DNA was purified by resins inside a column.

Large scale plasmid isolation from DH5α (maxi-prep)

QIAGEN Plasmid Maxi kits were used to prepare plasmid DNA at large scale. An overnight culture of transformed DH5α (500 ml of LB^{amp}) was transferred into a 500-ml conical tube and centrifuged at 6000 g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml of P1 solution. 10 ml of P2 solution was applied to the resuspended culture and incubated at room temperature for 5 minutes. To neutralize the lysate, 10 ml of P3 solution was pipetted into the lysate, followed by incubation on ice for 10 minutes. The cell debris was separated from the plasmid DNA by centrifugation in a 30-ml centrifuge tube at 13 k rpm in a desktop centrifuge for 30 minutes. To further remove the debris, the supernatant containing the plasmid DNA was transferred to a new 30-ml centrifuge tube and centrifuged for further 30 minutes. QBT solution was added to the maxi- prep column to activate the resin at the bottom of the column. After all the QBT has gone through the column, the cell lysate from above was pour into the column until all of the lysate has eluted out. To wash the resin, one column volume of QC solution was added to the column twice. To elute the DNA from the resin, the column was assembled to a 15-ml centrifuge tube containing 15 ml of 100% isopropanol; then 15 ml of QF solution was added to the column. The eluant was mixed with the isopropanol by inversion. To precipitate the DNA, the solution was centrifuged at 10,000 g for 45 minutes; then the supernatant was discarded and the pellet was dried at room temperature. To resuspend the dried pellet, 400 µl of MQ water was added to the tube.

Construction of *XRE* activated luciferase reporter gene

A fragment with five XRE elements from rat *CYP1A1* 5'-flanking region was amplified using primers (XRE FOR and XRE REV) specific for rat genomic DNA as described by Backlund et al. (1997). No other response elements were identified in this fragment. The PCR product was digested with SmaI and BamHI and subcloned into a firefly luciferase reporter vector pTA–Luc (Clontech, Palo Alto, CA, USA).

Measurement of DMBA-DNA adduct formation

This assay was performed as previously described (Ciolino et al., 1999). MCF-7 cells were plated in 6-well plates at 5×10^5 cells per well and allowed to attached for 24 h. Then 0.1 μg / ml of [^{3}H]-DMBA (Amersham, Arlington Height, IL, USA) was administered with or without phytochemicals. After 16 h, cells were washed twice with cold phosphate-buffered saline (PBS), trypsinized and pelleted. Nuclei were separated by incubating the cells for 10 min on ice in lysis buffer A (10 mM Tris-HCl, pH7.5, 320 mM sucrose, 5mM magnesium chloride and 1 % Triton X-100). The nuclei were collected by centrifugation at 5000 rpm for 10 min at 4°C after the incubation. The nuclei were then lysed by 400 μl lysis buffer B (1 % sodium dodecyl sulphate (SDS) in 0.5 M Tris. 20 mM EDTA and 10 mM NaCl, pH 9), followed by the treatment of 20 μl 20 mg / ml Proteinase K for 2 h at 48°C. After that, the samples were allowed to cool to room temperature and the residual protein was salted out by adding 150 μl of saturated NaCl. The samples were then subjected to centrifugation at 13,000 rpm for 30 min at 4°C. Genomic DNA was isolated from supernatant by ethanol precipitation, and redissolved in MQ water. Absorbance at 260 nm and 280 nm were employed to determine the amount and purity of the extracted DNA. DNA samples attained a 260nm/280nm ratio of > 1.9 was used for scintillation counting.

Semi-quantitative RT-PCR Assay

Isolation of RNA using TRIzol® Reagent(Life Technology, USA)

Total RNA was isolated from cells grown in 6-well Costar plates in triplicates by Trizol Reagent as described by the manufacturers (Life Technology, USA). Cells were washed with cold PBS twice. 1 ml of TRIzol reagent was added in each well and aspirated for several times to lyse the cells. The homogenous sample was incubated at room temperature for 5 min and transferred to a microtube. 0.2 ml of chloroform was added. Then the sample was mixed vigorously by shaking for 15 s and was allowed to stand at R.T. for 3 min. The sample was then subjected to a centrifugation at 12 000 g for 15 min at 4°C. Afterward, the upper aqueous layer was transferred to a new microtube containing 0.5 ml isopropanol. The sample was incubated at room temperature for another 10 min and centrifuged at 12 000 g for 10 min at 4°C. The supernatant was removed and the RNA pellet was mixed with 1 ml of 75 % ethanol. Then the RNA was re-pelleted and air-dried. 30 µl of DEPC water was added to redissolve the RNA which was then stored at –80°C. The amount and purity of RNA were determined by measuring its absorbance at 260nm and 280nm. The amount was calculated by the formula: OD_{260} * 40 mg ml^{-1} = Amount of RNA (mg ml^{-1}). RNA isolated by this method exhibited a 260/280 ratio of > 1.6.

RT-PCR

A reverse transcription-polymerase chain reaction (RT-PCR) assay was used to quantitate mRNA level. 5 µg of RNA was used for cDNA synthesis, and the final volume was diluted to 20 µl. Primers of CYP1A1, CYP1B1 and β-actin, sequences as published formerly (Dohr et al.,1995) and a Perkin Elmer Thermocycler (GeneAmp PCR System 2400) was utilized to amplify the target cDNAs separately after the first strand reaction. All PCR reactions consisted of 0.2 mmol/L dNTP, 2 µl

cDNA, 0.2 μmol/L of each primer, 1×PCR buffer and 1 U of Taq polymerase. The conditions were 94°C for 45 s, 65°C for 45 s, 72°C for 1 min, and a final extension period of 7 min at 72°C. The amplification cycles were 25 for CYP1A1, 23 for CYP1B1, and 19 for β-actin. The PCR products were separated on 2% agarose gel, stained with ethidium bromide, and photographed. A scanner equipped with Scion Image software (Scion Corporation, Frederick, MD, U.S.A.) was used to compare the optical density of the amplified fragments. The linearity of signals was verified in separate experiments.

Enzyme Activities

Isolation of microsomes

MCF-7 cells were treated with 1 μM of DMBA to induce CYP1A1 and 1B1 activities. After 24 h these cells were trypsinized, centrifuged at 1000 rpm for 5 min at 4°C. The cells were then suspended in storage buffer (10 mM Tris-HCl, 0.25 M sucrose, pH 7.5, with 100 μg/ml phenylmethylsulphonylfluoride, 300 μg/ml EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin 0.7 μg/ml Pepstatin A), and sonicated on ice for 30s. The lysate was centrifuged at 5,000 rpm for 15 min, and the supernatant was collected and centrifuged again at 45,000 rpm for 30 min at 4°C. The microsomal pellet was resuspended in the storage buffer, aliquoted and stored at –80°C. The concentration of microsomal protein was determined by a Bradford-based BioRad protein assay as described above.

EROD activities in intact cells

The assay method was performed as previously described (Cholino et al., 1999). In brief, MCF-7 cells in 96-well were treated with 1 μM DMBA and various

concentration of phytochemicals. The medium was then removed and the cells were washed twice by 100 µl PBS. Ethoxyresorufin-O-deethylase (EROD) activities, which are indicatives of CYP1A1 and CYP1B1, were then carried out. 50 µl of 5 µM ethoxyresorufin (ER) in PBS with 1.5 mM salicyclamide was added in each well, and incubated at 37°C for 15 min. The reaction was stopped by 50 µl of ice-cold methanol, and the resorufin generated was measured by a FLUOstar Galaxy microplate reader (BMG Labtechnologies, Offenburg, Germany) with an excitation of 544 nm and emission at 590 nm. The activities were quantitated against resorufin standards.

EROD inhibition assay

A reaction mixture containing 10 µg microsomal protein, or 1.6 pmol human recombinant CYP1A1, or 2 pmol CYP1B1 was incubated in 100 µl PBS, pH 7.2 with 400 nM ER/MR and phytochemicals in different concentrations. The reaction was initiated by 500 µM NADPH, and stopped by 100 µl of ice-cold methanol. After 15 min of incubation, the fluorescence was measured as described above.

To determine the kinetic of CYP1 enzyme inhibition, 280 µl of 125 – 1600 nM ER/MR were prepared in the presence of various concentrations of drugs. Three 80 µl of aliquots of each (yielding final concentration of 100nM – 1600 nM) was placed in a 96-well plate and the reaction was initiated by the addition of 20 µl of 2500 µM of NADPH (final concentration 500 µM) and 10 µg microsomes per well. After 15 min, 100 µl of methanol was added and the reaction was assayed as above.

Statistical Analysis

A Prism® 3.0 software package (GraphPad Software, Inc., CA, U.S.A) was utilized for statistical analysis. The results, whenever applicable, were analyzed by

One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p<0.05$) were observed. T-test was also performed to compare the cell cytotoxicity between DMBA-treated samples and those samples treated with DMBA plus phytochemicals.

CHAPTER 3 BAICALEIN INHIBITS DMBA-DNA ADDUCT FORMATION BY MODULATING CYP1A1 AND 1B1 ACTIVITIES

Introduction

Baicalein is a flavone isolated from the root of *Scutellariae radix* 黃芩. (Figure 3.1) The flavonoid is present in the popular Japanese herbal mixture Sho-saiko-to, which is widely used for the treatment of liver disease and carcinoma. In China, it has been used for thousands of years for curing inflammatory diseases and hypertension. Baicalein has been demonstrated to possess antiproliferative activity on prostate cancer cells (Chen et al., 2001), hepatoma cells (Motoo et al., 1994), vascular smooth muscle cells (Huang et al., 1994), etc. Baicalein has also been described as an antioxidant (Chen et al., 2000), anti-inflammatory agent (Lin et al., 1996), and an inhibitor to prostaglandin E2 (Nakahata et al., 1998). Previous animal studies have demonstrated the modulating effects of baicalein on CYP1A1 enzyme in mice (Ueng et al., 2000) while Kim et al. (2002) suggested that baicalein might protect the liver through the inhibition of CYPIA2-mediated procarcinogens activation. However, the possible role of baicalein on CYP1 enzymes and carcinogen induced DNA damage still remains unclear.

In this study, we investigated the effects of baicalein on DMBA-induced AhR transactivation, CYP1 enzymes activities and induction. Our hypothesis was that baicalein could reduce DMBA-DNA adduct formation by modulating the biotransformation enzyme activities.

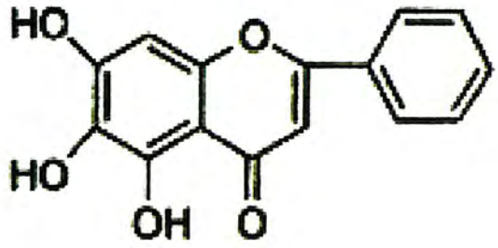


Figure 3.1 Picture of *Scutellariae radix* and the structure of baicalein.

Results

EROD activities in MCF-7 cells and inhibition assay

EROD activities could be the indicatives of CYP1A1 and CYP1B1. The significance of both CYP enzymes has been implicated in chemical carcinogenesis in recent researches. EROD activities in intact cells that had been exposed to 1 μM DMBA in the presence of baicalein for 24 h were measured. DMBA-induced EROD activities in cell cultures were reduced by baicalein in a dose-dependent manner. The IC_{50} was around 1 μM as shown in Figure 3.2 To further explore the inhibition mechanism for baicalein, microsomal EROD activity with or without baicalein was measured in a different substrate concentration and the kinetics of enzymes inhibition by baicalein were analyzed by double-reciprocal (Lineweaver-Burk) analysis (Figure 3.3) Baicalein was proved to be a competitive inhibitor of EROD with an apparent K_i of 0.54 μM. These results showed that baicalein could modulate the EROD activities induced by DMBA at the enzyme level, and the biotransformation of DMBA could be affected by the reduced enzyme activities.

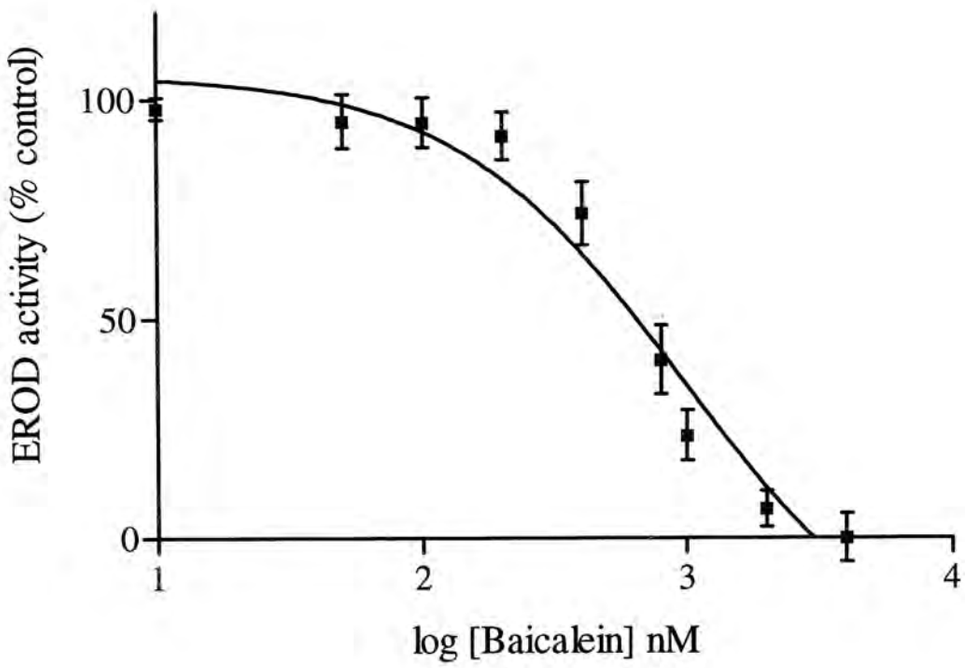


Figure 3.2 Baicalein inhibited DMBA-induced EROD activity in MCF-7 cells.

MCF-7 cells were seeded in 96-well culture plates and treated with 1 μM DMBA and various concentrations of baicalein. After 24 h of treatment, cells were assayed for EROD activity as described. The estimated EC_{50} value is 0.97 μM, and the values are means ±SE, n=6.

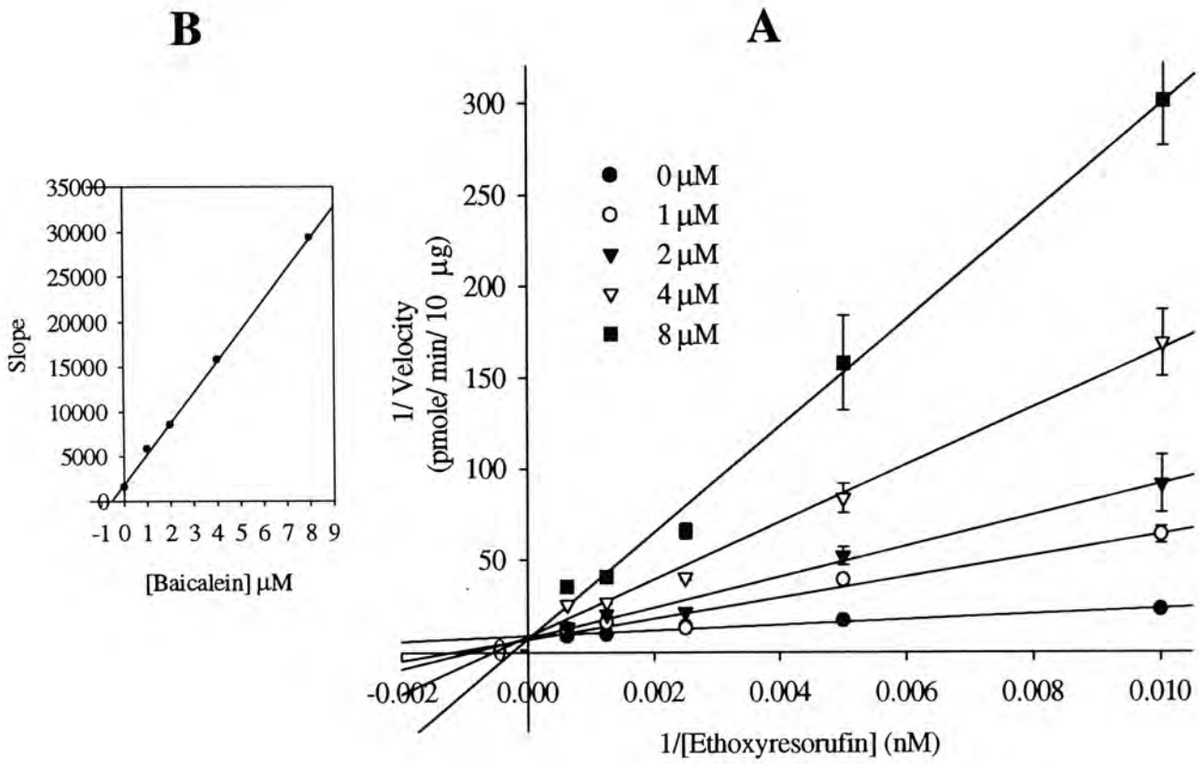


Figure 3.3 Baicalein competitively inhibited microsomal EROD activity.

MCF-7 cells were cultured in T-75 flasks and treated with 1 μM DMBA. After 24 h of treatment, microsomes were isolated and used for enzyme kinetic study. (A) EROD activity in 10 μg of microsomes was measured in the presence of 400nM ER and the indicated concentration of baicalein.. Values are means ±SE, n=3. (B) Lines shown were determined by linear regression of the reciprocal data. *Inset*, replot of the slopes obtained by linear regression of data from the Lineaweaver-Burk plot, with derivation of Ki Values shown here are mean ±SE, n=3.

Baicalein suppressed DMBA-induced XRE-driven luciferase activities.

Many studies indicate that some phytochemicals can compete with xenobiotics for Ah receptor binding (Ashida et al., 2000; Lee et al., 2000; Christopher et al., 2001). Because the DNA-binding domain of the activated receptor recognized XRE sequence, this transient transfection assay could signal the receptor status. Baicalein, by itself, was a weak agonist for XRE binding at 1 μM, but the agonistic activities were not seen at the other concentrations tested (Figure 3.4). On the other hand, DMBA could induce the luciferase activities by 4.2 folds as shown in Figure 3.5, and the luciferase activity was reduced in a dose dependent manner when baicalein was co-administered in the cultures. This indicated that the DMBA-induced XRE transactivation activities could be suppressed by baicalein. Gene promoters that contained XRE regions, such as CYP1A1 and CYP1B1, could be affected by baicalein treatment.

Baicalein inhibited DMBA-induced CYP1A1 and CYP1B1 mRNA expression

As the XRE transactivation activities were repressed by baicalein, the mRNA expressions of CYP1A1 and CYP1B1 were studied. The RT-PCR results as illustrated in Figure 3.6A indicated a dose dependent decline in both CYP1A1 and CYP1B1 expressions. The optical density readings (Figure 3.6B & 3.6C) showed 4.5 and 2.6 fold increase in CYP1A1 and CYP1B1 expressions in cells treated with 1 μM DMBA, and significant ($P<0.05$) inhibitions were seen at 5 and 10 μM of baicalein in CYP1A1 and CYP1B1 expressions, respectively. CYP1A1 expression appeared to be more responsive than that of CYP1B1.

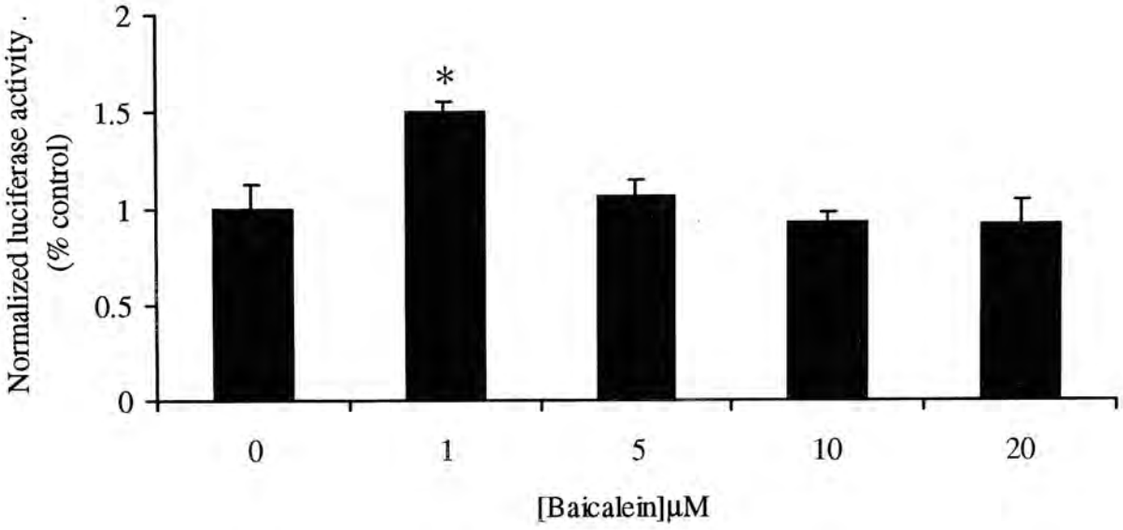


Figure 3.4 Effect of baicalein on XRE-driven luciferase activities.

MCF-7 cells were transient transfected with XRE-Luc reporter plasmids, and treated with baicalein at concentrations of 0, 1, 5, 10, and 20 μM for 24 h. Values are means ±SEM, n=3. Means marked (*) significantly ($P<0.05$) differ from control (0 μM).

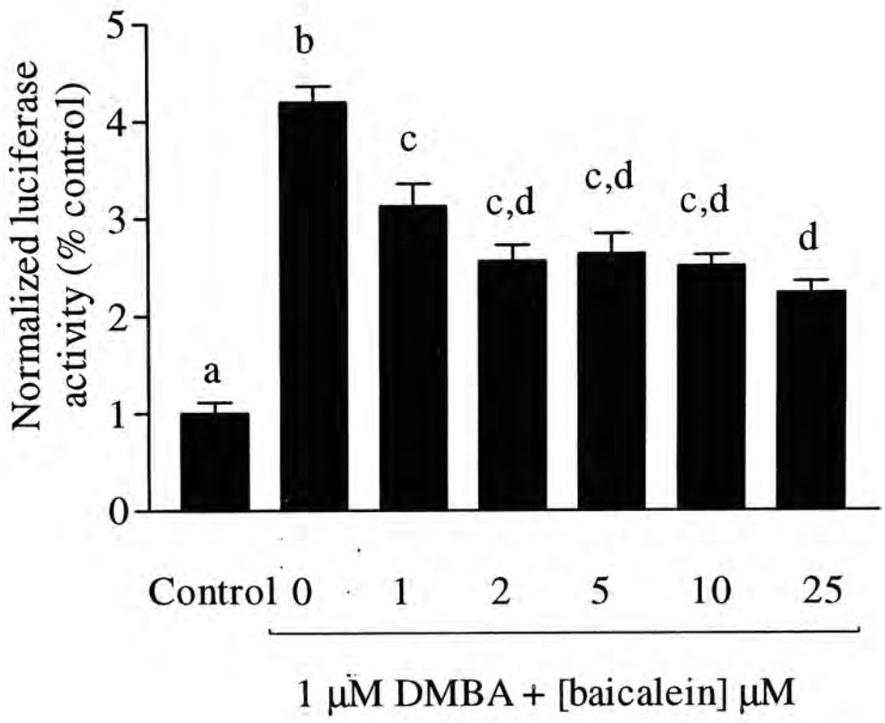


Figure 3.5 Effect of baicalein on DMBA-induced XRE-driven luciferase activities.

MCF-7 cells were transient transfected with XRE-Luc reporter plasmids, and treated with 1 μM DMBA and baicalein at concentrations of 0, 1, 5, 10, and 20 μM for 24 h. Values are means ±SEM, n=3. Means with different letters differ ($P<0.05$).

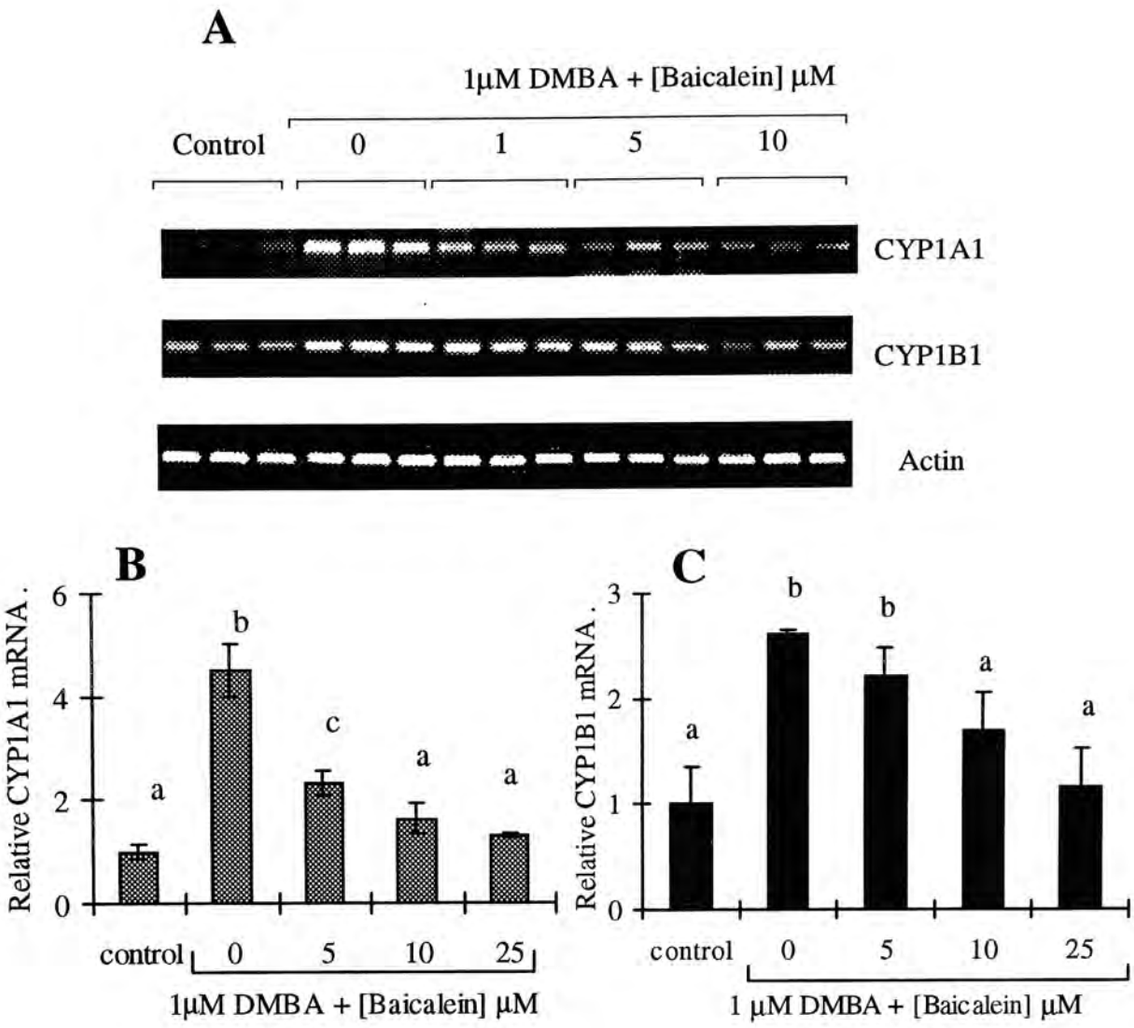


Figure 3.6 The influences of baicalein on mRNA abundance of CYP1A1 and CYP1B1

MCF-7 cells were seeded in 6-well culture plates and treated with 1 μM DMBA and DMSO (control) or various concentrations of baicalein for 24 h. Total RNA was then isolated and semi-quantitative RT-PCR was used to determine the relative mRNA expressions. Figure 5.7A is the gel image of the ethidium bromide-stained PCR fragments. Figure 3.6B and C were optical density results of the CYP1A1 and CYP1B1 fragments from RT-PCR. Values are means ±SEM, n=3. Means with different letters differ ($P<0.05$).

The cytotoxic effect of DMBA was reduced by baicalein

The metabolites of DMBA were genotoxic agents that could induce death to the MCF-7 cells in a span of 72 h. At all the concentrations tested, the percentage of cell survival was significantly lower than the control as shown in Figure 3.7 Baicalein administered at 2.5 μM could reverse some of the damages done to the MCF-7 cells by DMBA. By itself, 24-h baicalein treatment was not cytotoxic to the cultures until the concentration reached 50 μM (Figure 3.8).

Inhibition of DMBA-DNA adduct formation after baicalein treatment

Because of the inhibition at the enzyme as well as at the expression levels of CYP1A1 and CYP1B1, the ultimate carcinogenic form of DMBA might be affected. Figure 3.9 illustrated that a dose-response decrease was observed in the DMBA-DNA adduct formation in MCF-7 cells treated with baicalein. 1 μM baicalein could reduce the adduct formation by 20%, and 70% by 5 μM.

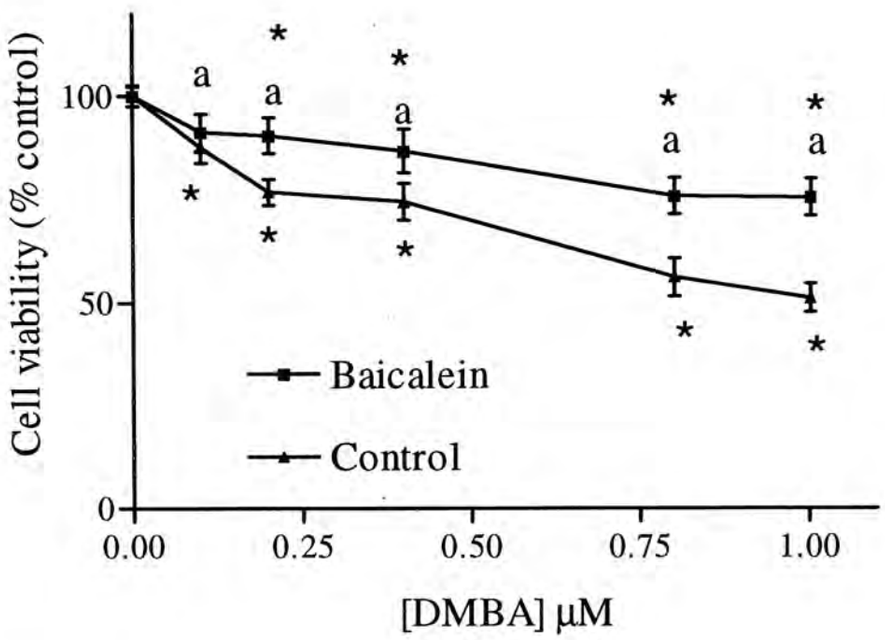


Figure 3.7 Cytotoxicity of DMBA and baicalein co-treatment.

MCF-7 cells were cultured in 96-well plates. DMBA ranged from 0 to 1 μM was added to the cultures, and 0 μM (Control) or 2.5 μM baicalein was co-administered with DMBA. After 72 h of treatment, the cell viability percentage was assayed by MTT. Values are means ±SE, n=3. Means marked (*) are significantly (*P<0.05) lower than the control; means of co-treatment marked (a) are higher than the means of their DMBA counterparts.

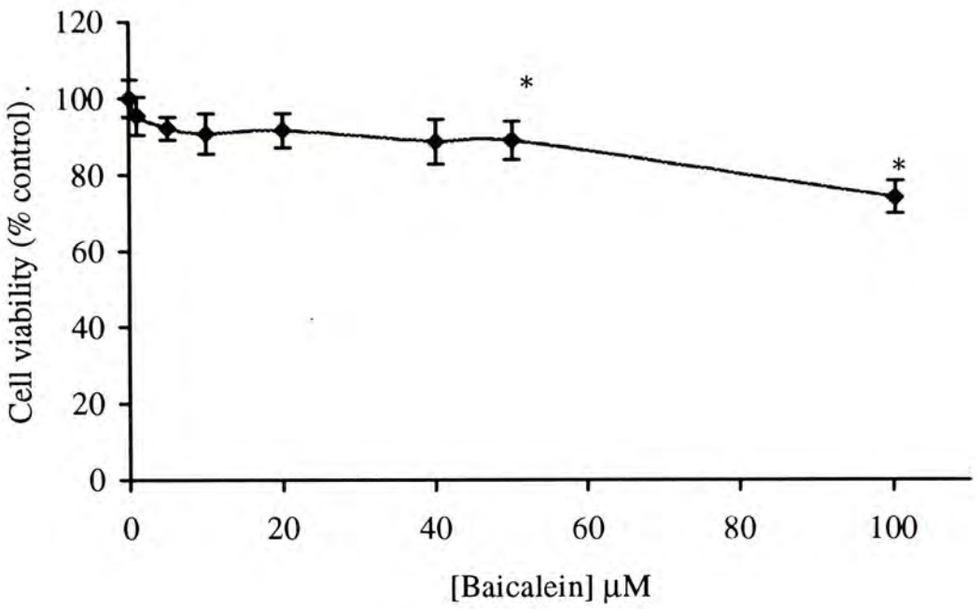


Figure 3.8 The cytotoxic effect of baicalein alone on MCF-7 cells.

MCF-7 cells were cultured in 96-wells plate and baicalein ranged from 0 to 100 μM was added to the cultures. After 24 h of treatment, the cell viability percentage was assayed. Values are means ±SE, n=3. Means marked (*) are significantly (*P<0.05) lower than the control.

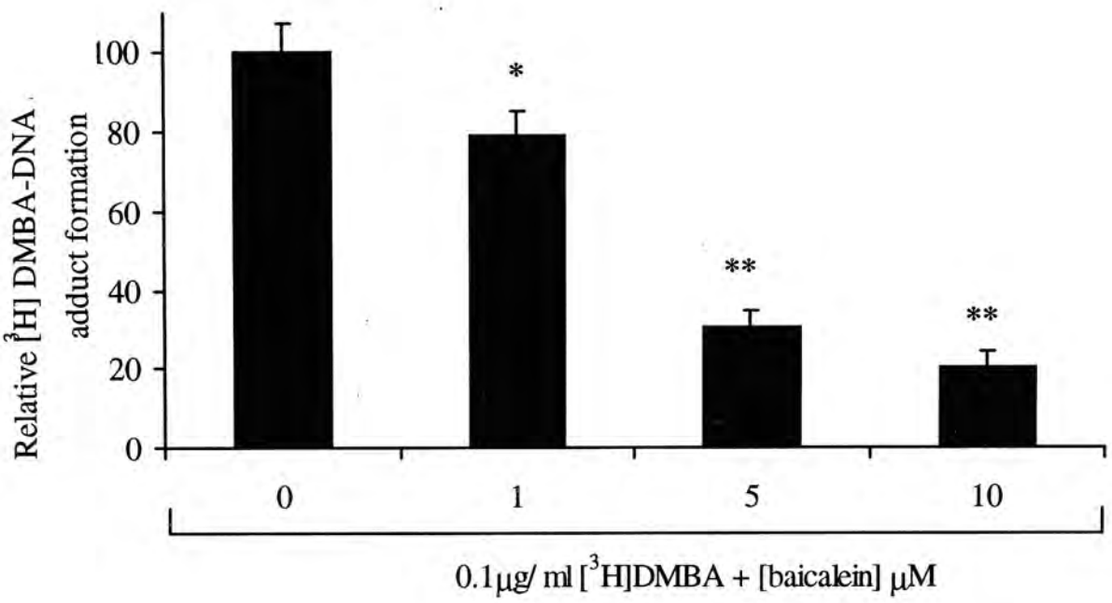


Figure 3.9 DMBA-DNA adduct formation in MCF-7 cells after baicalein treatment.

MCF-7 cells were cultured in 6-well plates and treated with tritiated DMBA in 0.1 µg/ml and one of four baicalein concentrations, 0, 1, 5, 10 µM, was co-administered. After 16 h of treatment, genomic DNA was isolated and the DMBA-DNA lesions were determined by scintillation counting. Values are means ±SE, n=3. Means marked (*, **) significantly (*P<0.05, **P<0.001) differ from control (0 µM).

Discussion

In this study, we demonstrated that baicalein reduced DMBA-DNA adduct formation in the breast cancer cell line MCF-7. The mechanism was related to the inhibition of CYP1A1 and CYP1B1 enzyme activities and gene expressions. EROD, the indicative of CYP1A1 and CYP1B1, was competitively inhibited by baicalein as shown in the enzyme kinetic study. In addition, the mRNA expressions of CYP1A1 and 1B1 induced by DMBA were also suppressed by baicalein. Previous studies have shown that AhR ligands, such as PAHs, can induce both CYPs (Lusska et al., 1992; Alexander et al., 1997). Our XRE-transfection results indicated that the suppression of mRNA expressions could have occurred in the interruption of Ah receptor transactivation. The overall DMBA-induced EROD activities in cell cultures, which were significantly reduced by baicalein at submicromolar concentrations, could be due to enzyme inhibition and decreased expression.

This study demonstrated baicalein inhibited xenobiotic-induced EROD activities in MCF-7 cells. By contrast, an animal study has shown that baicalein induces EROD activities in mouse lung without affecting EROD activities in the liver (Ueng et al., 2000). The difference between this and our study is that PAH is not administered in this rat study. Moreover, the tissue distribution and species variation may also account for the differences. The same research group uses the same model and demonstrates that baicalein prevents DNA damage caused by benzo[a]pyrene and aflatoxin B-1 (Ueng et al., 2001). Although the roles of CYP1A1 and CYP1B1 have not been addressed, the results of this animal study illustrates that baicalein can modulate the PAH metabolism. Since many XRE sites have been identified in the 5'-flanking regions in the human as well as rat CYP1A1 and

CYP1B1 promoters, the interruption of AhR transactivation can affect the gene expressions (Tang et al., 1996; Corchero et al., 2001). Our study verified that baicalein decreased the expressions of CYP1A1 and CYP1B1, through the interruption of AhR transactivation.

Many phytochemicals affects CYP1A1 either at the mRNA or enzyme level, or both. The flavonoid galangin (Ciolino et al., 1999), curcumin (Ciolino et al., 1998) and quercetin (Ciolino et al., 1999) are ligands of AhR. They can stimulate CYP1A1 mRNA expression by themselves, but they also inhibit the same expression induced by DMBA. Similar effects at the enzyme level are also observed. Kaempferol (Ciolino et al., 1999) and resveratrol (Ciolino et al., 1999), on the other hand, reduce PAH-induced CYP1A1 mRNA, but they are not AhR ligands. In current study, we demonstrated that baicalein was an inhibitor of EROD and a modulator of CYP1A1 as well as CYP1B1 expressions. Baicalein appeared to be a weak agonist to AhR because of the weak induction of EROD activities and XRE driven luciferase activities when administered alone. As suggested by Ciolino et al. (1999) individual flavonoid might have differential effects on the CYP protein at the enzyme and expression levels.

In summary, we demonstrated that DMBA-DNA adduct formation in the breast cancer cells MCF-7 was reduced by baicalein. The mechanisms by which these DNA lesions were decreased might be mediated through the modulation of CYP1A1 and 1B1 gene expressions and enzyme inhibitions.

CHAPTER 4 INHIBITION OF DMBA-DNA ADDUCT FORMATION BY (–)-EPIGALLOCATECHIN GALLATE AND THEAFLAVINS

INTRODUCTION

Tea, an extract of the leaves of the plant Camellia sinensis has long been considered as a healthy beverage worldwide. Epidemiological and experimental studies have demonstrated that tea diminishes cancer risk in breast, lung, esophagus, colon and skin (See reviews on Dreosti et al., 1997; Katiyar et al., 1997; Yang et al., 2001, 2002); Although green tea has shown chemopreventive properties, black tea also inhibits carcinogenesis with lower effectiveness than green tea in some studies. They inhibit tumorigenesis at the initiation, promotion, and progression stages in different animal models. The chemopreventive effects of tea have been attributed to the biological activities of tea polyphenolics.

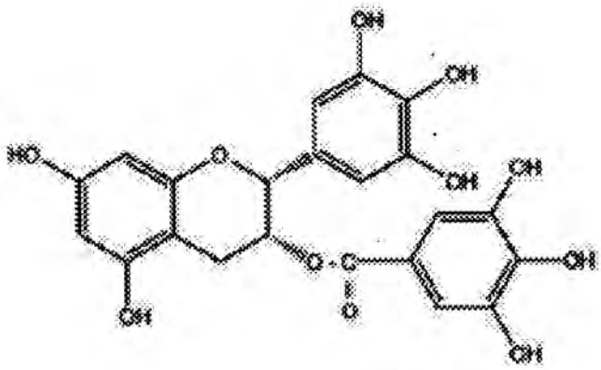
Being a major polyphenol in green tea, (–)-epigallocatechin gallate (EGCG) possesses higher anti-proliferative, anti-neoplastic, and anti-mutagenic activities in numerous models than other green tea polyphenols including (-)-epicatechin (EC), (-)-epicatechin gallate (EGC) (Liang et al., 1999; Ahmad et al., 1998; Mukhtar and Ahmad, 1999). EGCG inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) -induced lung tumors (Xu et al., 1992), 7,12-Dimethylbenz[a]anthracene (DMBA) induced-breast cancer (Kavanagh et al., 2001), and azoxymethane (AOM)-induced colon cancer (Ohishi et al., 2002). These indicate that EGCG exhibits protective effects against chemical induced carcinogenesis. Also, a reduction of

carcinogen-DNA adduct formation has been observed with human DNA in the presence of EGCG (Katiyar et al., 1992; Wang et al., 1989). Studies have demonstrated that EGCG can intervene the biotransformation of carcinogens by modulating some enzyme activities. Induction of phase II enzymes such as glutathione-S-transferase and NAD(P)H:quinone reductase (QR) is enhanced by EGCG and the removal of activated carcinogens is sped up (Stoner et al., 1998; Chou et al., 2000; Katiyar et al., 1993). Researches have shown that EGCG also modulates P450 enzymes activities and reduces ultimate carcinogen formation (Xu et al., 1999). CYP1 enzymes transform carcinogen that attack DNA and cause mutation. EGCG suppresses the AhR-mediated transcription of CYP1A gene expression and CYP1A enzyme activities, and toxicity of chemical carcinogens can be reduced (Williams et al., 2000). It is interesting to note that other green tea polyphenols act synergistically on the anticancer activities of EGCG but the mechanisms are still unclear (Suganuma et al., 1999; Williams et al., 2000).

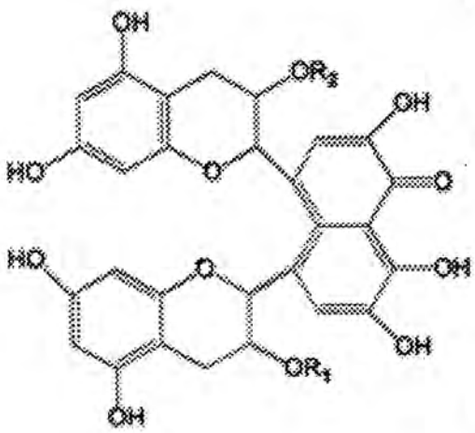
The typical polyphenols in black tea are theaflavins: TF1, TF2A, TF2B and TF3. (Figure 4.1) Although theaflavins also exhibit anti-carcinogenesis effects in different models, little is known about their chemoprevention mechanism compared with that of green tea. Theaflavins inhibit neoplastic transformation in human lung cells (Sazuka et al., 1997), mammary cells and rat tracheal epithelial cells (Steele et al., 2000). Similar to green tea polyphenols, they are strong antioxidants that are capable of inhibiting DNA-carcinogen lesions (Leung et al., 2001; Schut et al., 2000). Some researches have suggested that theaflavins may play a role in modulating carcinogens activation (Apostolides et al., 1997). Benzo[a]pyrene metabolism is inhibited in the presence of black tea in rat liver microsomes (Hammons et al., 1999). Steele et al. (2000) show that the inhibition of free radical formation rather than the phase II

isozyme induction contributes to the protective mechanism of theaflavins against the development of tumors *in vitro*. On the other hand, Feng et al. (2002) showed that theaflavins inhibit omeprazole (OME)-induced CYP1A1 expression. Instead of inducing CYP1A1 by the AhR cascade, OME upregulates CYP1A1 from kinase-mediated signal transduction pathway. Thus, the protective role of theaflavins may partly arise from their ability to inhibit the formation of activated carcinogens.

In the present study, we have investigated the effects of theaflavins on DMBA-induced DNA-adduct formation in the mammary cancer cell line MCF-7. Also, the possible role of theaflavins on AhR mediated CYP1 enzyme expression and activities was studied.



(–)-epigallocatechin gallate



Theaflavins		Substitutions	
		R1	R2
Theaflavin -1	TF1	H	H
Theaflavin -3-gallate-A	TF2A	H	galloyl
Theaflavin –3'–gallate-B	TF2B	galloyl	H
Theaflavin –3,3' -digallate	TF3	galloyl	galloyl

Galloyl group

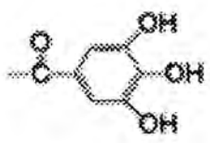


Figure 4.1 Chemical structures of theaflavins and EGCG.

RESULTS

Persistence of DMBA-induced DNA adducts

The anticarcinogenic effects of theaflavins and EGCG have been demonstrated in rats and various cell models. Nevertheless, their effects on DMBA-DNA adduct formation in mammalian cell culture remain unknown. To explore their effects on DMBA toxicity, 1 μg/ml [^{3}H] DMBA was added to MCF-7 cells seeded in 6-well plate for 16h in the presence of various concentration of theaflavins and EGCG. Genomic DNA was then isolated and subjected to scintillation counting. Among the 4 theaflavins, TF2A was the most effective agents that suppressed approximately 50% of DMBA-DNA adduct formation at a concentration of 50 μM (Figure 4.2). Nevertheless, TF1 at 50 μM suppressed only 30% of DMBA-DNA adduct formation. EGCG showed a 20 % reduction in DMBA-DNA adduct formation at 10 μM, while EGC and EC did not have significant effects (Figure 4.3).

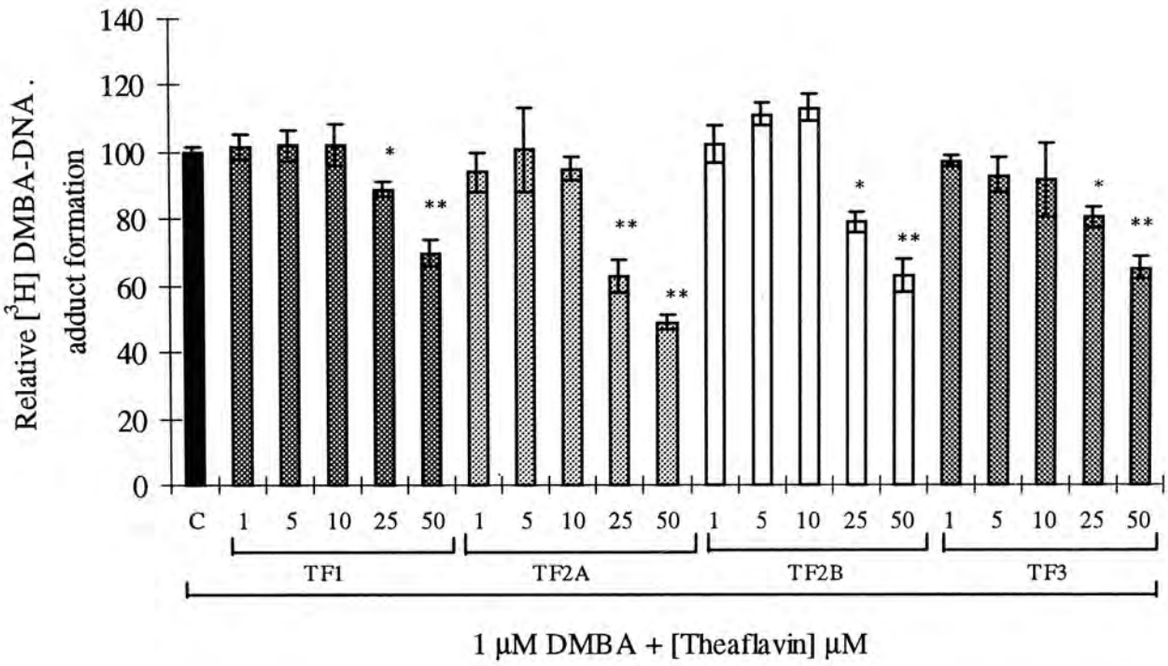


Figure 4.2 Pattern of DMBA-DNA adduct formation in MCF-7 cells treated with theaflavins.

MCF-7 cells were cultured in 6-well plates and treated with tritiated DMBA in 0.1 μg/ml and one of six theaflavins concentrations, 0, 1, 5, 10, 25, 50 μM, was co-administered. After 16 h of treatment, genomic DNA was isolated and the DMBA-DNA lesions were determined by scintillation counting. Values are means ±SE, n=3. Means marked (*, **) significantly (*P<0.05, **P<0.001) differ from control (0 μM).

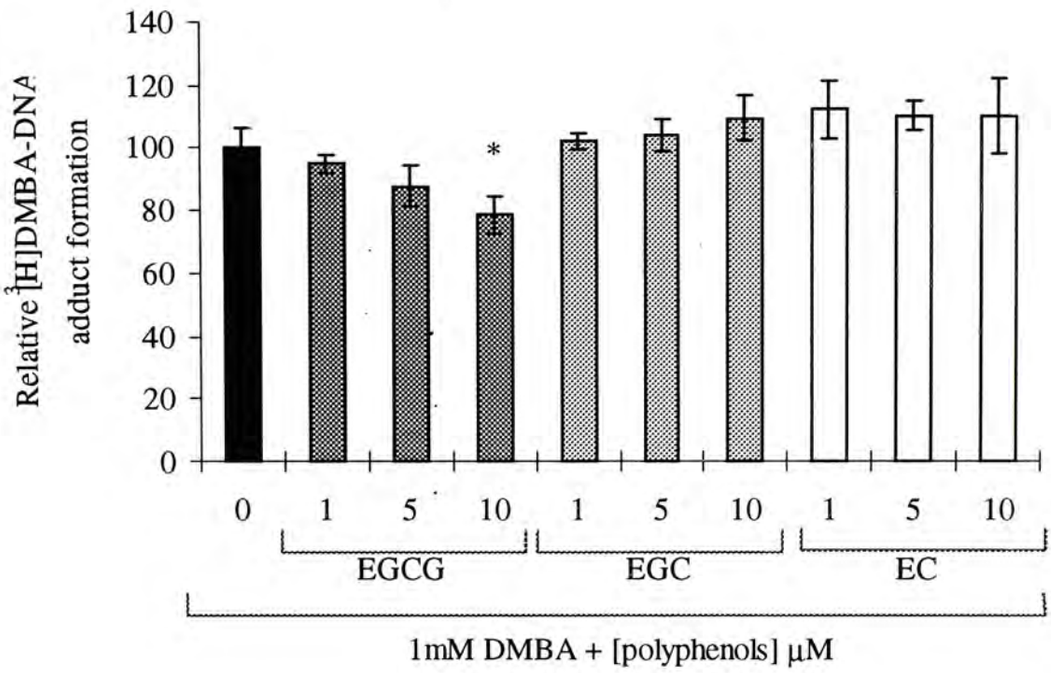


Figure 4.3 EGCG suppressed DMBA-DNA adduct formation.

The effect of green tea polyphenols on DMBA-DNA adduct formation was assayed as described in figure 3.6. Values are means ±SE, n=3. Means marked (*) significantly (*P<0.05) differ from control (0 μM).

Inhibition of theaflavins and EGCG on human recombinant CYP1A1 and CYP1B1 enzyme activities

As EGC and EC did not modulate DMBA-DNA adduct formation, we focused on EGCG and theaflavins only in the following experiments. To investigate their effects on the catalytic activities of CYP1A1 and CYP1B1, enzyme kinetic assays were carried out with recombinant enzymes and various concentrations of theaflavins and EGCG (Figure 4.4). It was found that inhibitory effects of the 4 theaflavins on CYP1A1 and CYP1B1 enzymes were stronger than that of EGCG. Among the 4 theaflavins, TF2B seemed to be the most effective in the inhibition of recombinant CYP1A1 and CYP1B1 activities.

EGCG suppressed DMBA-induced EROD activity while thealfavin had no significant effect on this

Previous studies have demonstrated that phytochemicals may exert their anticarcinogenic effects by inhibiting DMBA-induced CYP1 enzyme activities. Thus, we would like to investigate if theaflavins and EGCG could modulate the activities of CYP1 enzymes after DMBA adminstration. MCF-7 cells were plated in 96-well plate in the presence of 1 μM DMBA and various concentrations of theaflavins or EGCG were administerd. EROD activity, an indicator for CYP1A1/1B1 enzyme activities was then measured after 24 h. Results showed that EGCG but not the 4 theaflavins had significant effects on DMBA-induced EROD activities (Figure 4.5) with an IC_{50} value of ~40 μM (Figure 4.6). Moreover, EGCG alone caused slight increase on EROD activities in MCF-7 cells (Figure 4.7).

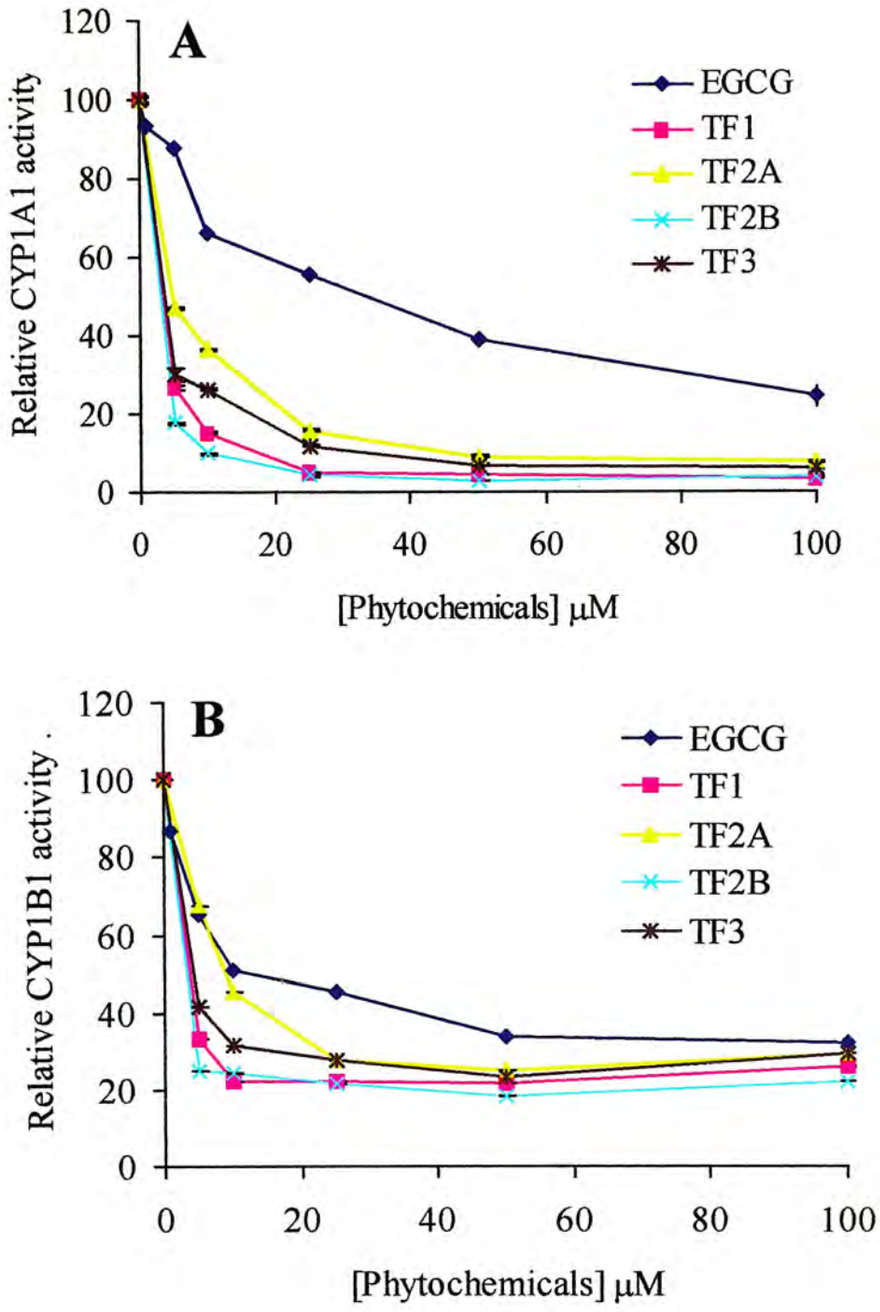


Figure 4.4 Effects of theaflavins and EGCG on recombinant CYP1A1 and CYP1B1 EROD activities

EROD assay (400nM ER) was performed with 2.5 pmole of CYP1A1 (A) and 2 pmole of CYP1B1 (B) enzymes and varying concentration of theaflavins and EGCG. The values are means ±SE, n=3. All values significantly differ (P >0.05) from the control (0 μM).

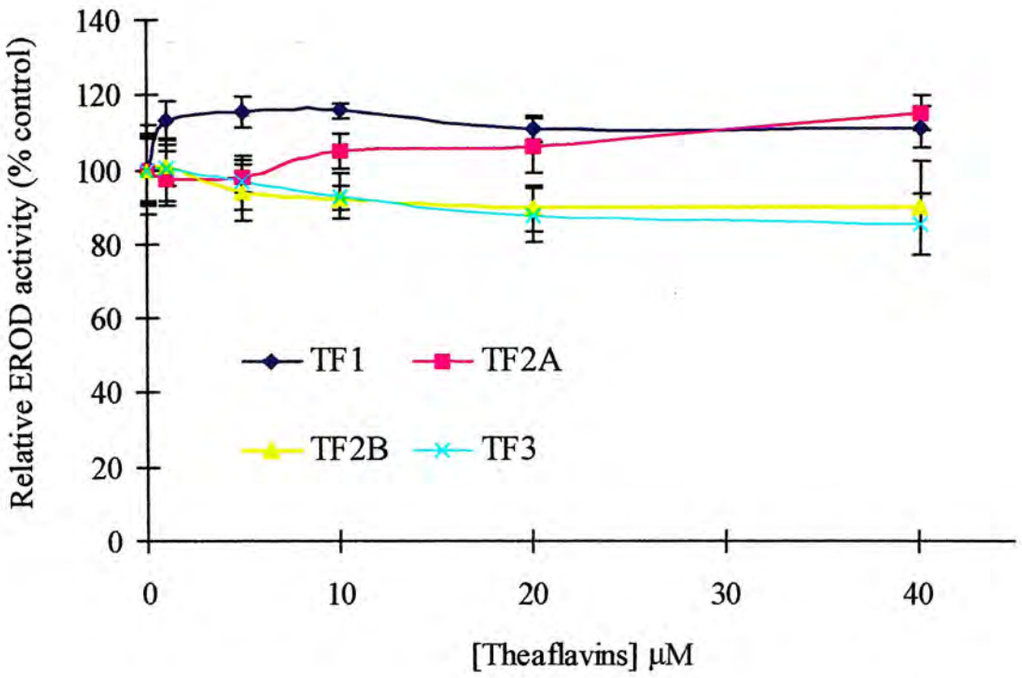


Figure 4.5 Effects of theaflavins on DMBA-induced EROD activities

MCF-7 cells were seeded in 96-well culture plates and treated with 1 μM DMBA and various concentrations of baicalein. After 24 h of treatment, cells were assayed for EROD activity as described. The values are means ±SE, n=6. The values at 40 μM for the four theaflavins are significantly differ (P >0.05) from the control (0 μM).

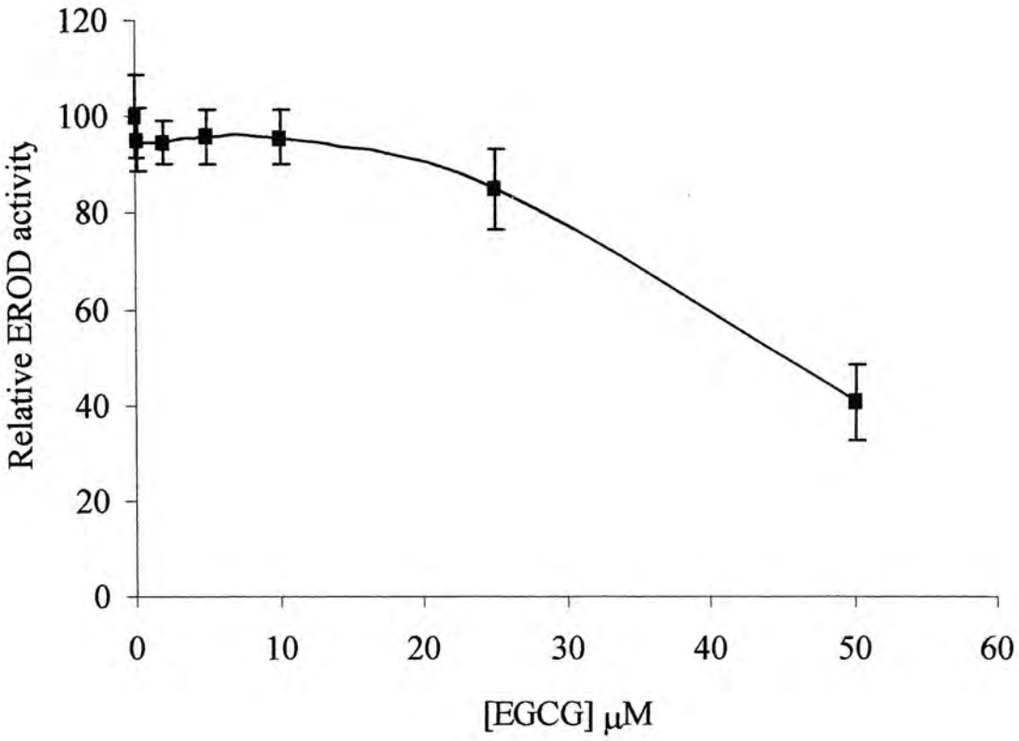


Figure 4.6 Effect of theaflavins on DMBA-induced EROD activities.

The effect of EGCG on DMBA-induced activity was assayed as described in figure 4.1. The values are means ±SE, n=6. All values are significantly different (P >0.05) form the control (0 μM).

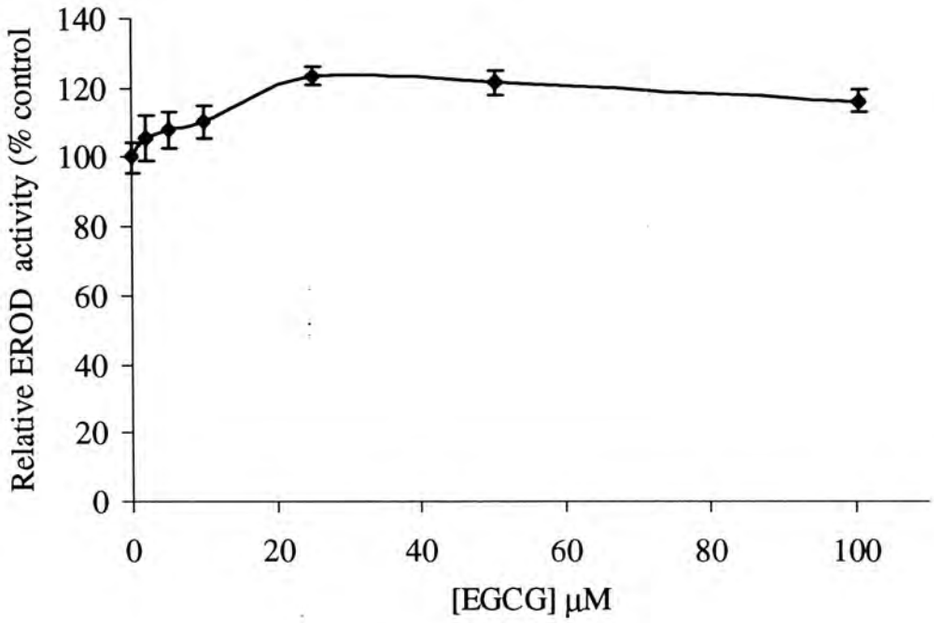


Figure 4.7 EROD activities in MCF-7 cells treated with EGCG alone.

MCF-7 cells were treated with 1 to 100 μM of EGCG for 24 h. EROD activities were determined as described. The values are means ±SE, n=6. All data are significantly difference from the control ($P< 0.05$).

Kinetic analysis of EGCG on CYP1A1 and CYP1B1 activities

As EGCG was shown to inhibit DMBA-induced CYP1 enzymes in MCF-7 cells, we would like to investigate it inhibitory pattern on CYP1A1 and CYP1B1 enzymes. Thus, kinetic analyses with recombinant CYP1A1 and CYP1B1 enzymes in the presence of various concentrations of ER and EGCG were carried out. Results showed that EGCG competitively inhibited CYP1A1 enzyme with a Ki value of ~20 μM (Figure 4.8A). A mixed type inhibition was observed in CYP1B1 activities and the Ki value was 3 μM (Figure 4.8B). It showed that EGCG preferably inhibited CYP1B1 enzyme.

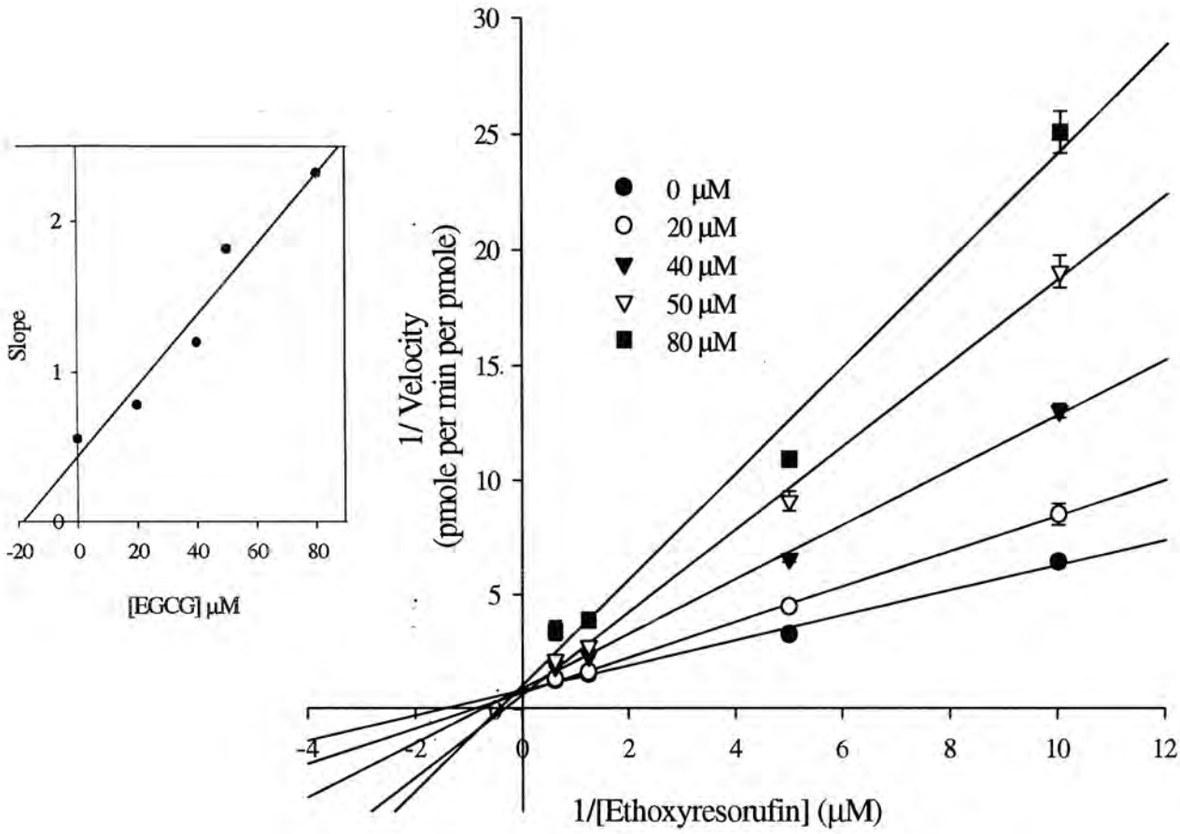


Figure 4.8A Lineweaver-Burk plot of EGCG on CYP1A1 enzymes inhibition.

EROD assay was performed with human recombinant CYP1A1 at indicated concentration of EGCG and 100-1600 nM ER as described under *Material and Methods*. Lineweaver-Burk plot were generated by linear regression of the reciprocal data. Inset, replot of the slopes from the Lineaweaver-Burk plot, with derivation of K which is equal to 18.34 μM_i. Values shown here are mean ±SE, n=3.

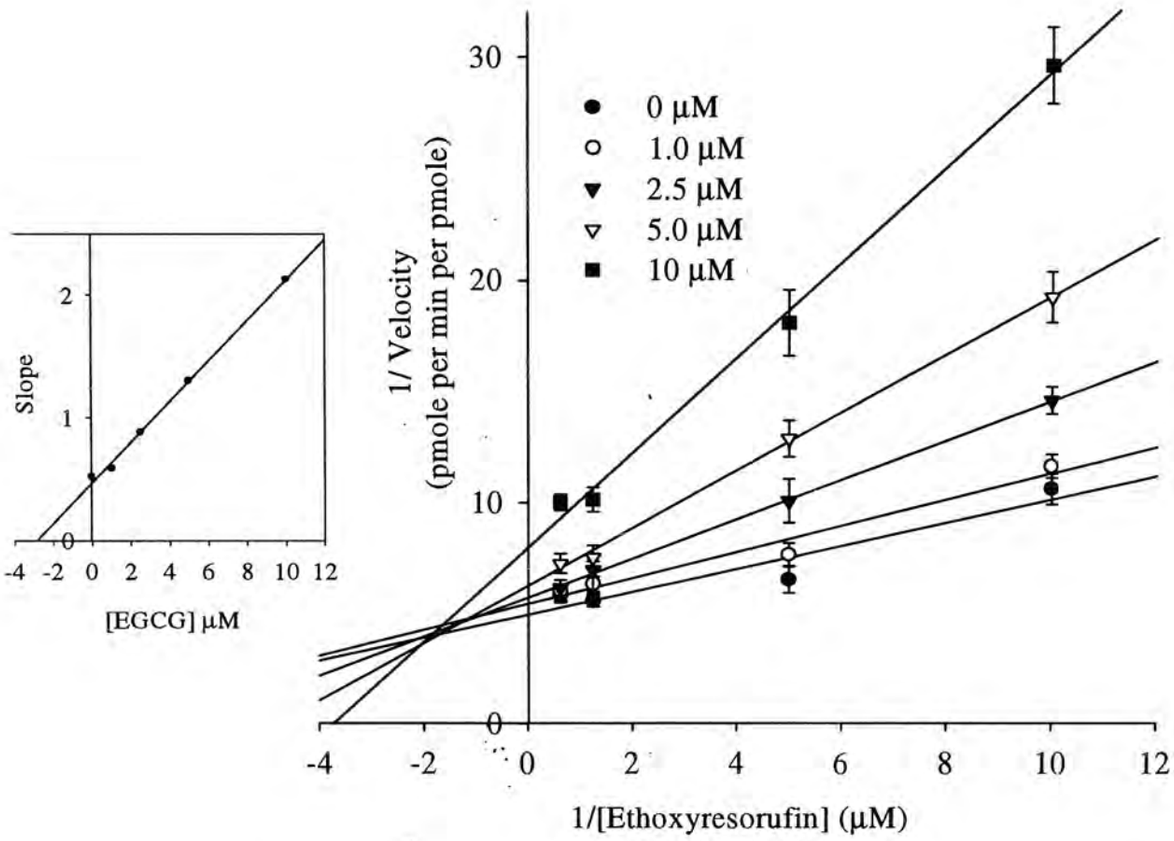


Figure 4.8B Lineweaver-Burk plot of EGCG on CYP1A1 enzymes inhibition.

EROD assay was performed with human recombinant CYP1B1 at indicated concentration of EGCG and 100-1600 nM ER as described in figure 4.5A. Ki value is equal to 2.85 μM.

Modulation of DMBA-induced XRE-driven luciferase activities by theaflavins and EGCG

In addition to the evaluation of the effects of theaflavins and EGCG on DMBA-induced transcription of *CYP1*, XRE-driven luciferase reporter assay was also performed. This reporter assay signaled the transcription of *CYP1* and the adminstration of DMBA induced the luciferase activity by 3-fold as shown in figure 4.6. Cotreatment with theaflavins had no effects on the induced luciferase activities (Figure 4.9A-C) except that TF3 caused an increase of activity (Figure 4.9D). By contrast, co-administration of EGCG resulted in a significant reduction of DMBA-induced luciferase activity (Figure 4.10). EGCG alone caused a slight but significant suppression at >2.5 μM (Figure 4.11).

The influence of theaflavins and EGCG on CYP1A1 and CYP1B1 abundance

To further confirm the results from the transient transfection assay, RT-PCR were carried out to study CYP1A1 and CYP1B1 mRNA abundances. DMBA caused approximately 4-fold and 2-fold induction on CYP1A1 and CYP1B1 mRNA, respectively. The 4 theaflavins did not have significant effects on DMBA-induced CYP1A1 and CYP1B1 mRNA expressions (Figure 4.12-4.16). Nevertheless, all of them caused a slight induction on DMBA-induced CYP1A1 mRNA expression at 20 μM. In contrast, EGCG caused a 25% reduction on the mRNA expression (Figure 4.14).

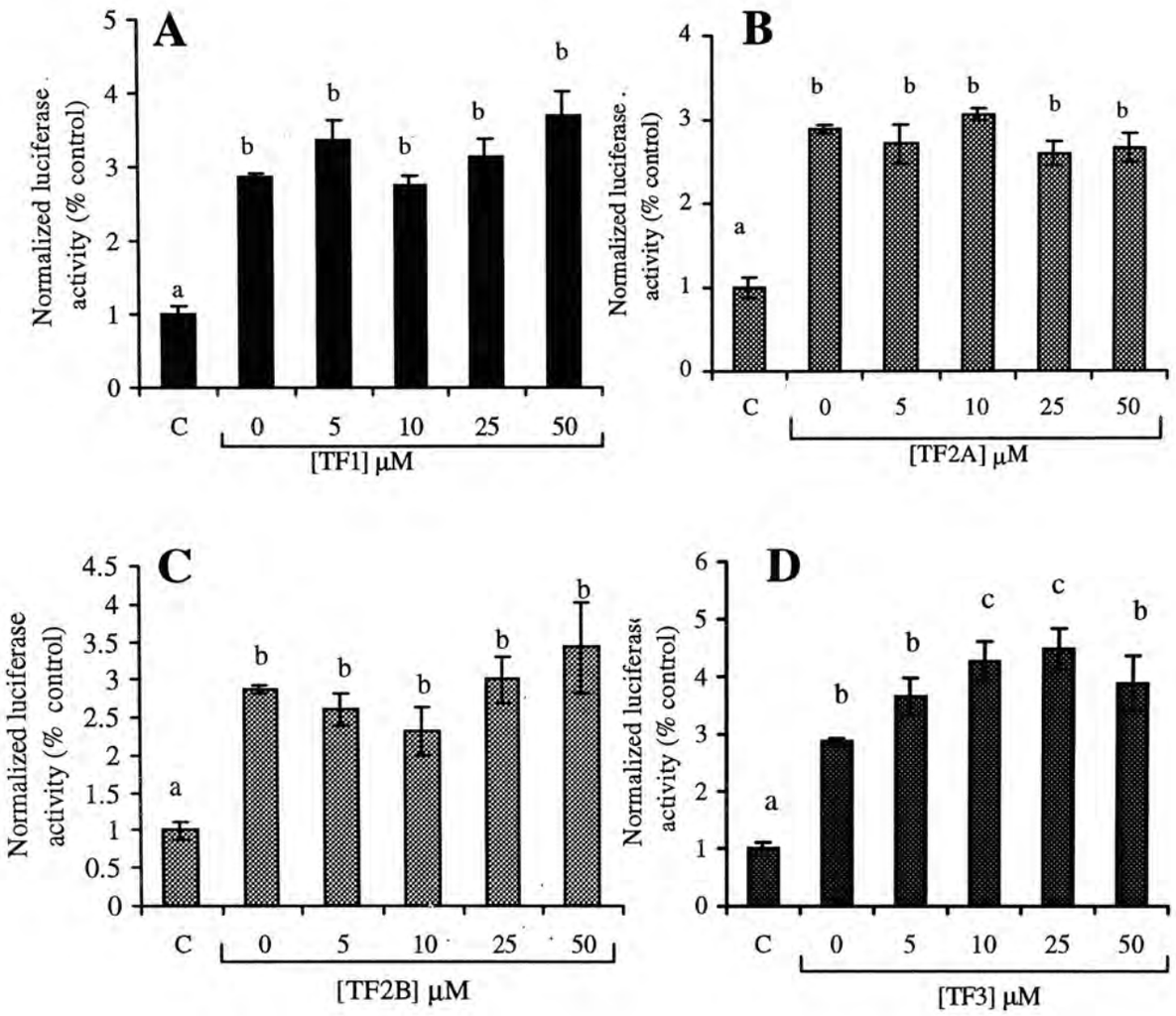


Figure 4.9 Effects of theaflavins on DMBA-induced XRE-mediated *CYP1* transcription.

MCF-7 cells were transient transfected with XRE-Luc reporter plasmid, and treated with 1 μM DMBA and theaflavins: TF1 (A), TF2A (B), TF2B (C) and TF3 (D) at concentrations of 0, 1, 5, 10, 25 and 50 μM for 24 h. Values are means ±SEM, n=3. Means with different letters differ ($P<0.05$) from control (0 μM).

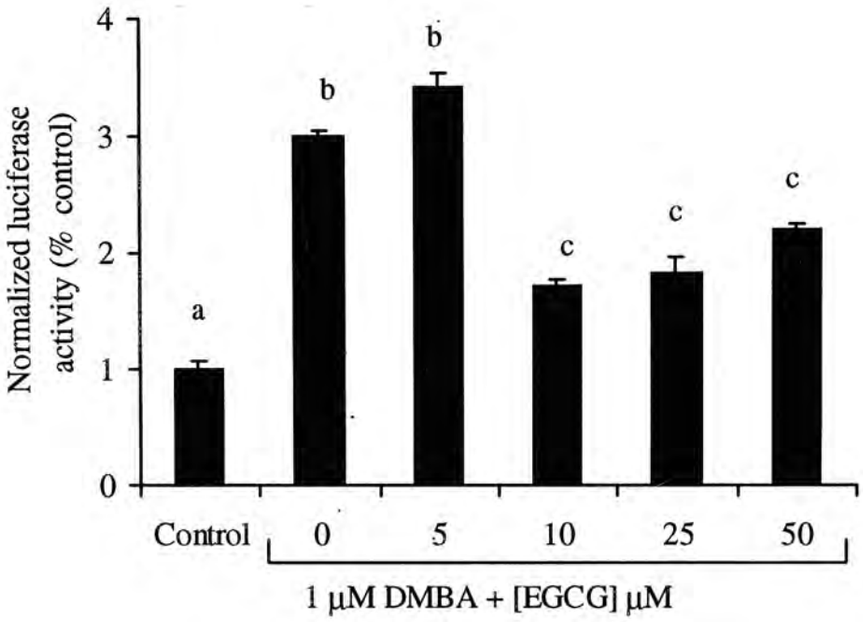


Figure 4.10 Effect of EGCG on DMBA-induced XRE-mediated luciferase activity.

The effect of EGCG on DMBA-induced XRE-driven luciferase activities was assayed as described. Values are means ±SEM, n=3. Means with different letters differ ($P<0.05$) from control (0 μM).

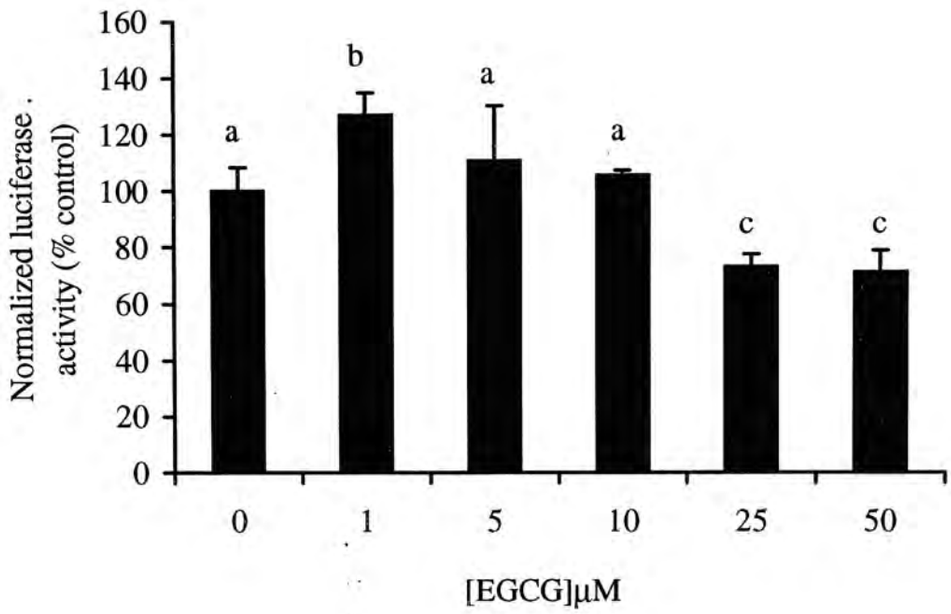


Figure 4.11 Effects of EGCG alone on XRE-driven luciferase activities.

MCF-7 cells were transiently transfected with a luciferase reporter gene containing XRE and a pRL. Transfected cells were treated with 1, 5, 10, 25 and 50 μM of EGCG for 24 h. Firefly luciferase activities were normalized with renilla luciferase. Values are means ±SEM, n=3. Means without same letters differ ($P<0.05$).

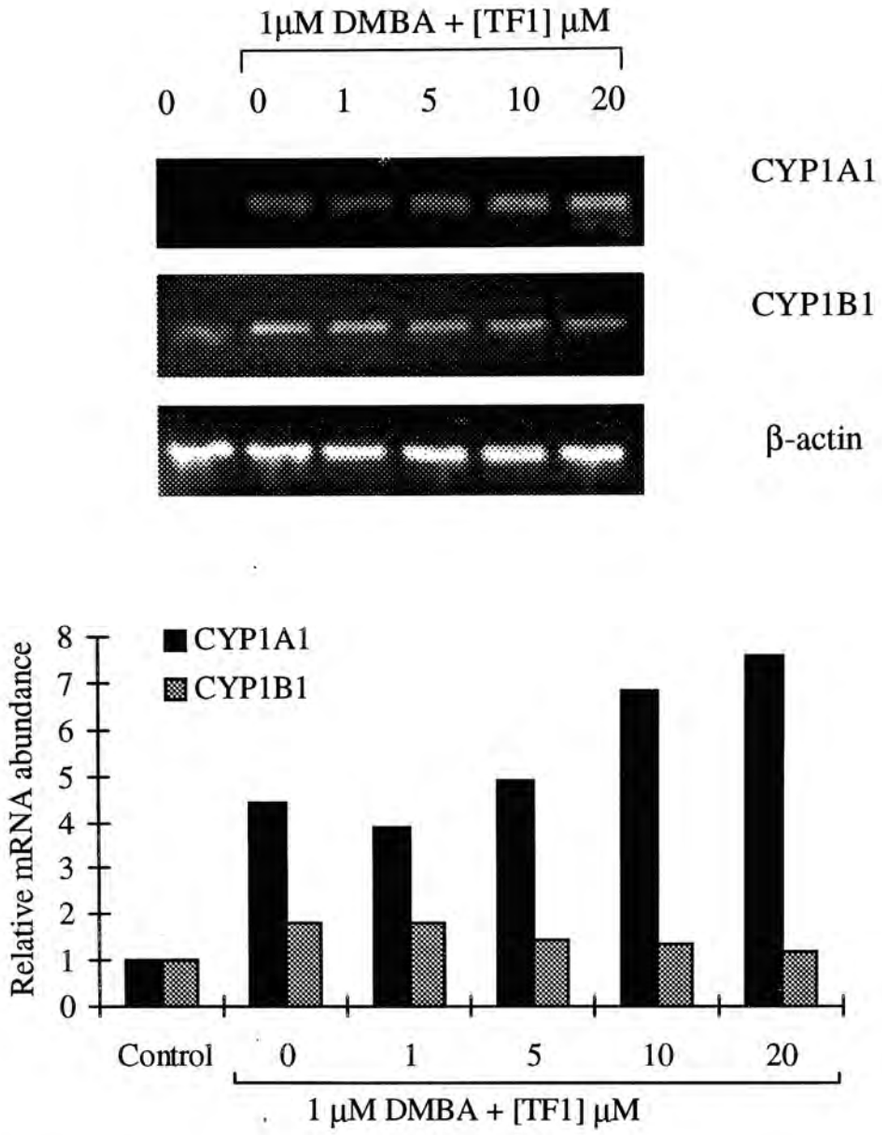


Figure 4.12 CYP1A1/CYP1B1 mRNA expressions in MCF-7 cells treated with TF1 and DMBA.

MCF-7 cells were seeded in 6-well culture plates and treated with 1 μM DMBA and various concentrations of baicalein for 24 h. After 24 h of treatment, total RNA was isolated and semi-quantitative RT-PCR was used to determine the relative mRNA expressions. The upper panel is the gel image of the ethidium bromide-stained PCR fragments. The lower panel is the optical density results of the CYP1A1 and CYP1B1 fragments.

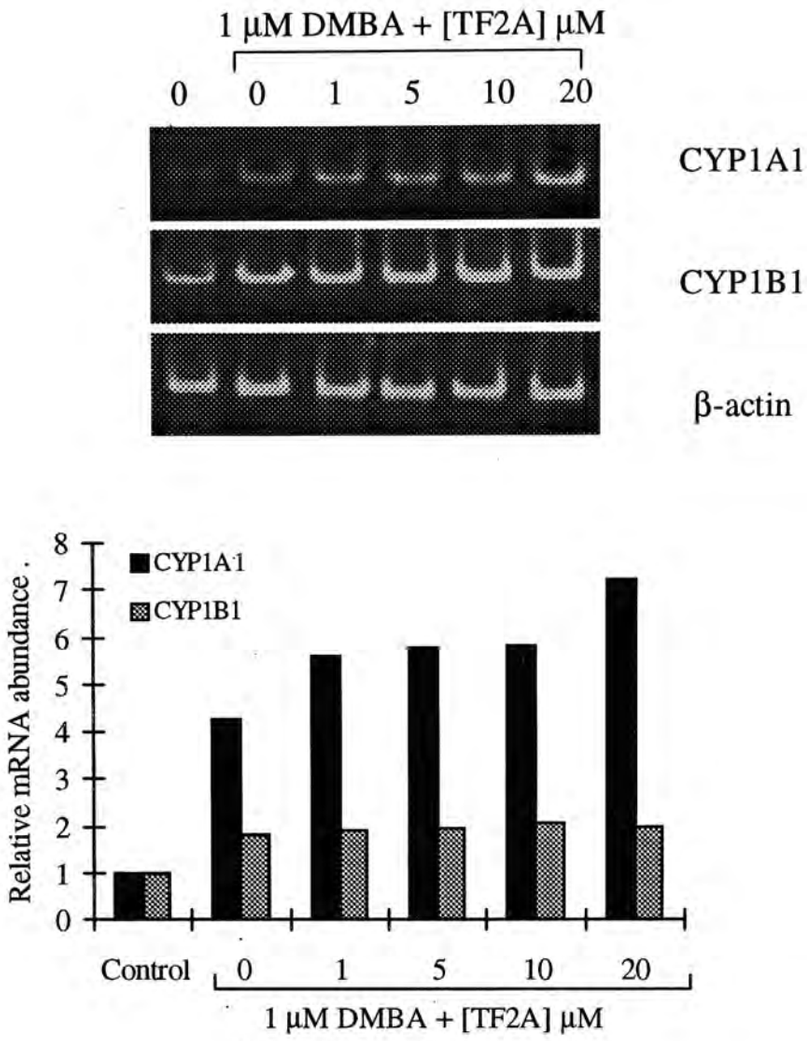


Figure 4.13 CYP1A1/CYP1B1 mRNA expressions in MCF-7 cells treated with TF2A and DMBA.

The effects of TF2A on CYP1A1 and CYP1B1 mRNA expression were assayed as described in figure 4.12. The upper panel is the gel image of the ethidium bromide-stained PCR fragments. The lower panel is the optical density results of the CYP1A1 and CYP1B1 fragments.

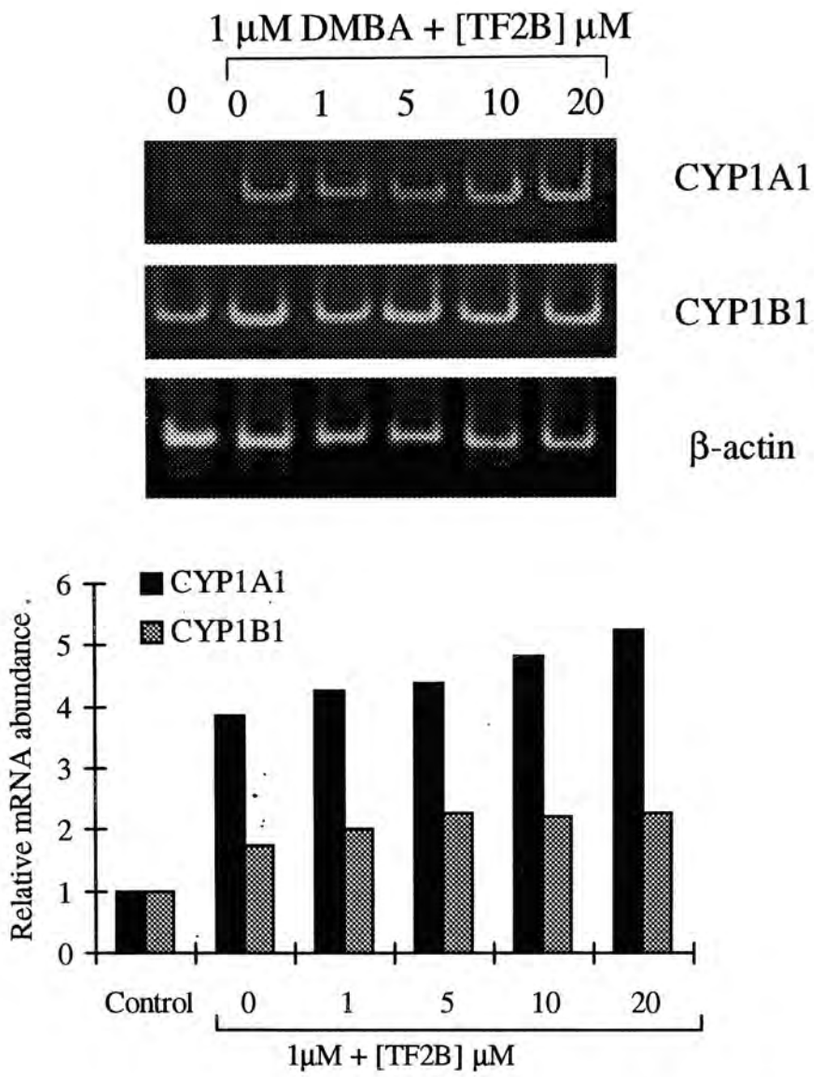


Figure 4.14 CYP1A1/CYP1B1 mRNA expressions in MCF-7 cells treated with TF2B and DMBA.

The effect of TF2B on CYP1A1 and CYP1B1 mRNA expression was assayed as described in figure 4.12. The upper panel is the gel image of the ethidium bromide-stained PCR fragments. The lower panel is the optical density results of the CYP1A1 and CYP1B1 fragments.

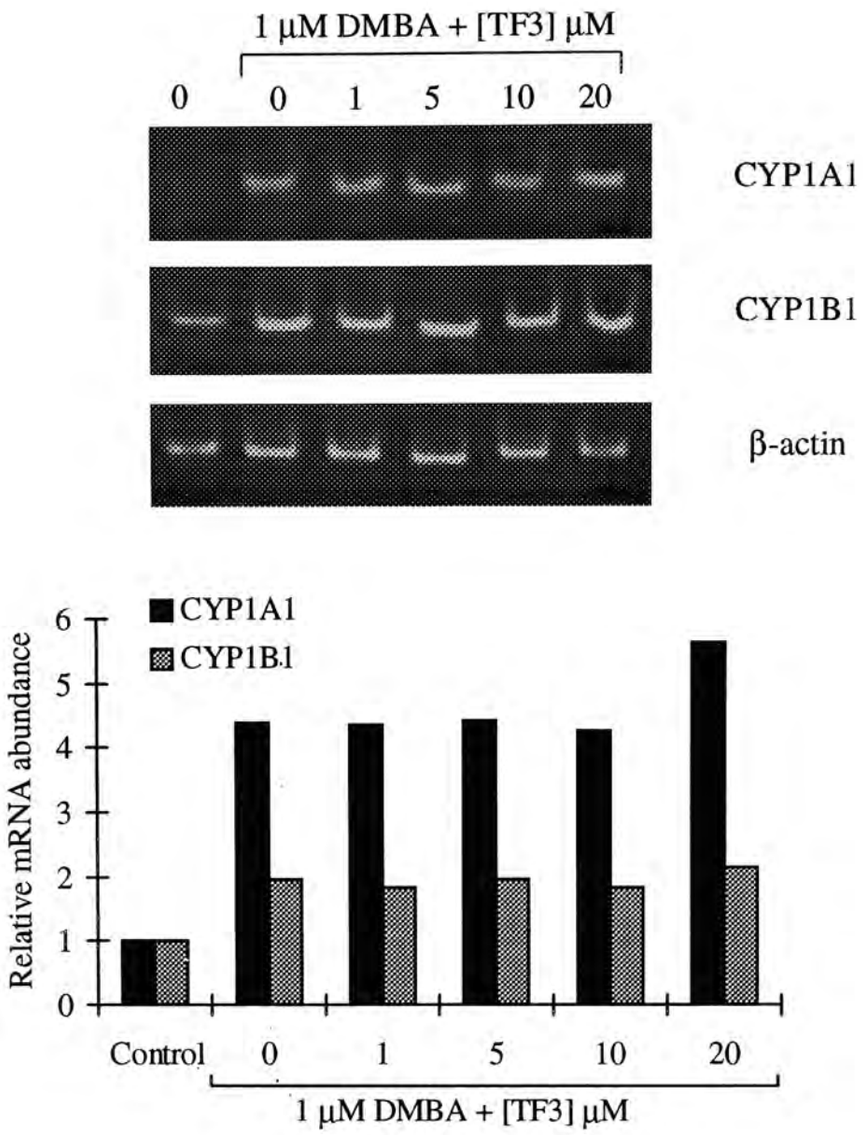


Figure 4.15 CYP1A1/CYP1B1 mRNA expressions in MCF-7 cells treated with TF3 and DMBA.

The effect of TF3 on CYP1A1 and CYP1B1 mRNA expression was assayed as described in figure 4.12. The upper panel is the gel image of the ethidium bromide-stained PCR fragments. The lower panel is the optical density results of the CYP1A1 and CYP1B1 fragments.

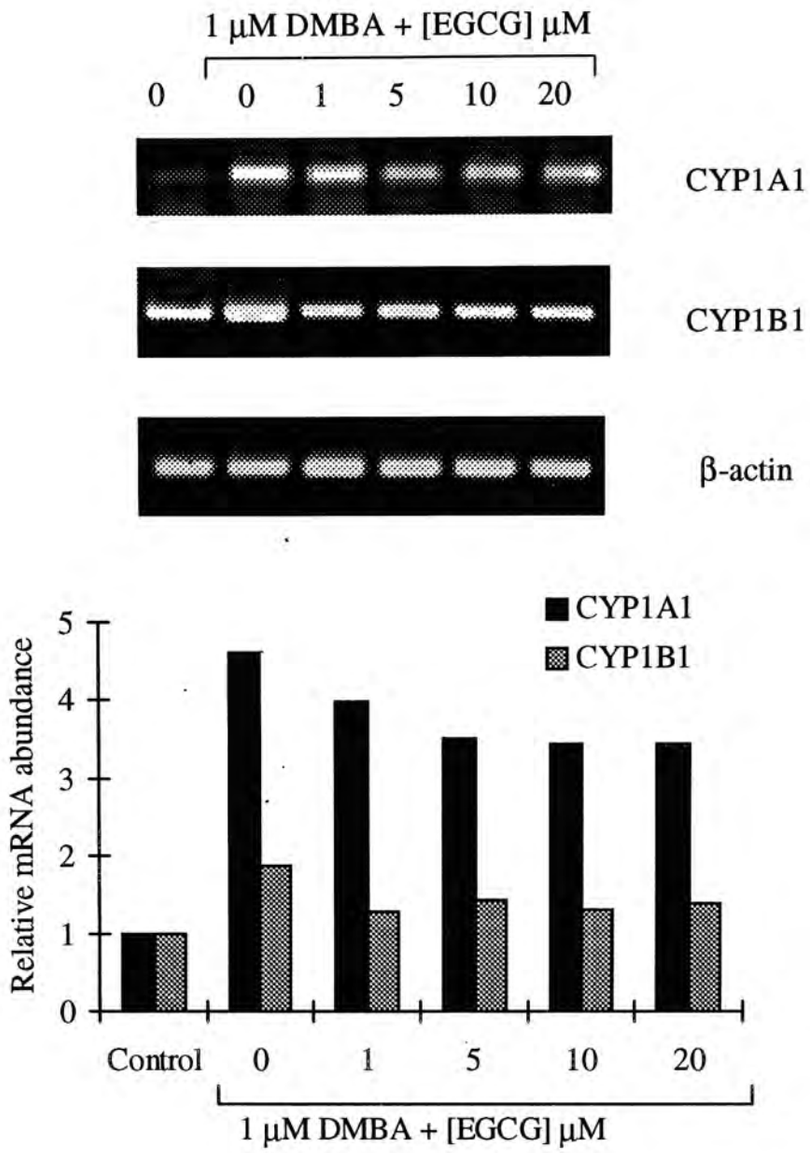


Figure 4.16 DMBA-induced CYP1A1 and CYP1B1 mRNA expression were inhibited by EGCG cotreatment.

The effect of EGCG on CYP1A1 and CYP1B1 mRNA expression was assayed as described in figure 4.12. The upper panel is the gel image of the ethidium bromide-stained PCR fragments. The lower panel is the optical density results of the CYP1A1 and CYP1B1 fragments.

Discussion

The present study showed that theaflavins and EGCG inhibited DMBA-DNA adduct formation in MCF-7 cells, indicating that they may protect against chemical carcinogenesis because activated carcinogen-DNA adduct formation is correlated to a high risk of cancer (Hemminki, 2000). EGCG was shown to be more effective in preventing DMBA-DNA adduct formation than theaflavins. The protective properties of theaflavins against DMBA toxicity are TF2A> TF2B>=TF3> TF1.

Many studies suggest that the chemoprotective effects of tea against PAH-induced mutagenesis are due to their abilities to modulate carcinogen metabolizing enzymes such as CYP1 enzymes. Inhibition of CYP1 enzymes may reduce the formation of activated carcinogens that leads to DNA damages. While numerous evidences show that green tea polyphenols inhibit carcinogens-induced CYP1 enzyme expression, no conclusive result is obtained for the effects of black tea on P450 enzymes (Dashwood et al., 1999). Previous studies have demonstrated that green tea or black tea alone induced phase I P450 and phase II conjugating enzymes. Weisburger et al. (1998) report that black tea does not alter the amount of CYP2E1 that is required for the activation metabolism of AOM. The present studies showed that both theaflavins and EGCG inhibited human recombinant CYP1A1 and CYP1B1 enzyme activities in vitro. Surprising, theaflavins had no significant effects on DMBA-induced CYP1 activities in intact MCF-7 cells and this disagreed with the *in vivo* experiment. In contrast with theaflavins, EGCG was found to be capable to inhibit DMBA-induced CYP1 activities.

Certain phytochemicals have been demonstrated to affect AhR-mediated induction of CYP1A1. Resveratrol (Ciolino et al., 1998), quercetin (Ciolino et al., 1999) and curcumin (Oetari et al., 1996) are naturally occurring ligands to the AhR

that produce either agonist or antagonist activities. Consistent with other reports (Williams et al., 2000), our finding showed that EGCG antagonized induced CYP1 gene expression. Nevertheless, the 4 theaflavins did not produce significant effects on XRE-mediated gene transcription, whereas TF3 and DMBA co-administration led to a slight induction. Subsequent semi-quantitative RT-PCR experiments confirmed that theaflavins did not inhibit AHR mediated CYP1 expression. Cotreatment of DMBA and theaflavins even led to a slight increase in CYP1A1 mRNA expression. It was suggested that the chemoprotective activities of theaflavins on DMBA-induced carcinogenesis were not related to their modulating ability on CYP1 activities. On the other hand, EGCG inhibited DMBA-induced CYP1A1 and CYP1B1 mRNA expressions.

Feng et al. (2002) report that theaflavins suppress OME-induced CYP1A1 activities. The difference between this and our studies is that we use DMBA to induce CYP1 activities. DMBA is a mammary carcinogen that stimulates the transcription of CYP1 enzymes via AhR, a signal transduction pathway distinct from the protein tyrosine kinase-mediated pathway. As suggested by Feng et al. (2002), theaflavins may stimulate tyrosine receptor kinase activity to block omeprazole-induced CYP1A1.

In conclusion, this report showed that EGCG from green tea is a potent chemopreventive agent that antagonises AHR-mediated CYP1 induction and inhibits CYP1 enzyme activities. Theaflavins from black tea also possess inhibitory effects on DMBA-DNA adduct formation, however, they do not reduce DMBA-induced CYP1 expression.

CHAPTER 5 ISOFLAVONES PREVENT DMBA-INDUCED CARCINOGENESIS BY INHIBITING CYP1A1 AND CYP1B1 ACTIVITIES

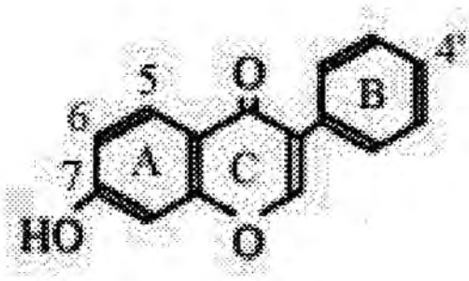
Introduction

Of all the phytochemicals that are currently under intense investigation, perhaps none has received as much attention as isoflavones found in soybean, chick peas and other legumes. Numerous studies have shown that isoflavones have multi-biological and pharmacological properties. They are estrogenic and antiestrogenic compounds (Ren et al., 2001). Others like antiproliferative effect (Kuntz et al., 1999), induction of cell-cycle arrest, apoptosis (Matsukawa et al., 1993; Shapiro et al., 1999) as well as cellular signaling (Agawal, 2000) are also described. Based on these properties, isoflavones have been associated with reduced incidences of breast and prostate cancers (Busby et al., 2002), cardiovascular diseases or osteoporosis (Goldwyn et al., 2000; Pollard et al., 2000), and exhibit some other favorable effects.

Genistein, daidzein are primary aglycone isoflavones in soybeans while biochanin A is a methylated derivative of genistein isolated from red clover, *Trifolium pratense* (Table 5.1). The anticarcinogenic effects of these isoflavones are well-documented (Rosenberg et al., 2002; Birt et al., 2001). They have the potential in protecting against cancers on several fronts. Being weak estrogens, they are postulated to reduce the risk of estrogen-related cancers by receptor competition (Naik et al., 1994). They possess antiproliferative and apoptotic properties (Zhou et al., 1998). Moreover, they are effective antioxidants that scavenge free-radicals and prevent oxidative DNA-damage. Another proposed mechanism for protection against

cancer by these isoflavones may include modification of carcinogen metabolizing enzymes that results in the reduction of activated carcinogens. This can be accomplished by coupling the inhibition of phase I activating enzymes and induction of phase II detoxification enzymes. Genistein and biochanin A are inducers of the phase II enzyme NAD(P)H: quinone reductase (QR), while daidzein has no significant effect on it (Wang et al., 1998; Sun et al., 1998). To date, few researches have been carried out to investigate the effects of biochanin A and daidzein on phase I activating enzymes. Kishida et al. (2000) have shown that genistein and daidzein do not induce CYP content in liver microsomes of mice. By contrast, Helsby et al. (1998) have demonstrated that genistein inhibits mouse CYP1A1 and CYP2E1-dependent substrate metabolism *in vitro*. Furthermore, genistein has been reported to be a tyrosine kinase inhibitor that suppresses both dioxin and OME-induced CYP1A1 but not CYP1B1 expression (Kikuchi et al., 1999). Daidzein inhibits CYP1A1 enzymes activities in mice (Shertzer et al., 1999), while the role of biochanin A on CYP1 enzyme inhibition remains unclear.

It has been demonstrated that genistein and daidzein have potent chemopreventive activities against DMBA-induced carcinogenesis in rats (Wei et al., 1995; Giri et al., 1995), whereas biochanin A reduces B[a]P-induced toxicity in mice and mammalian cell culture (Cassady et al., 1888; Chae et al., 1991). This effect is attributed to, in part, the ability of isoflavones to inhibit carcinogen-DNA adduct formation. However, the possible roles of them on CYP1 metabolizing enzymes have not been fully elucidated. Therefore, in the present study, we would like to investigate the effects of these isoflavones on the expression and activities of CYP1 enzymes in mammary cell cultures.



Isoflavones	Substitutions			
	5	6	7	4'
Biochanin A	OH	H	OH	OMe
Genistein	OH	H	OH	OH
Daidzein	H	H	OH	OH

Table 5.1 Chemical structures of isoflavones.

Results

Isoflavones inhibited DMBA-induced EROD activity in MCF-7 cells

CYP1A1 and CYP1B1 activities were measured as EROD activity in intact cells. DMBA caused approximately 2-fold increase in EROD activity compared with controls (Data not shown). Co-treatment with genistein and biochanin A suppressed this induction in a concentration dependent manner, with IC_{50} of approximately 12 μM and 0.57 μM respectively (Figure 5.2A and B). Nevertheless, the suppressive effect of daidzein was less effective. Only <30% of DMBA-induced EROD activity was inhibited by 50 μM of daidzein (Figure 5.2C). The above data indicated that biochanin A was the most effective in suppressing DMBA-induced EROD activity among the three. Treatments with isoflavones alone only have slight inhibition on EROD activities in MCF-7 cells (Figure 5.3).

Inhibition of MCF-7 microsomal EROD activities by isoflavones

The effects of isoflavones on EROD activities were determined on microsomal fractions from DMBA-treated MCF-7 cells (Figure 5.4). Isoflavones treatment resulted in a concentration-dependent decrease in EROD activities. Among them, biochanin A was the most potent inhibitor with IC50 value of ~8 μM, followed by genistein. Daidzein was the least potent inhibitor.

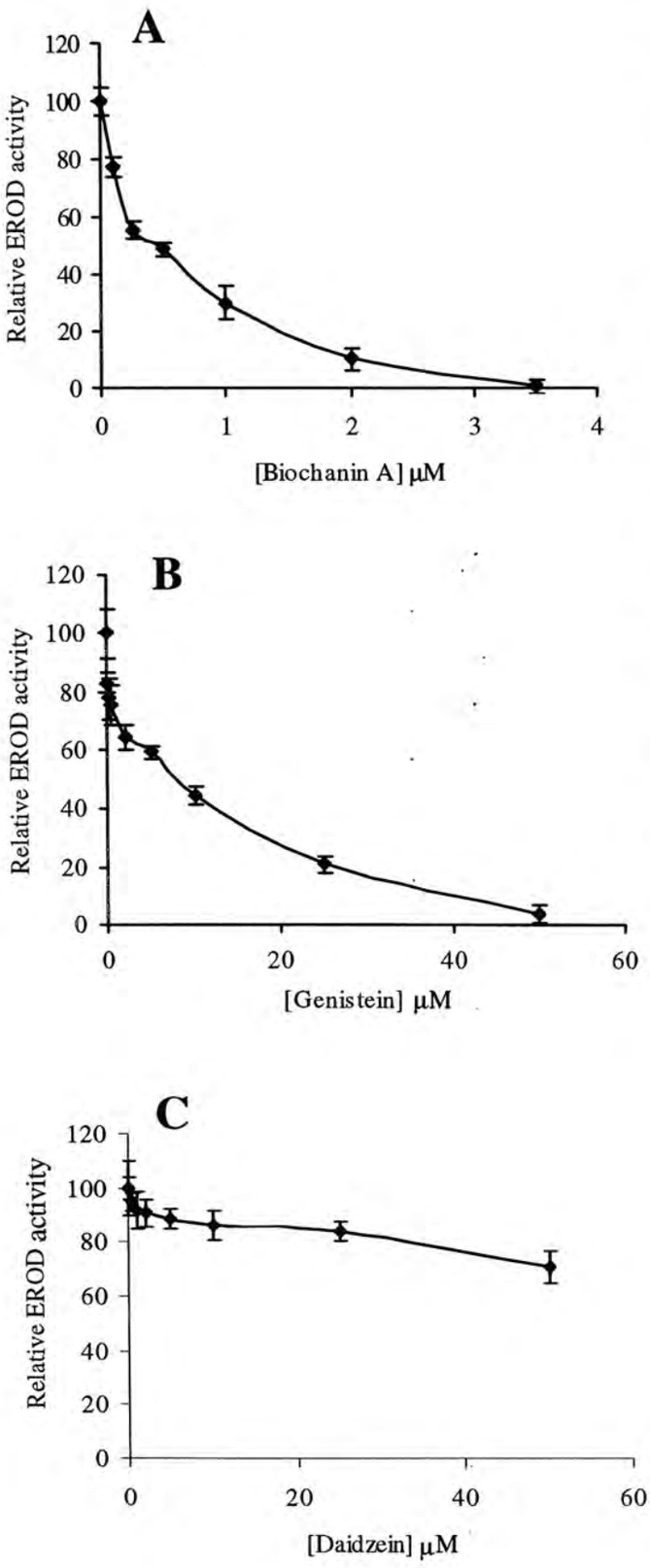


Figure 5.2 Diverse effects of isoflavones on DMBA-induced EROD activities.

MCF-7 cells were seeded in 96-well culture plates and treated with 1 μM DMBA and various concentrations of isoflavones; biochanin A (A), genistein (B) and daidzein (C). After 24 h of treatment, cells were assayed for EROD activity as described. The values are means ±SE, n=6. All data are significantly different from the control ($P< 0.05$).

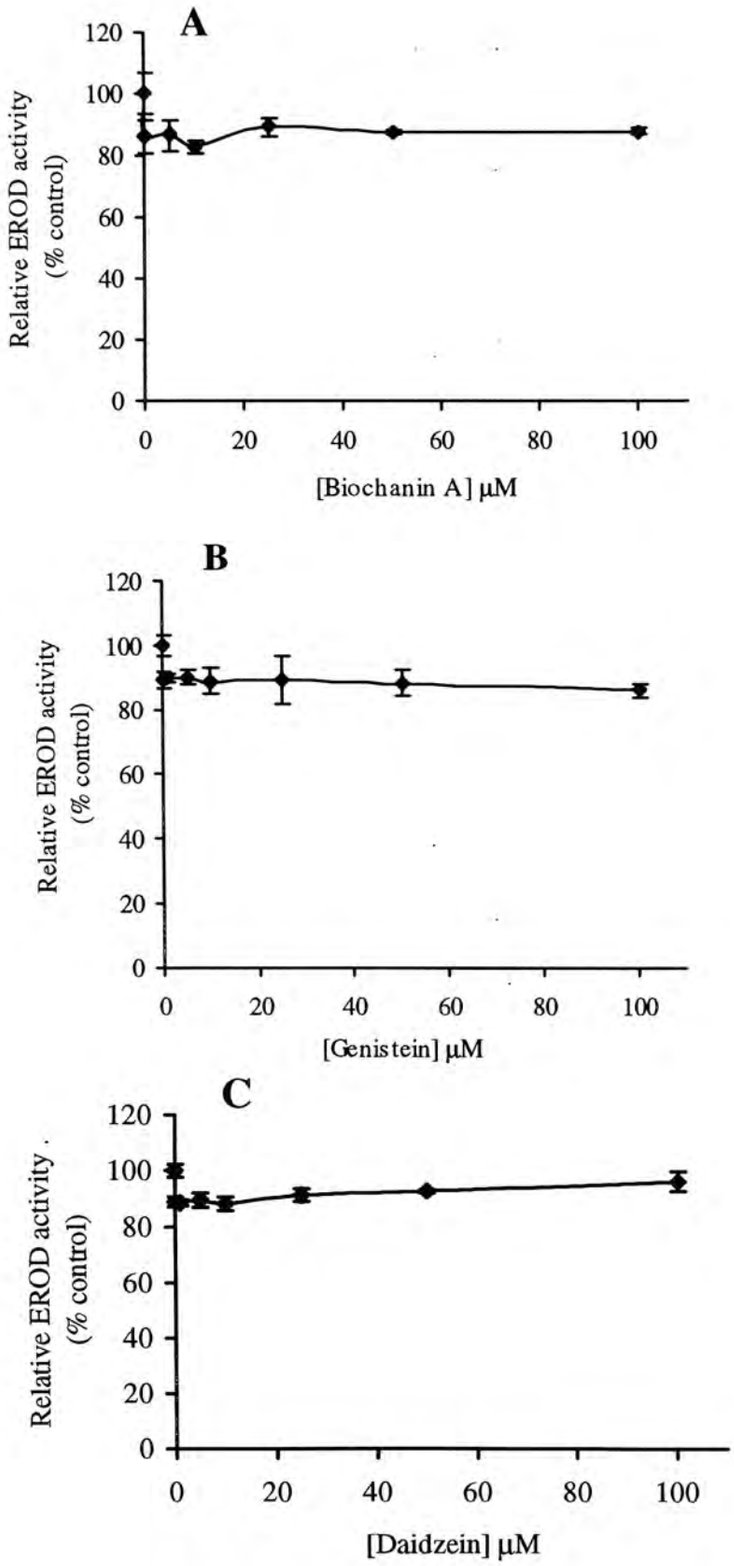


Figure 5.3 Inhibitory effects of isoflavones alone on EROD activities.

MCF-7 cells were treated with 0.1 to 100 μM of isoflavones for 24 h. EROD activities were determined as described. The values are means ±SE, n=6. All data are significantly difference from the control ($P< 0.05$).

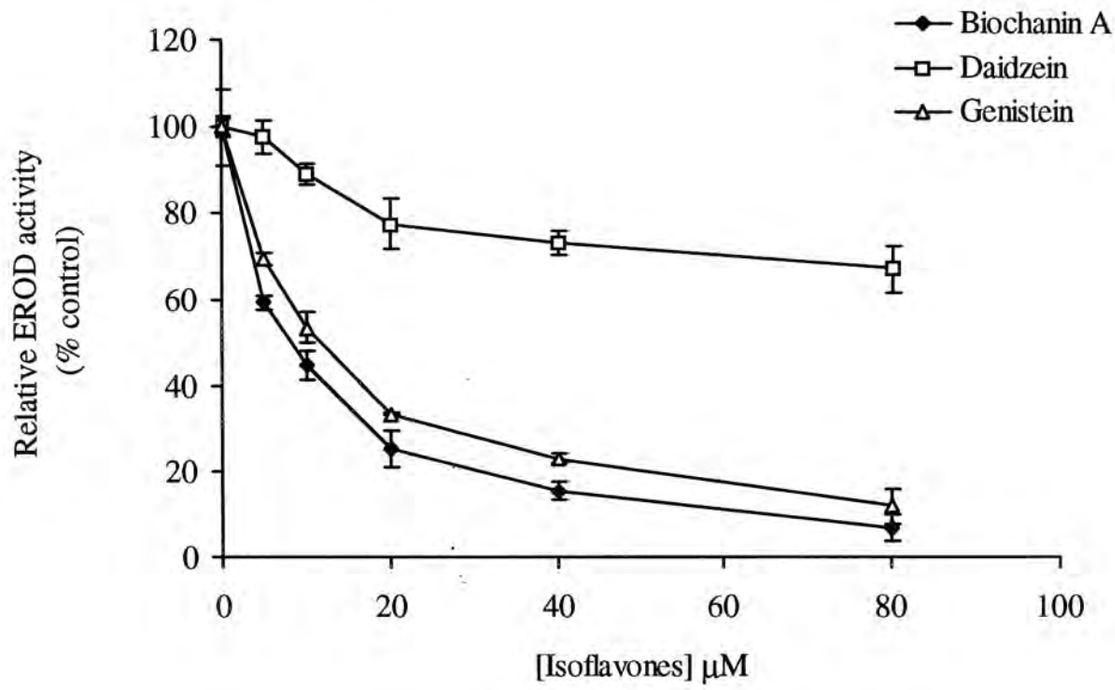


Figure 5.4 Effects of isoflavones on microsomal EROD activities.

Microsomes were isolated from MCF-7 cells treated with 1μM DMBA for 24 h. Then EROD activities in 10 μg microsomes were measured in the presence of indicated concentration of isoflavones. EROD activities in all isoflavones-treated samples were statistically different from the controls ($P < 0.05$). The values are means ±SE, n=3.

Kinetic analysis of the inhibition of human recombinant CYP1 enzymes by isoflavones

To further characterize the inhibition of CYP1A1 and CYP1B1 catalytic activities by isoflavones, enzymes kinetic experiments using human recombinant CYP1A1 and CYP1B1 enzymes were carried out. Figure 5.5 provided a general view on the inhibitory effects of various isoflavones on recombinant CYP1A1 and CYP1B1 enzymes. The inhibitory ability of isoflavones on both CYP1A1 and CYP1B1 activities were in the order biochanin A> genistein> daidzein. Lineweaver-Burk and inset plot showed that the 3 isoflavones exhibited mixed type inhibition on CYP1A1 enzymes whereas they competitively inhibited CYP1B1 enzymes. Based on the corresponding Ki values (Figure 5.9), we may conclude that biochanin A was the most effective inhibitor on both CYP1A1 and CYP1B1 activities among the 3 isoflavones.

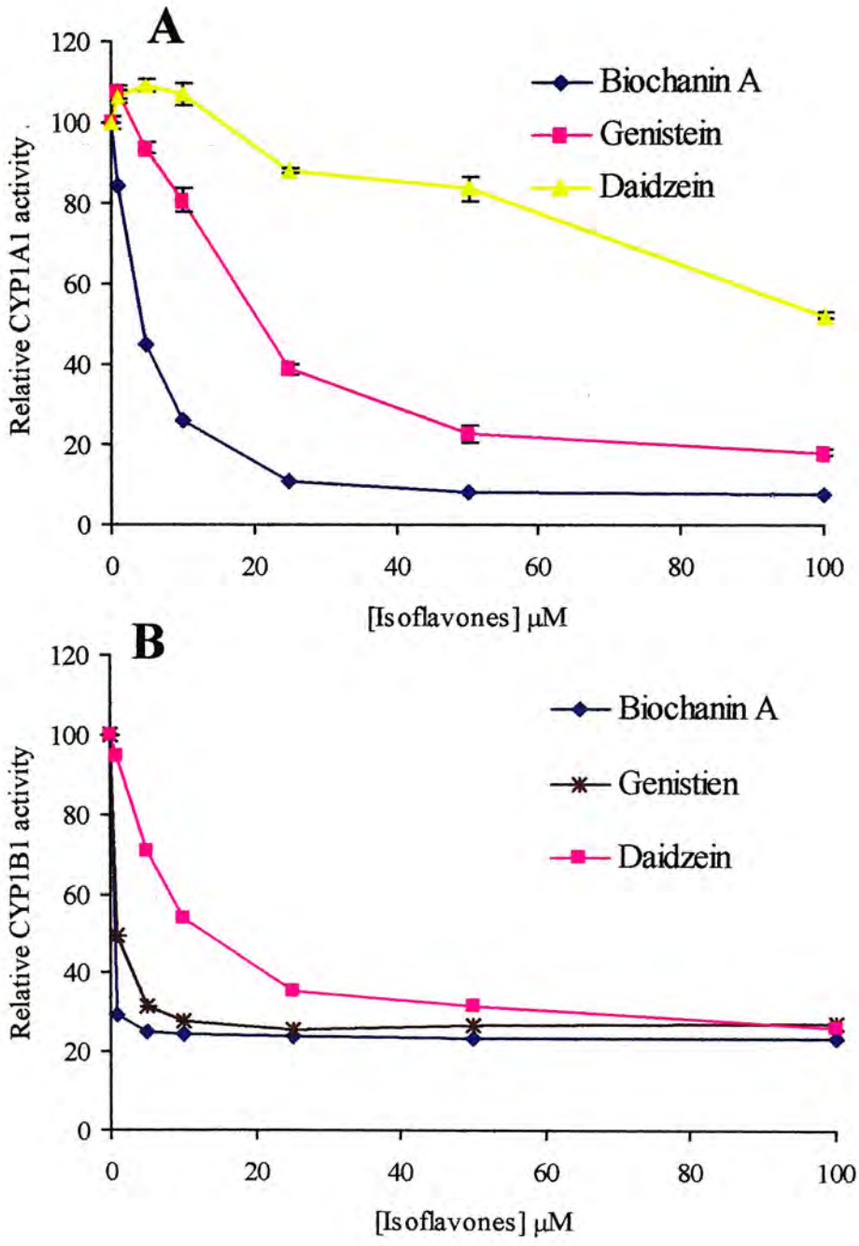


Figure 5.5 Inhibition of isoflavones on human recombinant CYP1A1 and CYP1B1 activities.

EROD assay (400nM ER) was performed with 2.5 pmole of CYP1A1 (A) and 2 pmole of CYP1B1 (B) enzymes and varying concentration of theaflavins and EGCG. The values are means ±SE, n=3. All values are significantly different (P >0.05) form the control (0 μM).

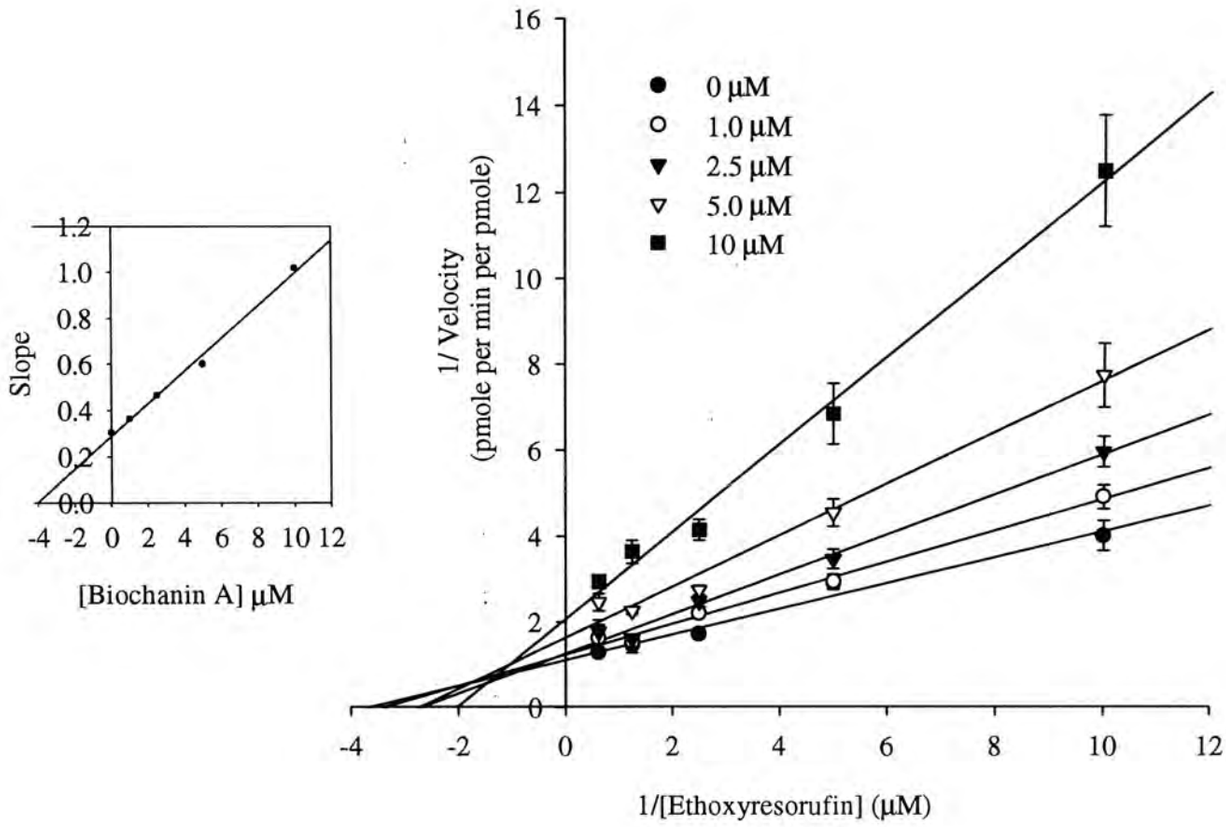


Figure 5.6A Lineweaver-Burk plot of biochanin A on CYP1A1 enzymes inhibition.

EROD assay was performed with human recombinant CYP1A1 at the indicated concentration of biochanin A and 100-1600 nM ER as described under *Material and Methods*. Lineweaver-Burk plot was generated by linear regression of the reciprocal data. Inset, replot of the slopes from the Lineaweaver-Burk plot, with derivation of Ki. Values shown are mean ±SE, n=3.

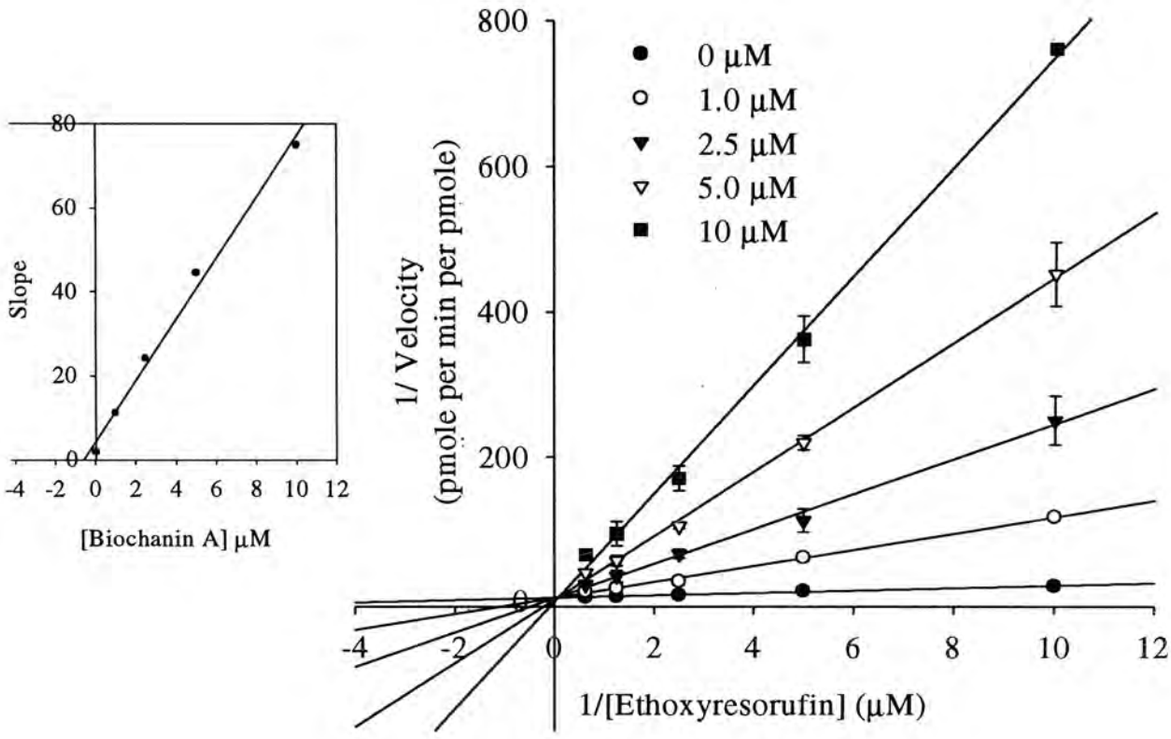


Figure 5.6B Lineweaver-Burk plot of biochanin A on CYP1B1 enzymes inhibition.

EROD assay was performed with human recombinant CYP1B1 at the indicated concentration of biochanin A and 100-1600 nM ER as described in figure 5.6A.

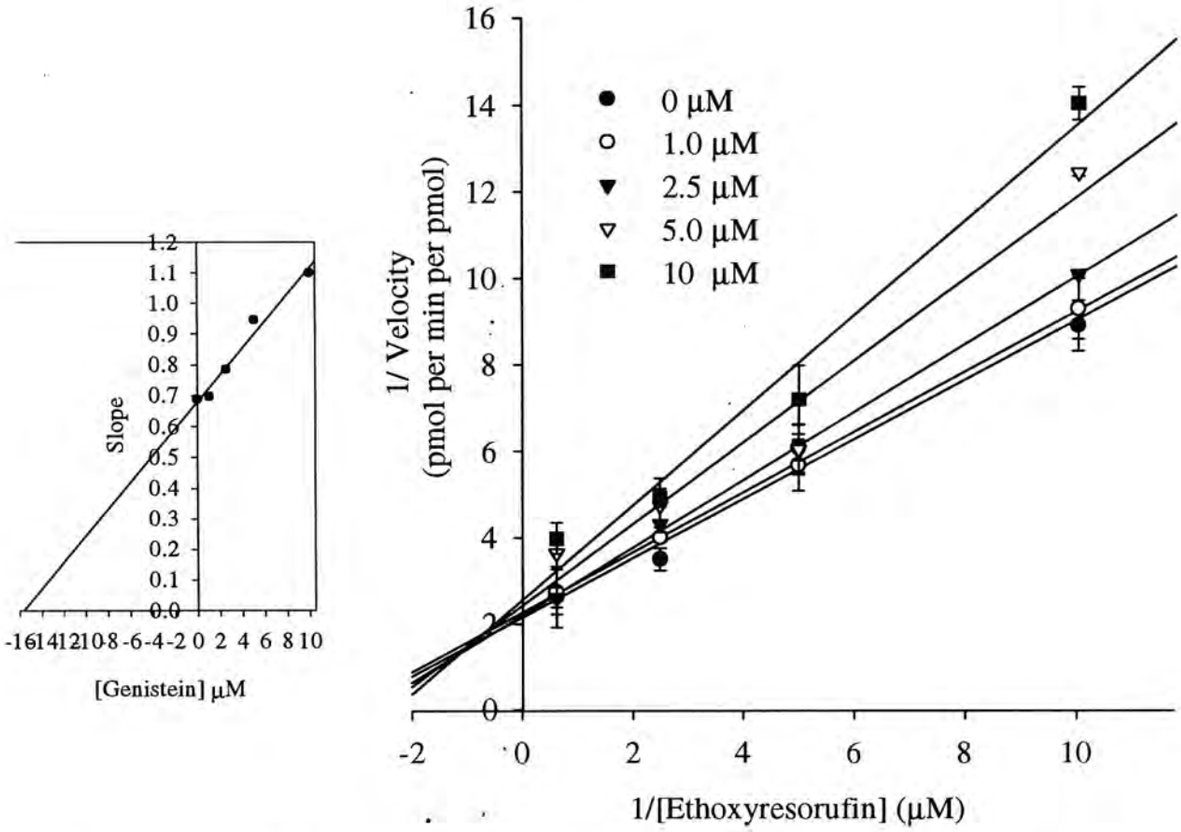


Figure 5.7A Lineweaver-Burk plot of genistein on CYP1A1 enzymes inhibition.

EROD assay was performed with human recombinant CYP1A1 at the indicated concentration of genistein and 100-1600 nM ER as described under *Material and Methods*. Lineweaver-Burk plot were generated by linear regression of the reciprocal data. Inset, replot of the slopes from the Lineaweaver-Burk plot, with derivation of Ki. Values shown are mean ±SE, n=3.

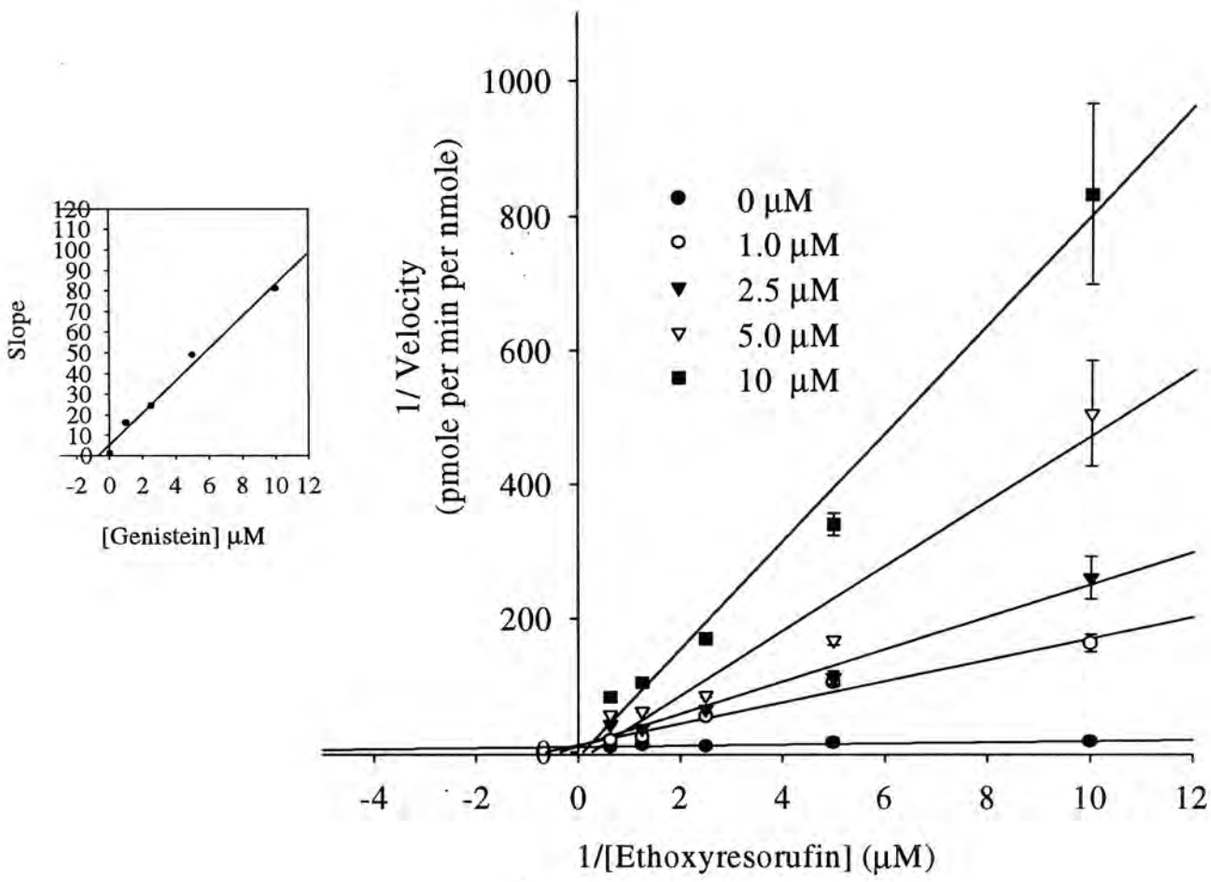


Figure 5.7B Lineweaver-Burk plot of genistein on CYP1B1 enzymes inhibition.

EROD assay was performed with human recombinant CYP1B1 at the indicated concentration of genistein and 100-1600 nM ER as described in figure 5.7A.

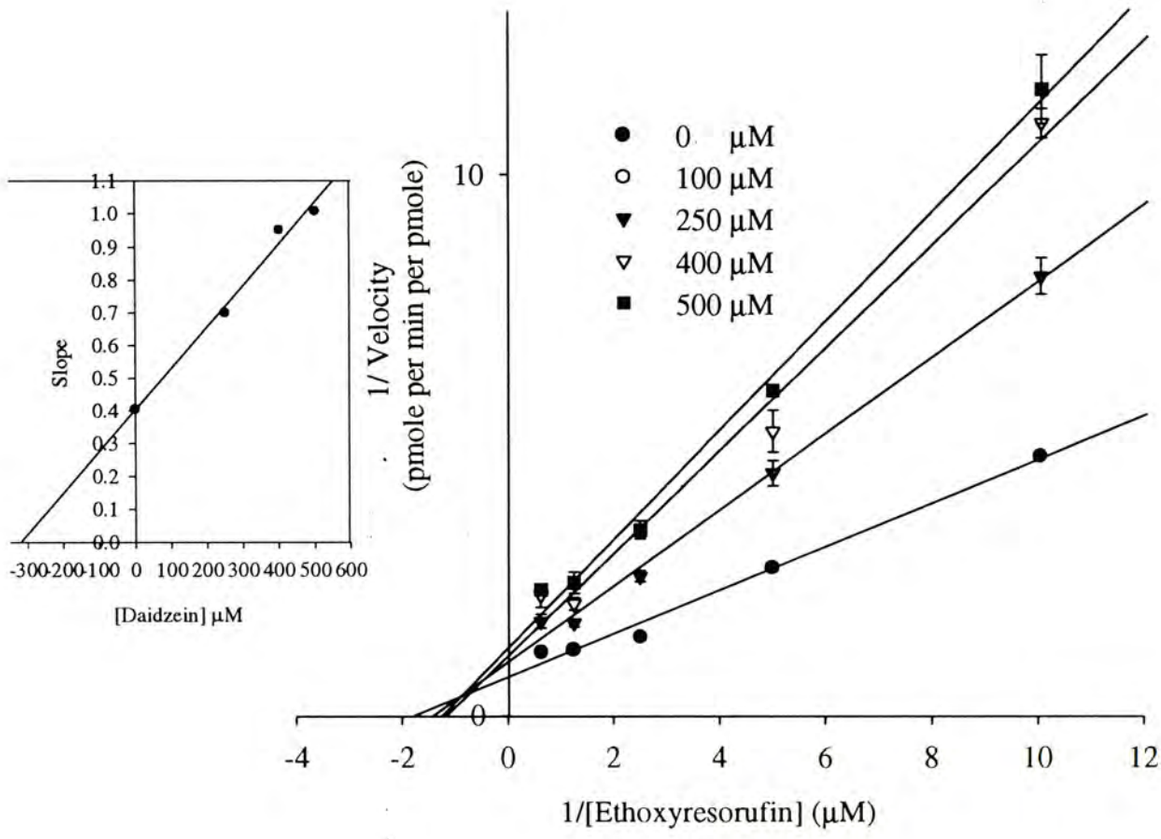


Figure 5.8A Lineweaver-Burk plot of daidzein on CYP1A1 enzymes inhibition.

EROD assay was performed with human recombinant CYP1A1 at the indicated concentration of daidzein and 100-1600 nM ER as described under *Material and Methods*. Lineweaver-Burk plot were generated by linear regression of the reciprocal data. Inset, replot of the slopes from the Lineaweaver-Burk plot, with derivation of Ki. Values shown are mean ±SE, n=3.

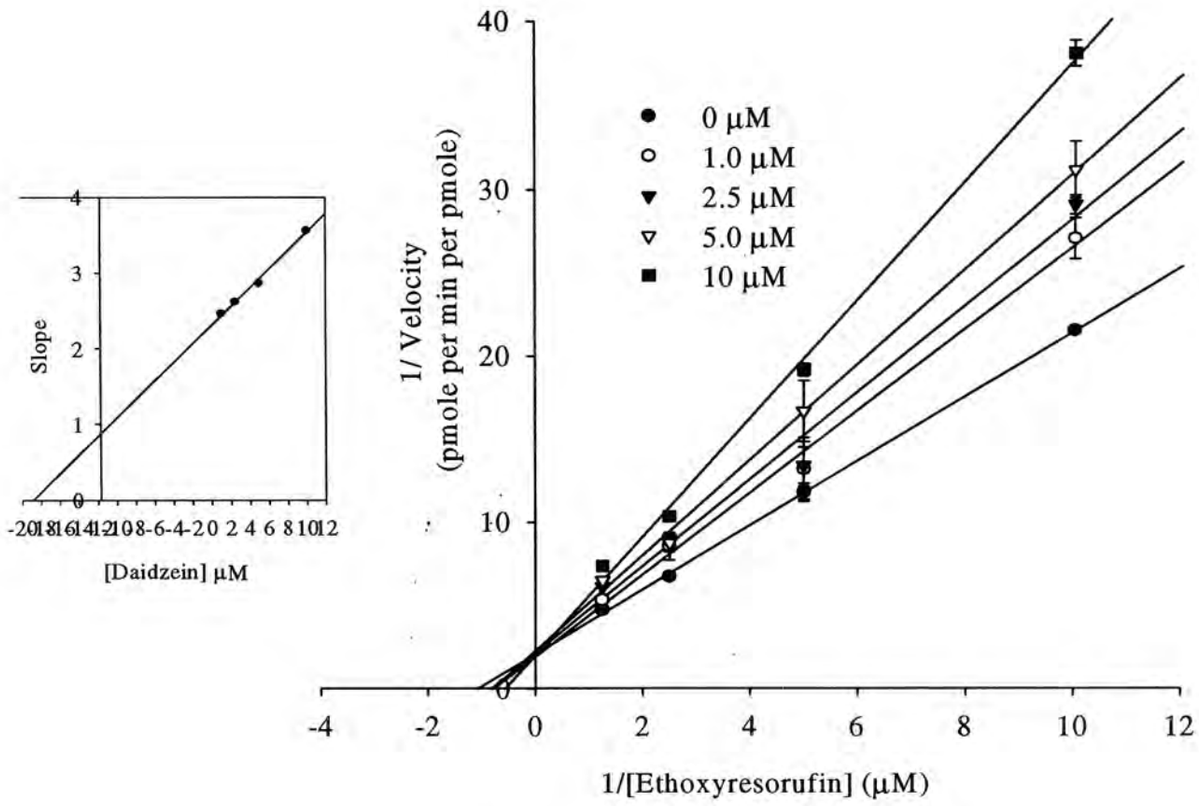


Figure 5.8B Lineweaver-Burk plot of daidzein on CYP1B1 enzymes inhibition.

EROD assay was performed with human recombinant CYP1B1 at the indicated concentration of daidzein and 100-1600 nM ER as described in figure 5.8A.

	Ki (μM)	
	CYP1A1	**CYP1B1**
Biochanin A	4	0.59
Genistein	15.35	0.677
Daidzein	319	18.82

Figure 5.9 Competitive Ki value for isoflavones on CYP1 inhibition.

This table concluded the competitive Ki value for isoflavones on CYP1A1 and CYP1B1 inhibition.

XRE-driven Luciferase activities

MCF-7 cells were transfected with XRE fused to a luciferase reporter gene and luciferase activity was subsequently measured to index AhR-mediated transcription. DMBA induced luciferase activity by 6.5-fold above control level. The addition of 5 μM biochanin A produced a 65% reduction in DMBA-induced luciferase activities (Figure 5.10A), while 25 μM of genistein resulted in a 99% reduction (Figure 5.10B). Nevertheless, daidzein had no effect at this level (Figure 5.10C). Treatment with daidzein alone led to a 3.2-fold increase in XRE-mediated luciferase activity compared with control, which indicated that daidzein was an agonist for XRE-dependent transactivation (Figure 5.11C). Both biochanin A (Figure 5.11A) and genistein (Figure 5.11B) induced XRE-driven luciferase activity at low concentration.

Both biochanin A and genistein suppressed DMBA-induced CYP1 mRNA expression

DMBA induced CYP1A1, CYP1B1 mRNA expression by 6.6- and 3.1-fold above basal levels, respectively. The increases were abated in a concentration dependent manner with genistein co-treatment (Figure 5.13). Treatment with 5 μM of biochanin A showed the greatest reduction on both DMBA-induced CYP1A1 and CYP1B1 mRNA levels (Figure 5.12). Daidzein had no effect on CYP1A1 mRNA level and caused a slight but significant ($P< 0.05$) increase on DMBA-induced CYP1B1 mRNA levels at 10 and 25 μM (Figure 5.14).

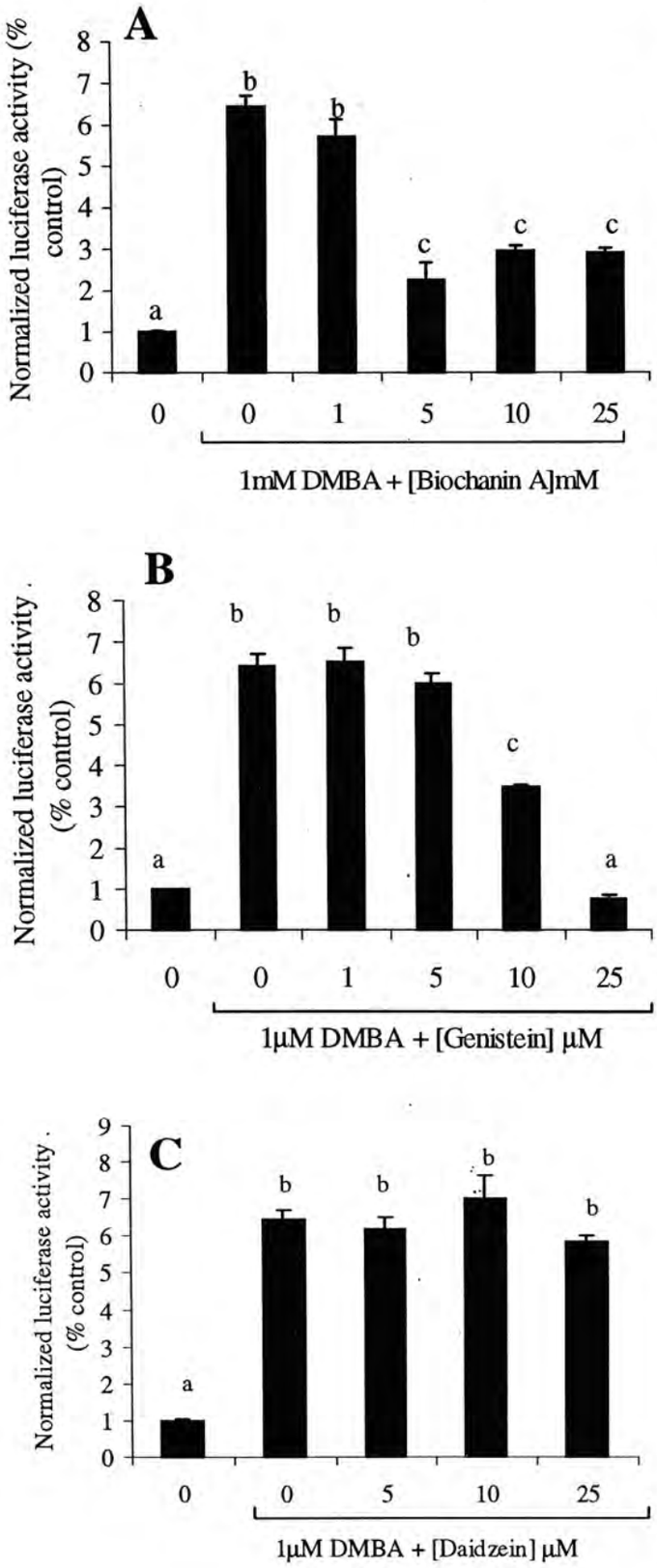


Figure 5.10 Differential effects of isoflavones on DMBA-induced XRE-driven luciferase activities.

MCF-7 cells were transiently transfected with a luciferase reporter gene containing XRE and a pRL. Transfected cells were treated with 1 μM DMBA in the presence of various concentration of isoflavones; biochanin A (A), genistein (B) and daidzein (C) for 24 h. Values are means ±SEM, n=3. Means without same letters differ ($P<0.05$).

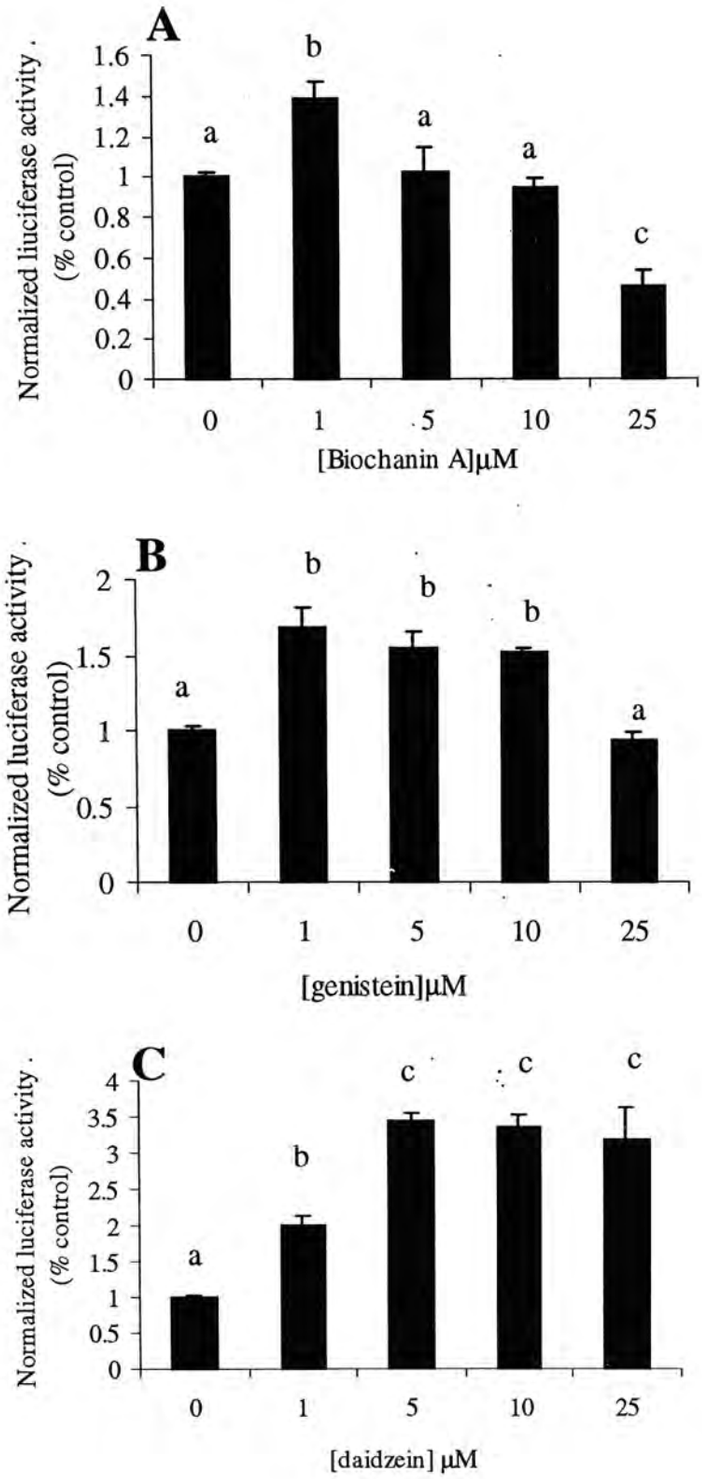


Figure 5.11 Effects of isoflavones alone on XRE-driven luciferase activities.

MCF-7 cells were transiently transfected with a luciferase reporter gene containing XRE and a pRL. Transfected cells were treated with 1, 5, 10, 25 μM of isoflavones; biochanin A (A), genistein (B) and daidzein (C) for 24 h. Firefly luciferase activities were normalized with renilla luciferase. Values are means ±SEM, n=3. Means without same letters differ ($P<0.05$).

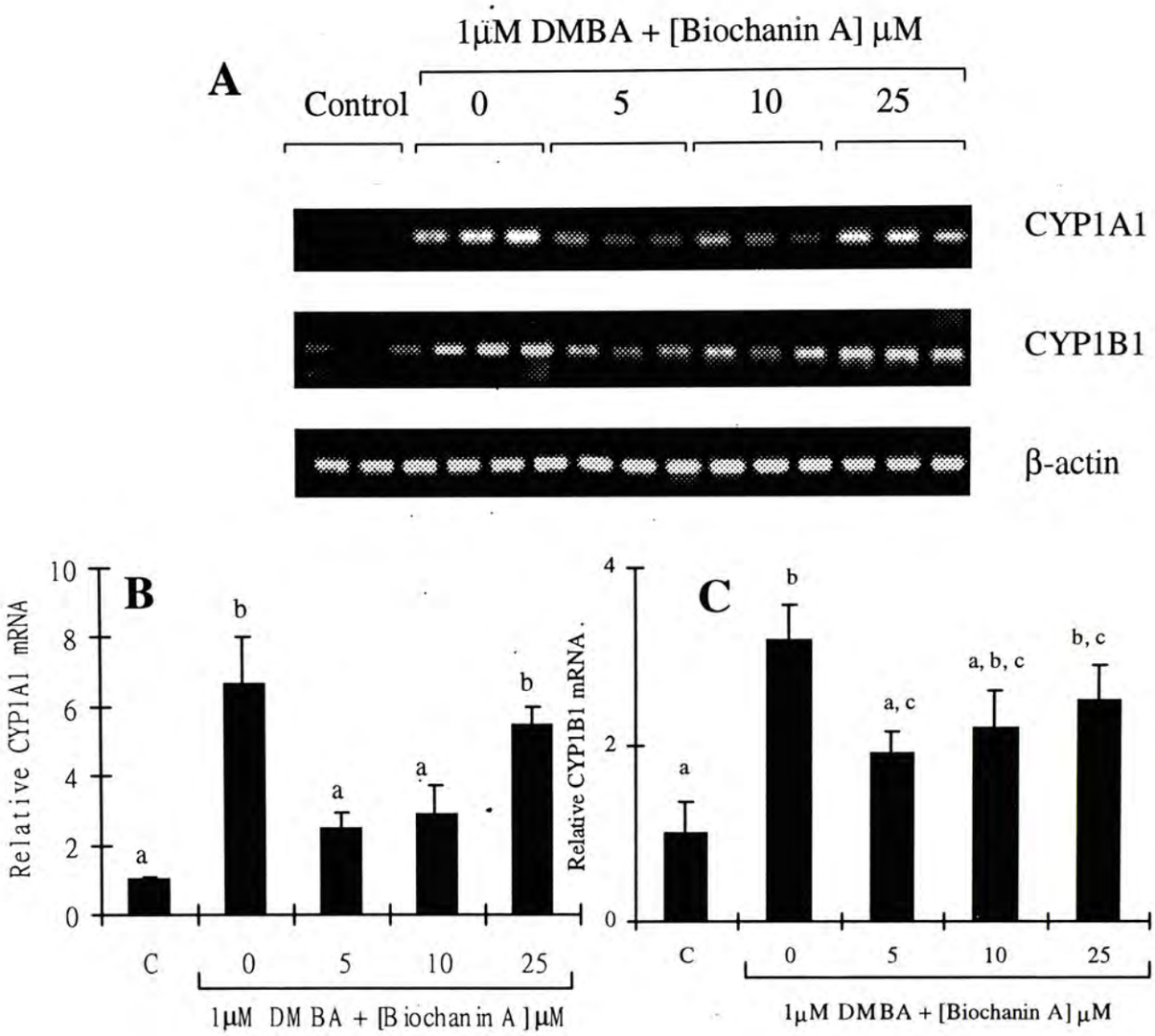


Figure 5.12 Effect of Biochanin A on DMBA-induced CYP1A1 and CYP1B1 mRNA expression.

MCF-7 cells were seeded in 6-well culture plates and treated with 1 μM DMBA and various concentrations of baicalein for 24 h. Total RNA was then isolated and semi-quantitative RT-PCR was used to determine the relative mRNA expressions. Figure 5.7A is the gel image of the ethidium bromide-stained PCR fragments. Figure 5.7B and C are the optical density readings of the CYP1A1 and CYP1B1 fragments. Values are means ±SE, n=3. Means without same letters differ ($P<0.05$).

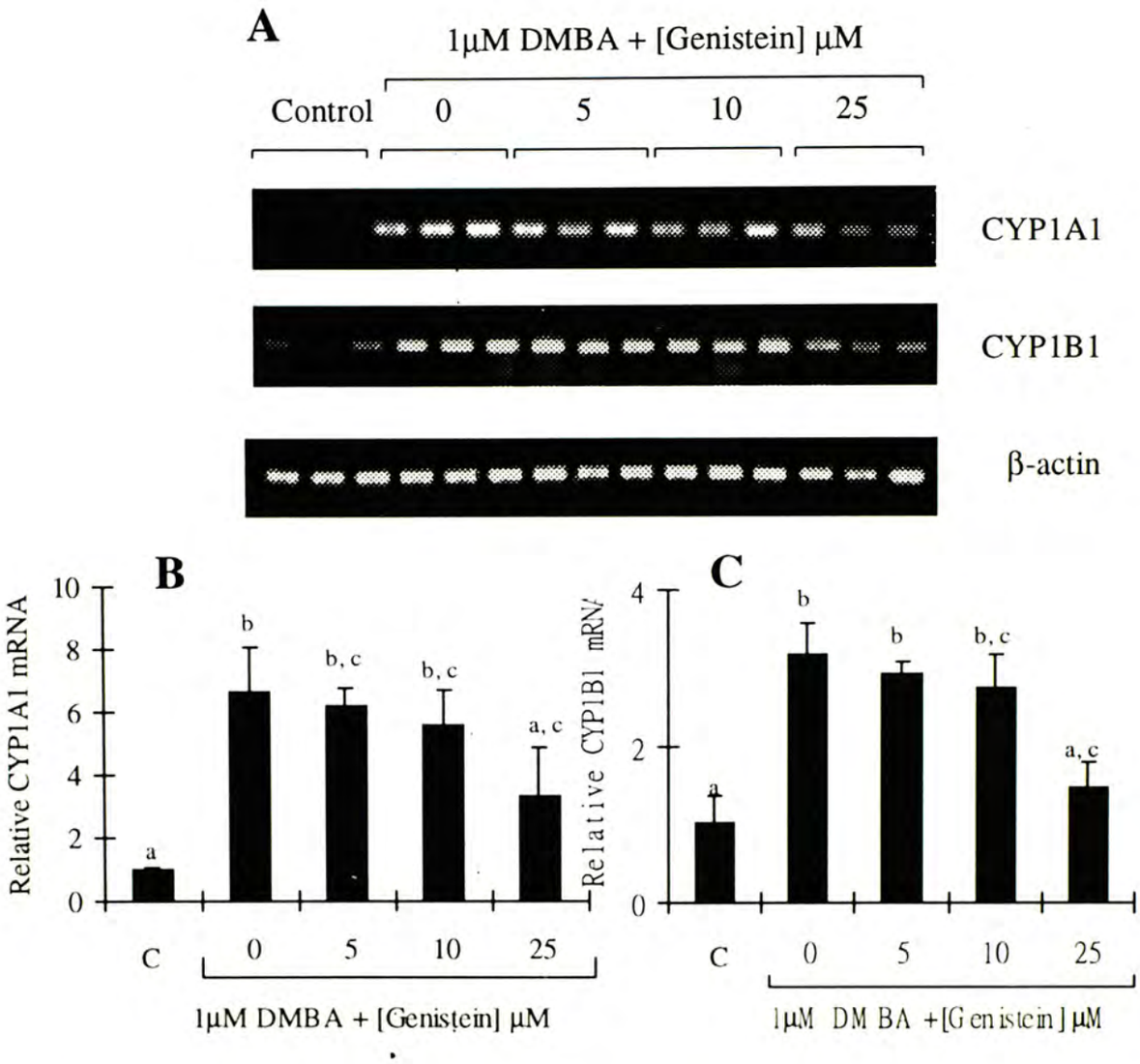


Figure 5.13 Effect of genistein on DMBA-induced CYP1 mRNA expression.

CYP1A1 and CYP1B1 mRNA expression after treatment with DMBA and genistein was investigated as described in figure 5.7. Figure 5.8A is the gel image of the ethidium bromide-stained PCR fragments. Figure 5.8B and C are the optical density readings. Values are means ±SE, n=3. Means without same letters differ ($P<0.05$).

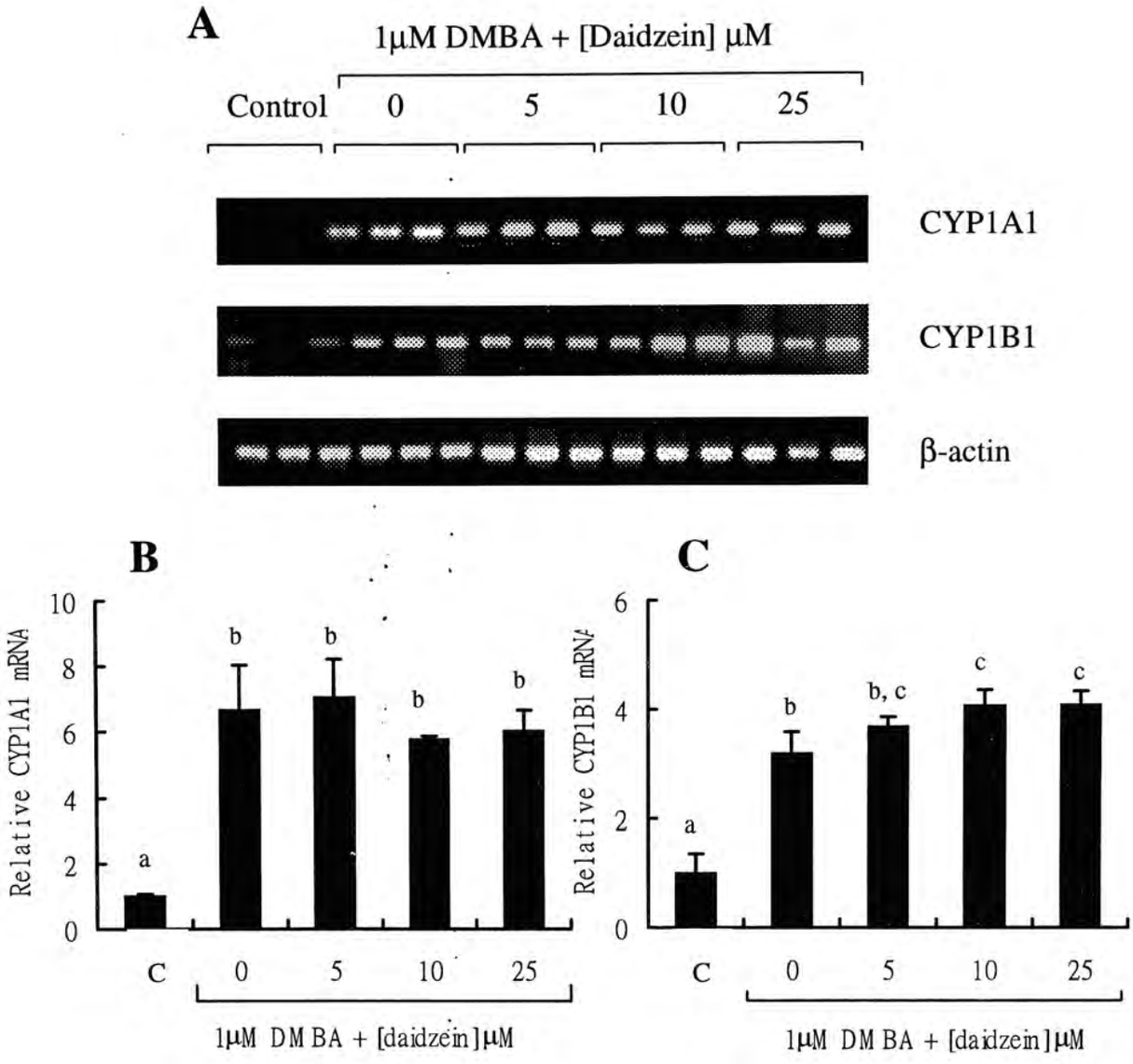


Figure 5.14 Effect of daidzein on DMBA-induced CYP1 mRNA expression.

CYP1A1 and CYP1B1 mRNA expression after treatment with DMBA and daidzein was investigated as described in figure 5.7. Figure 5.9A is the gel image of the ethidium bromide-stained PCR fragments. Figure 5.9B and C are the optical density readings. Values are means ±SE, n=3. Means with same letters differ ($P<0.05$).

Cytotoxicity of DMBA and isoflavones co-treatment

To test the effects of isoflavones on DMBA toxicity, the cell viability of MCF-7 was measured by MTT assay after 72 h of incubation with indicated concentration of DMBA in the presence or absence of 2.5 μM isoflavones. All 3 isoflavones inhibited DMBA-induced cytotoxicity (Figure 5.15). Biochanin A was the most effective agent that rescued MCF-7 cells from DMBA-induced cell death, followed by daidzein and finally genistein. All isoflavones, by themselves had no effect on cell viability after 24h of treatment until their concentration reached 100 μM (Figure 5.16).

Isoflavones reduced the binding of activated DMBA to DNA

The above results showed that isoflavones modulated the expression and activities of CYP1 enzymes. To analyze the effects of isoflavones on DMBA metabolism, tritiated DMBA was added to MCF-7 cells in the presence or absence of isoflavones and the amount of [^{3}H]DMBA-DNA adduct was measured by scintillation counting. Genistein treatment resulted in concentration dependent reduction of [^{3}H]DMBA-DNA adducts formation (Figure 5.17B) and 5 μM of biochanin A could reduce DNA-adduct formation by 50% (Figure 5.17A). However, daidzein inhibited less than 25% [^{3}H]DMBA-DNA adduct formation at a concentration of 50 μM (Figure 5.17C).

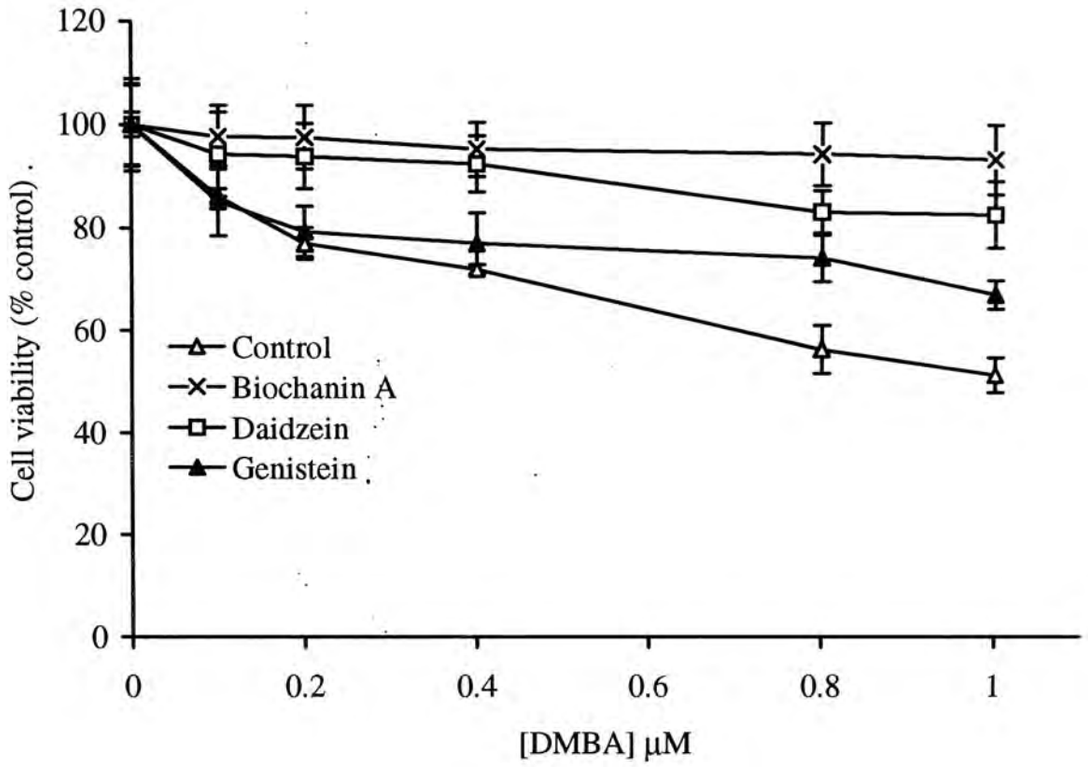


Figure 5.15 The cytotoxic effects of DMBA and isoflavones on MCF-7 cells.

MCF-7 cells were cultured in 96-well plates. DMBA ranged from 0 to 1 μM was added to the cultures, and 0 μM (Control) or 2.5 μM isoflavones; biochanin A (A), genistein (B) and daidzein (C) were co-administered with DMBA. After 72 h of treatment, the cell viability percentage was assayed. Values are means ±SE, n=6.

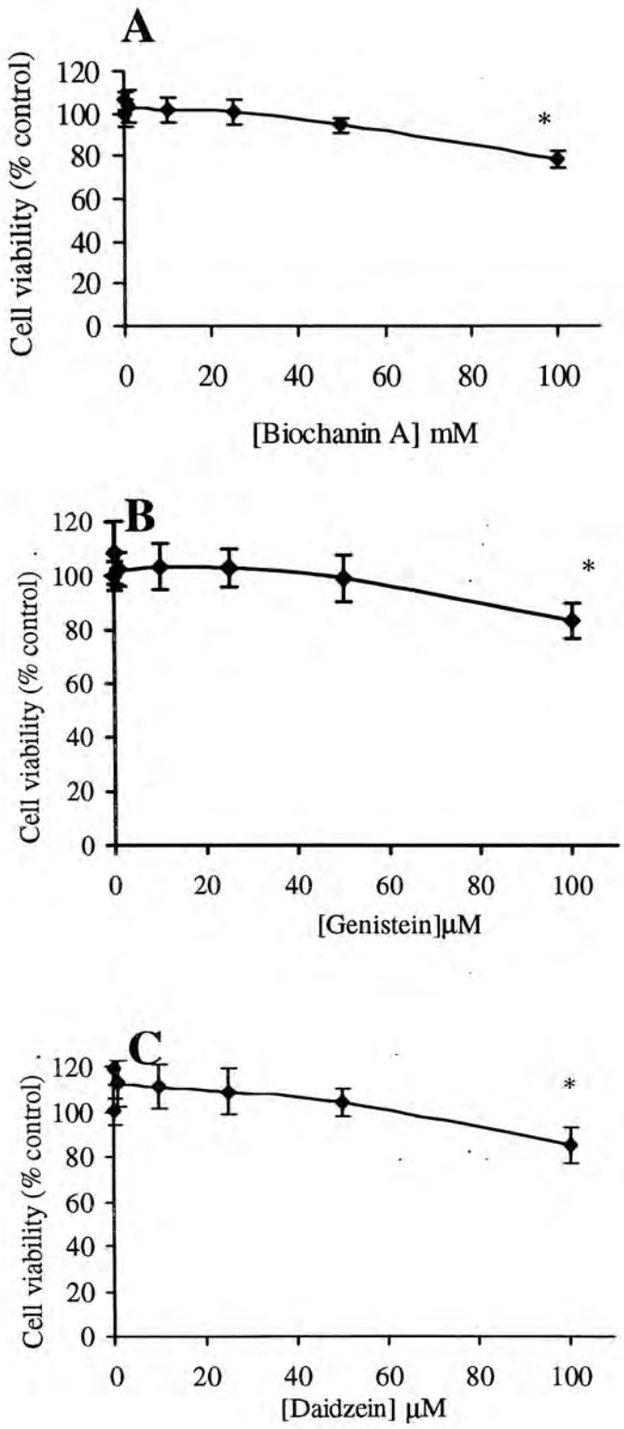


Figure 5.16 The cytotoxic effects of isoflavones on MCF-7 cells.

MCF-7 cells in 98 well plated were treated with various concentration of isoflavones; biochanin A (A), genistein (B) and daidzein (C) for 24 h. Cell viability was then measured by MTT assay. Values are means ±SE, n=6. Means marked (*) are significantly (*P<0.05) lower than the control.

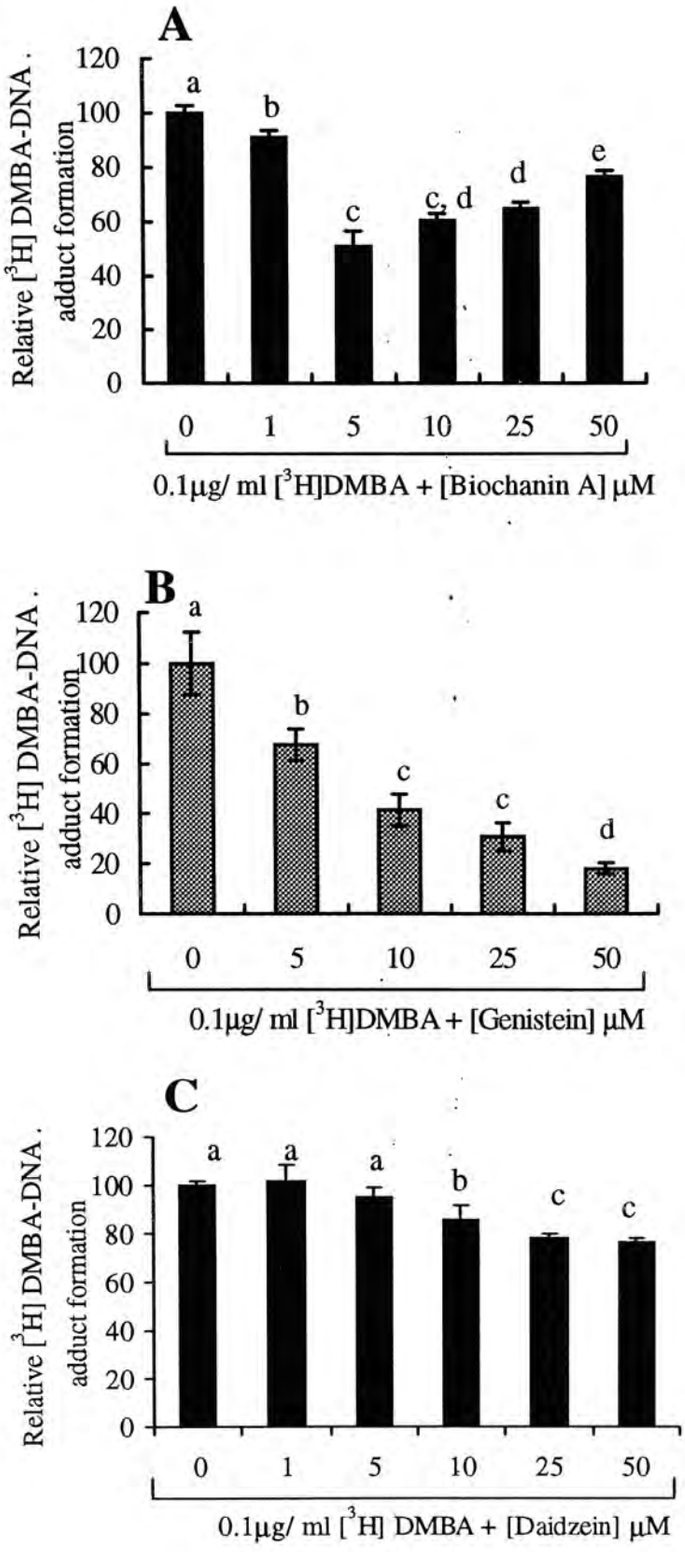


Figure 5.17 The influences of isoflavones on [^{3}H]DMBA -DNA adduct formation.

MCF-7 cells were cultured in 6-well plates and treated with tritiated DMBA in 0.1 μg/ml and various concentrations of isoflavones; biochanin A (A), genistein (B) or daidzein (C) was co-administered. After 16 h of treatment, genomic DNA was isolated and the DMBA-DNA lesions were determined by scintillation counting. Values are means ±SE, n=3. Means with same letters differ ($P<0.05$).

Discussion

Numerous reports have been demonstrated the board chemopreventive effects of isoflavones against cancer. They exhibit apoptotic, antiproliferative and antioxidative activities in different animal models. Most of the current researches are focusing on their estrogenic and antiestrogenic activities, which may attribute to their ability to prevent hormone-related cancers. Nevertheless, no conclusive results have yet been obtained because of substantial variations. Some suggest that isoflavones may only protect against hormone-related cancer when administered prepubertally. Others report the possible role of isoflavones on inhibiting chemical induced carcinogenesis. Previous studies demonstrate that isoflavones inhibit DMBA-DNA adduct formation in different models. The present report evaluated the role of isoflavones on PAHs metabolism by using MCF-7 cells.

MCF-7 cells may be regarded as an applicable model to study the effects of chemopreventive agents on carcinogens metabolism (Giri et al., 1995, Upadhyaya et al., 1998; Shertzer et al., 2001; Fritz et al., 1998 Lamartiniere et al., 1995). We showed that the 3 isoflavones reduced DMBA genotoxicity in various extents with biochanin A > genistein > daidzein. The chemopreventive properties of isoflavones might attribute to their activities in diminishing DMBA-metabolism mediated by CYP1 enzymes. CYP1 enzymes have been demonstrated to activate DMBA that may attack biological macromolecules and results in mutation (Gonzalez et al., 1994). Inhibition of CYP1 activities by phytochemicals has been suggested to inhibit xenobiotics-induced genotoxicity. The 3 isoflavones were shown to be either agonist or antagonist for microsomal and human recombinant CYP1 activities (Figure 5.4-5.5). Furthermore, genistein and biochanin A inhibited DMBA-induced CYP1 at the transcription level (Figure 5.10). By contrast, Shertzer et al. (1999) have

demonstrated that genistein and daidzein inhibit TCDD-induced CYP1A1 activities without affecting CYP1A1 gene transcription in a liver cancer cell line HepG2. In accord with our finding, genistein inhibits dioxin-dependent expression of CYP1A1 human keratinocytes, possibly by acting at the binding domain of the AHR (Gradin et al., 1994). On the other hand, Backlund et al. (1997) have shown that genistein does not inhibit B[a]P induced CYP1A1 in rat hepatoma cells. Isoflavones also induce the activities of phase II conjugating enzymes (Giri et al., 1995; Appelt et al., 1999). It is possible that isoflavones speed up the clearance of carcinogens by the induction of phase II detoxification. Isoflavones alone are weak agonists to AHR at low concentrations. Certain phytochemicals are natural ligands for AhR which compete with other AhR ligands such as DMBA or TCDD. This can give rise to the chemopreventive activities of these phytochemicals (Ciolino et al., (1998, 1999); McDonald et al., (2001).

The hydroxyl groups at 5 and 7 positions of the isoflavone molecules and the phenolic group at C-2 are critical for the inhibitory action of phytochemicals on xenobiotics-induced carcinogenesis (Chae et al., 1991; 1992; Lee et al., 1994). In the present studies, biochanin A with a methoxy group at the 4' position was more active than genistein. Daidzein, which has hydroxyl groups at position 4' and 7 but lacks a hydroxyl group at the 5th position, was not active. Thus, the hydroxyl group at the 5-position was essential for inhibition of DMBA metabolism. The presumed upper limit for the serum genistein concentration in those on a high soy diet was estimated to be 13.2 μmole/L (5 micrograms/mL) (Barnes, 1995). Although the effective dosage of genistein in inhibiting DMBA-induced CYP1 activities was shown to be ~10 μM, it was suggested that metabolism of genistein *in vivo* may potentiate their effects. So, the current cell culture experimental result may applicable in vivo (Boersma et al., 2001). Surely, further investigation is required to elucidate the

activities and metabolism of isoflavones *in vivo*.

Studies from Peterson et al. (1996) investigate the metabolism of biochanin A and genistein in MCF-7 cells. Both compounds were found to be extensively metabolized in MCF-7 cells. Biochanin A is converted into genistein 7-sulfate, genistein and another hydroxylated and methylated form of genistein 7-sulfate. On the other hand, 2 genistein metabolites are identified to be genistein and genistein 7-sulfate. Thus, the metabolism of biochanin A into genistein may account for its similar potency in preventing DMBA-induced carcinogenesis when compared to genistein in the present study. In addition, the presence of hydroxylated and methylated genistein 7-sulfate metabolite after biochanin A treatment may attribute to the relatively higher potency of biochanin A in inhibiting CYP1 activities. Actually, this compound is reported to be inhibitory to cancer cell proliferation (Peterson et al., 1998). Daidzein can be metabolized by gut microflora into equol (Setchell et al., 1984) and equol is reported to be a potent CYP1A inhibitor *in vitro* (Helsby et al., 1998). In the present study, daidzein may weakly antagonizes DMBA-induced CYP1 activities. Our results demonstrated that phytochemicals metabolism did play a crucial role on their bioavailability and bioactivity.

In conclusion, this report demonstrated genistein and biochanin A suppressed CYP1 expression and activities, and inhibited DMBA-DNA adduct formation in MCF-7 cells. Daidzein was less effective than the other two isoflavones.

CHAPTER 6 IN VITRO EFFECTS OF BAICALEIN AND THEAFLAVINS ON RAT HEPATIC P450 ACTIVITIES

Introduction

In the previous chapters, baicalein and theaflavins demonstrated antimutagenic properties and diverse effect on CYP1 enzymes in mammalian cell culture. Baicalein inhibited DMBA-induced CYP1 activities (EC_{50} = 0.97 μM) and the formation of DMBA-DNA adduct. Although theaflavins did also reduce DMBA toxicity, it had no effects on DMBA-induced CYP1 activities. In this chapter, their effects on hepatic P450 activities were determined by measuring ethoxyresorufin O-deethoxylase and methoxyresorufin O-demethylase activities in rat microsomes. Cytochrome P450 enzymes are involved in the metabolic activation and detoxification of environmental carcinogens. The differences between this and the previous studies were that no PAH was used to induce the CYP1 activities and rat microsomes instead of human microsomes were used. EROD and MROD activities from untreated rat liver were catalyzed mainly by CYP2C6 and CYP1A2 respectively (Burke et al., 1994; Levine et al., 1998). Constitutively expressed CYP2C6 are developmentally regulated P450 in rat liver. It plays a role in the biotransformation of various carcinogens including DMBA, thalidomide and sulfamethoxazole (Morrison et al., 1991; Ando et al., 2002; Cribb et al., 1995) while the resulting reactive metabolites was proposed to increase oxidative stress. Being the predominant CYP 450 enzyme in rat liver, CYP1A2 catalyse stereoselective epoxidation of a series of PAHs and nitrosamines to carcinogens (Shou et al., 1996) and plays a role in metabolizing important xenobiotics such as caffeine and propranolol (Brosen, 1995). Evaluating the

influence of baicalein and theaflavins on microsomal CYP2C6 and CYP1A2 enzymes could facilitate the study of phytochemicals on other P450 enzyme activities.

Results

Inhibition of EROD and MROD activities in rat liver microsomes by baicalein

To evaluate the effects of baicalein on rat hepatic CYP2C6 and CYP1A2 activities, microsomal EROD and MROD activities were assayed with various concentrations of baicalein. Both activities in microsomes isolated from rat liver were potently inhibited by baicalein in a dose-dependent manner. Baicalein was more effective in inhibiting MROD activity when compared with EROD activity. The IC_{50} values are 4 μM and 1 μM for EROD and MROD inhibition respectively. Figure 6.2 and 6.3 showed that baicalein was a competitive inhibitor for EROD activity by competing with ER for the active site without undergoing the catalytic steps. Replot of the slopes obtained by linear regression of the data indicated that the Ki for competitive inhibition of baicalein on EROD activity was 0.4 μM. Ki is the dissociation constant for inhibitor binding which represent the affinity of inhibitor to enzymes. The lower is the Ki; the higher is the affinity. Baicalein also acted as a competitive inhibitor for CYP1A2 activity and the Ki value was approximately 0.2 μM (Figure 6.3).

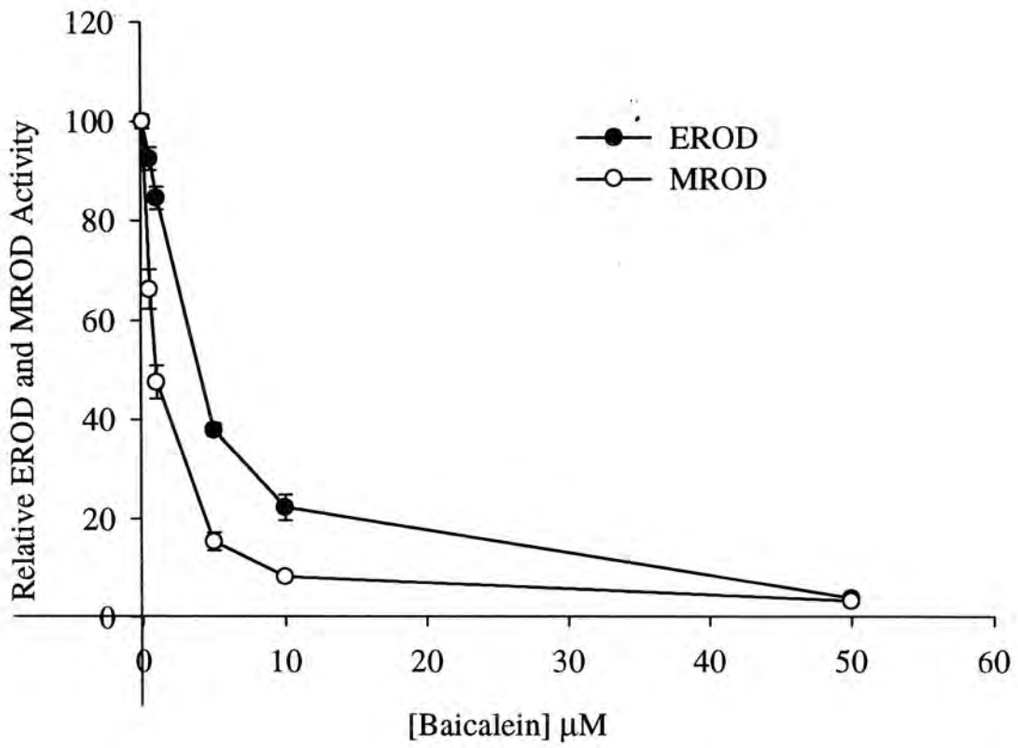


Figure 6.1 Direct effects of baicalein on microsomal EROD and MROD activity.

The EROD and MROD activities in 5 μg of microsomes isolated from rat livers were measured in the presence of 400 nM ER/MR and the indicated concentration of baicalein. The values are means ±SE, n=3.

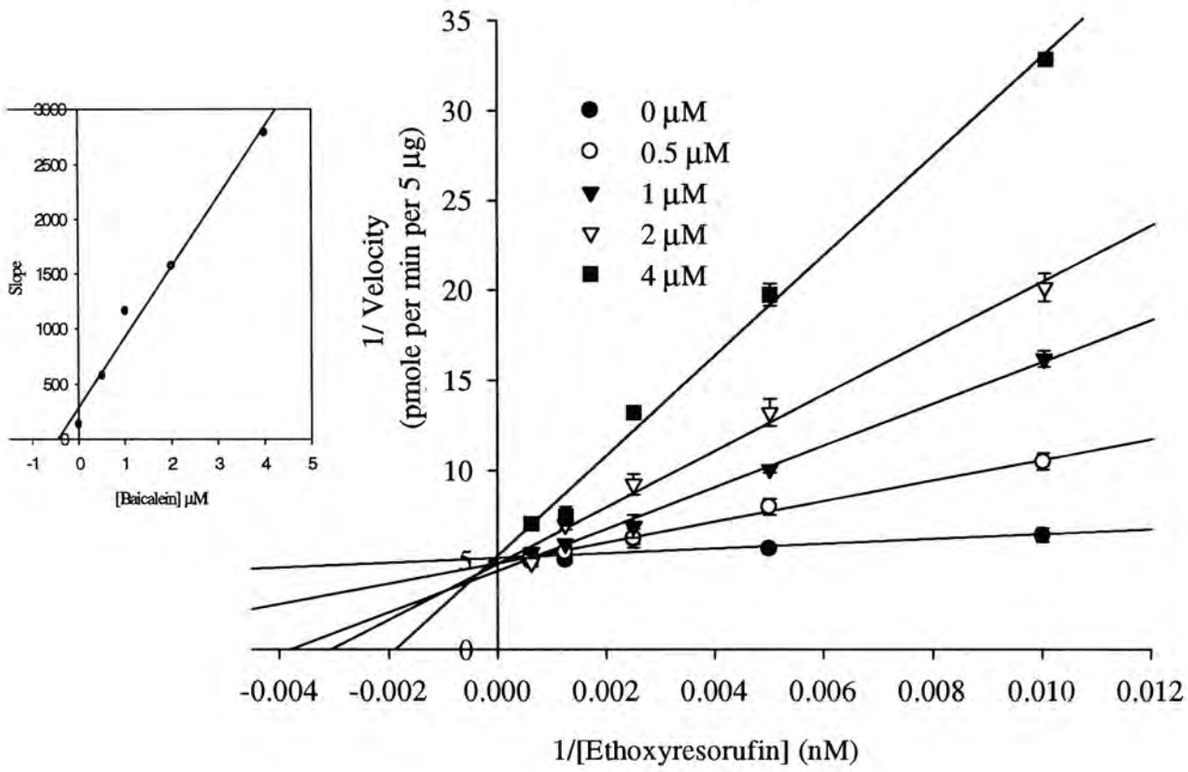


Figure 6.2 Lineweaver-Burk plot of baicalein on the EROD inhibition.

EROD activity in 5 µg of microsomes was measured in the presence of various concentrations of baicalein and 100-1600 nM ER and the double reciprocal plot was generated. The values are means ±SE, n=3. *Inset*, replot of the slopes obtained by linear regression of the data from the Lineweaver-Burk plot, with derivation of Ki, which was equal to 0.4 µM.

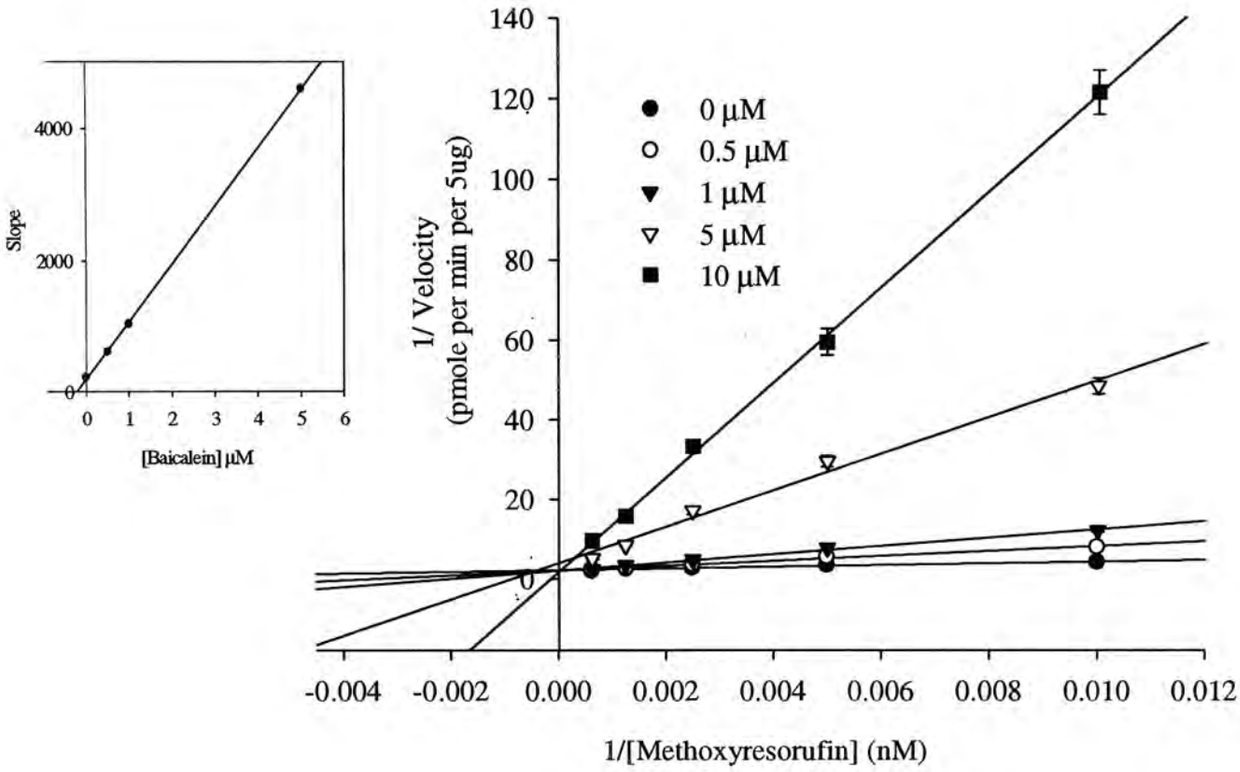


Figure 6.3 Lineweaver-Burk plot of baicalein on the MROD inhibition.

Lines shown here were determined by linear regression of the reciprocal data as described in figure 6.2. Ki value was equal to 0.2 µM.

Effects of theaflavins on EROD and MROD activities in rat liver microsomes

To elucidate the structural feature governing theaflavins interaction with CYP2C6 and CYP1A2 enzymes, the 4 theaflavins with various concentrations were assayed for their EROD and MROD activities in the presence of 400 nM of ER/MR. TF1 was the most potent inhibitor for CYP2C6, followed by TF2B, TF2A and TF3 (Figure 6.4A). All TFs showed a less potent inhibition for CYP1A2 compared with TF1 (Figure 6.4B). In figure 6.4 C, the IC_{50} values for EROD and MROD activities were plotted inversely in order to facilitate the comparison of their relative potencies. TF1 was the most potent CYP2C6 and 1A2 inhibitors and was approximately 2 fold greater than the other 3 theaflavins in rat liver microsomes

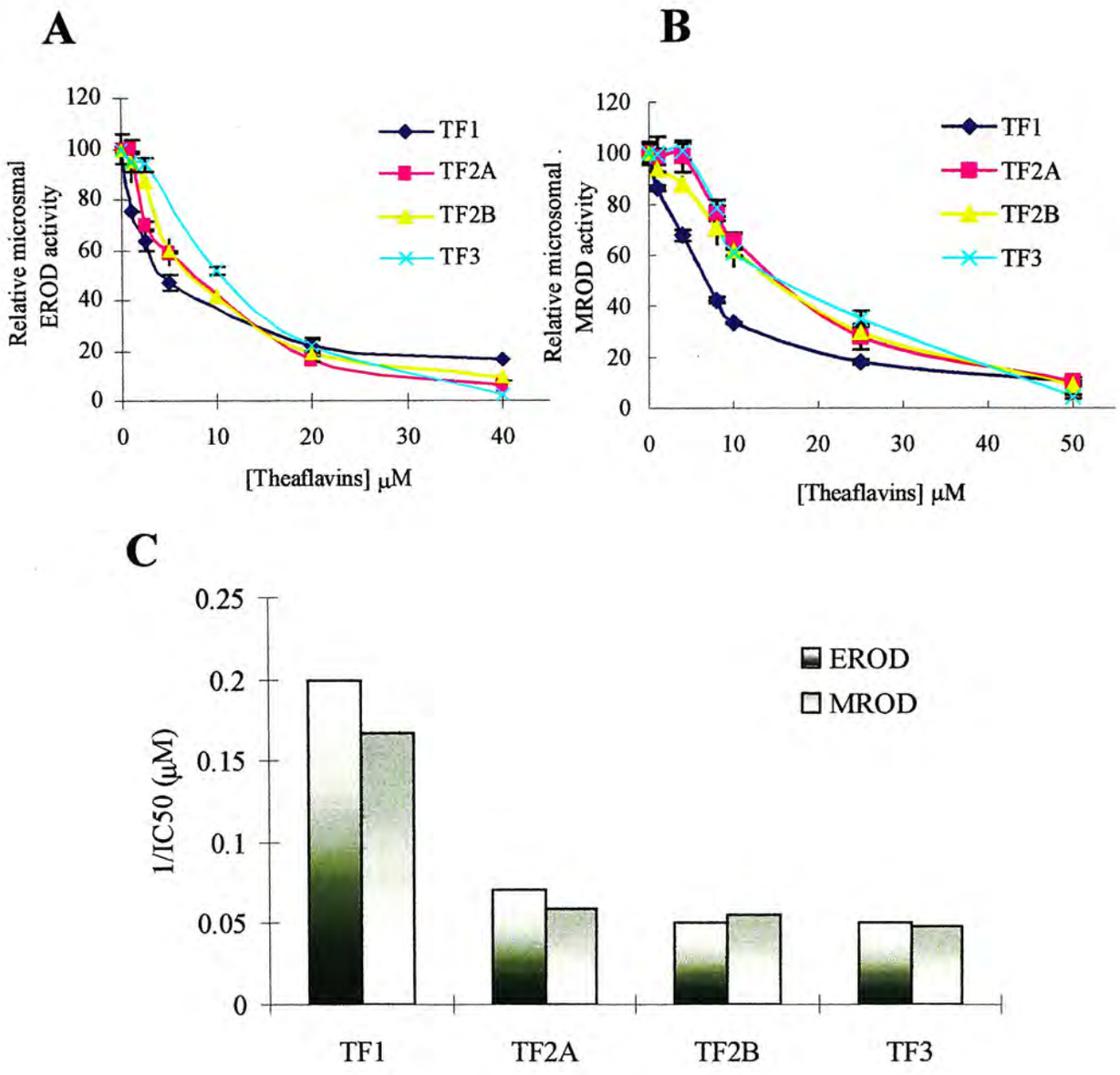


Figure 6.4 Direct effects of theaflavins on microsomal EROD and MROD activities.

The EROD (A) and MROD (B) activities in 5 μg of microsomes isolated from rat livers were measured in the presence of 400 nM ER/MR and the indicated concentration of theaflavins. The values are means ±SE, n=3. Figure 6.4C was the reciprocal plot of IC_{50} for EROD and MROD activities of theaflavins.

Kinetic studies for EROD and MROD activities of theaflavins

In addition, kinetic analyses of the EROD and MROD activities of rat liver microsomes were performed. With TF1, competitive inhibition was observed for CYP1A1 with a Ki value of 0.7 μM (Figure 6.5). Mixed-type inhibition of CYP1A2 by TF1 was observed (Figure 6.6). It indicated that this compounds could compete for substrate binding at the active site, and also might bind to a region that did not participate directly in substrate binding. For EROD activity, TF2A exhibited a competitive mode of inhibition (Figure 6.7). A 2-fold selectivity for CYP2C6 over mixed type inhibition of CYP1A2 (Figure 6.8) was observed by comparing their Ki value. The inhibition by TF2B on both EROD (Figure 6.9) and MROD (Figure 6.10) activities were mixed type with Ki value of 0.8 and 2 μM respectively. TF3 competitively inhibited EROD activities and the Ki value was as low as 0.8 μM (Figure 6.11). However, a large Ki value was observed in mixed type inhibition of CYP1A2-mediated MROD activity (Figure 6.12). Figure 6.13 illustrated the competitive Ki value for baicalein and theaflavins on EROD and MROD inhibition.

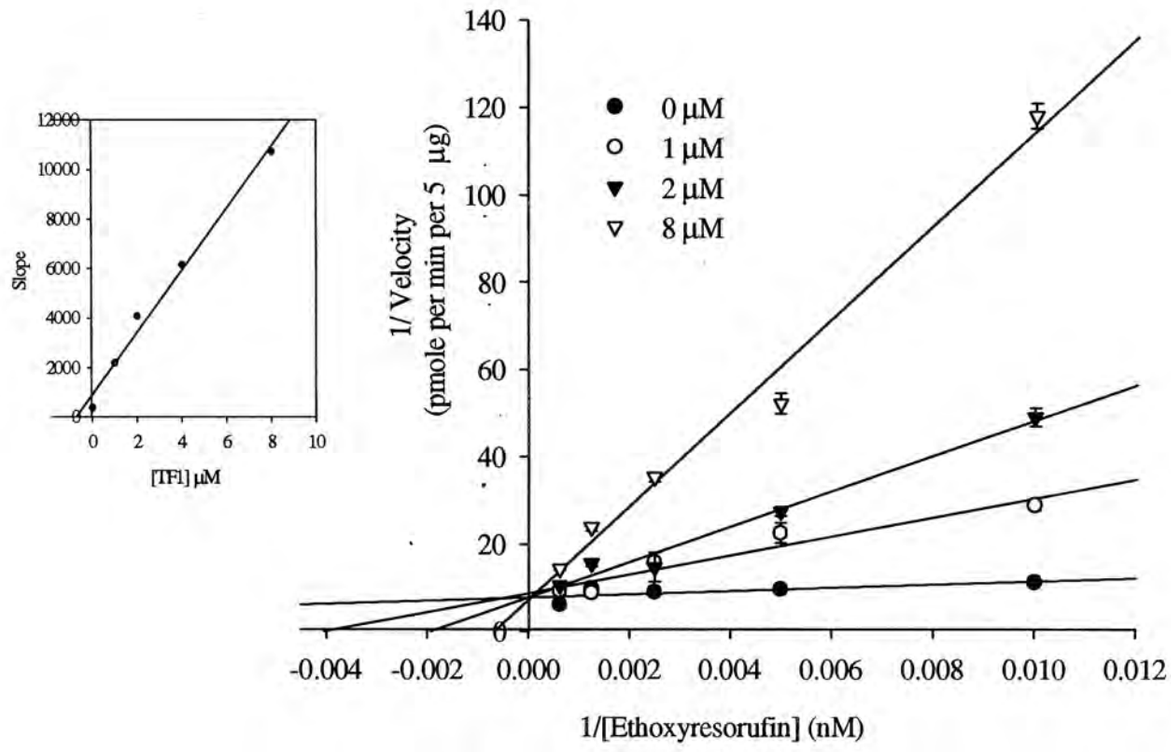


Figure 6.5 Lineweaver-Burk plot of TF1 on the EROD inhibition.

EROD activity in 5 µg of microsomes was measured in the presence of various concentrations of TF1 and 100-1600 nM ER and the double reciprocal plot was generated. The values are means ±SE, n=3. *Inset*, replot of the slopes obtained by linear regression of the data from the Lineweaver-Burk plot, with derivation of Ki, which was equal to 0.7 µM.

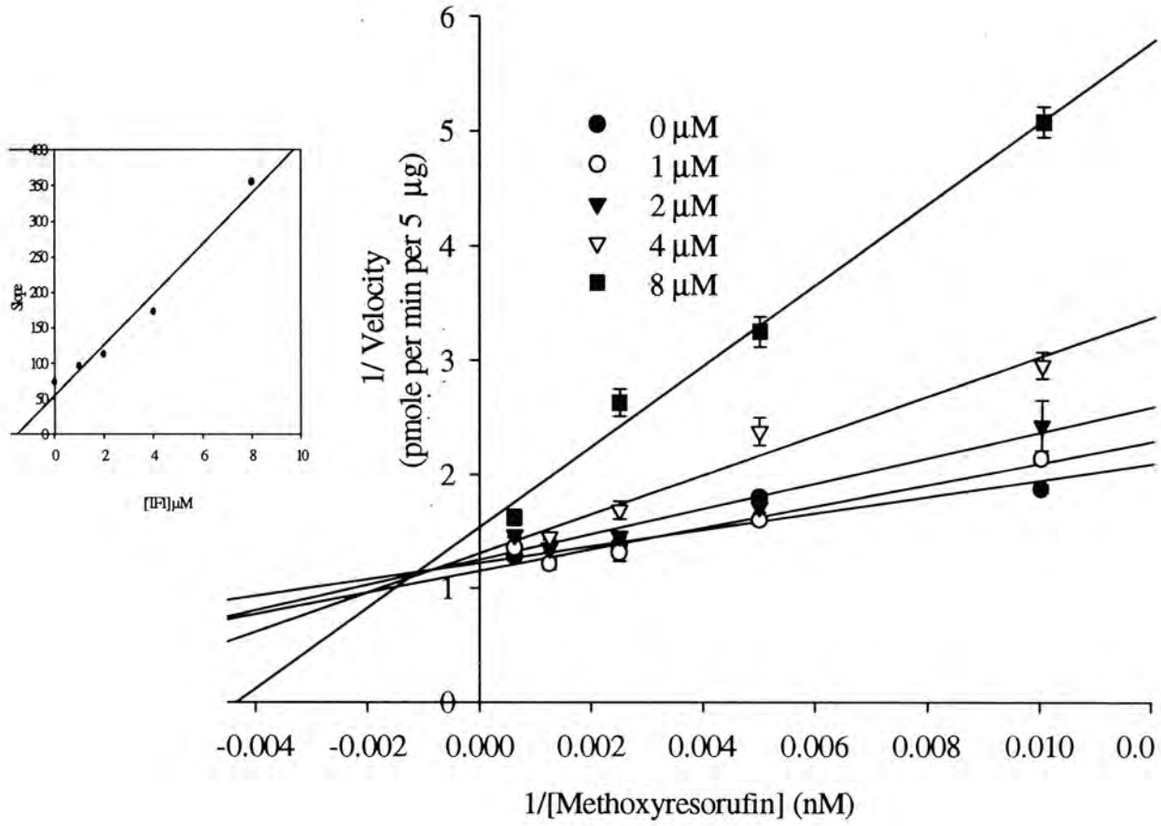


Figure 6.6 Lineweaver-Burk plot of TF1 on the MROD inhibition.

Lines shown here were determined by linear regression of the reciprocal data as described in figure 6.5 Ki value was equal to 1.5 µM.

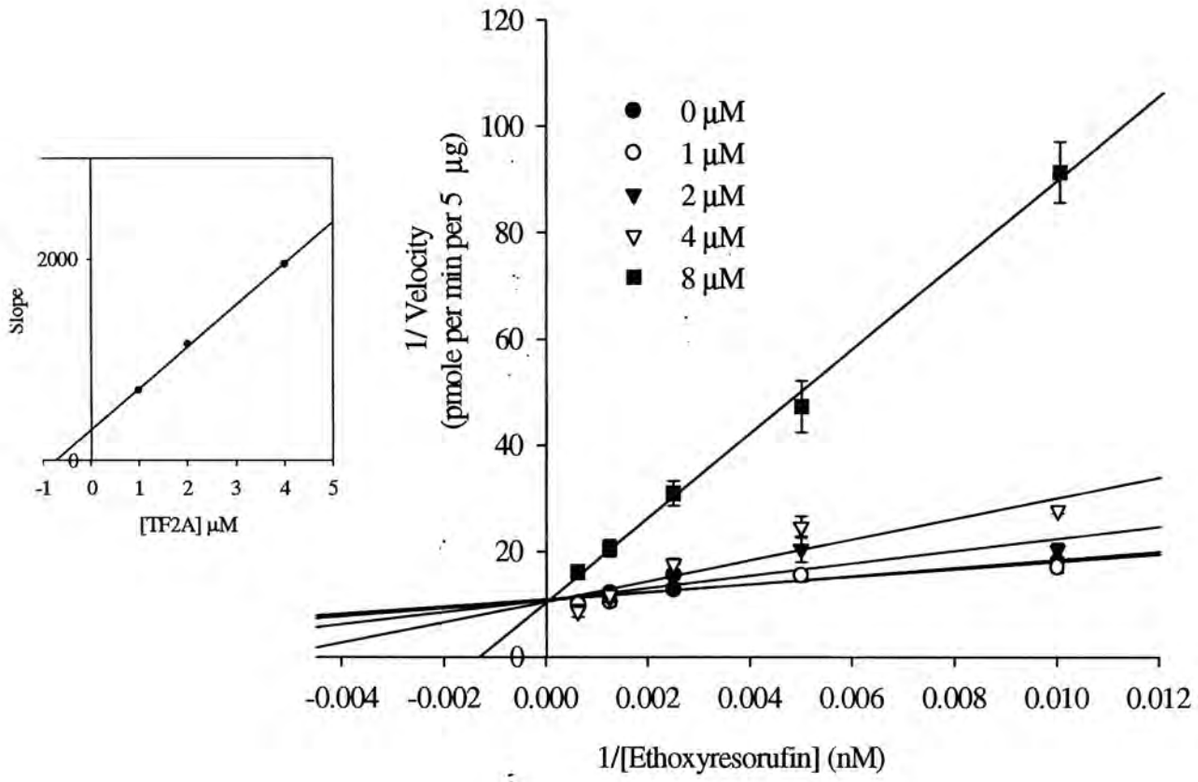


Figure 6.7 Lineweaver-Burk plot of TF2A on the EROD inhibition.

EROD activity in 5 µg of microsomes was measured in the presence of various concentrations of TF2A and 100-1600 nM ER and the double reciprocal plot was generated. The values are means ±SE, n=3. *Inset*, replot of the slopes obtained by linear regression of the data from the Lineweaver-Burk plot, with derivation of Ki, which was equal to 0.7 µM.

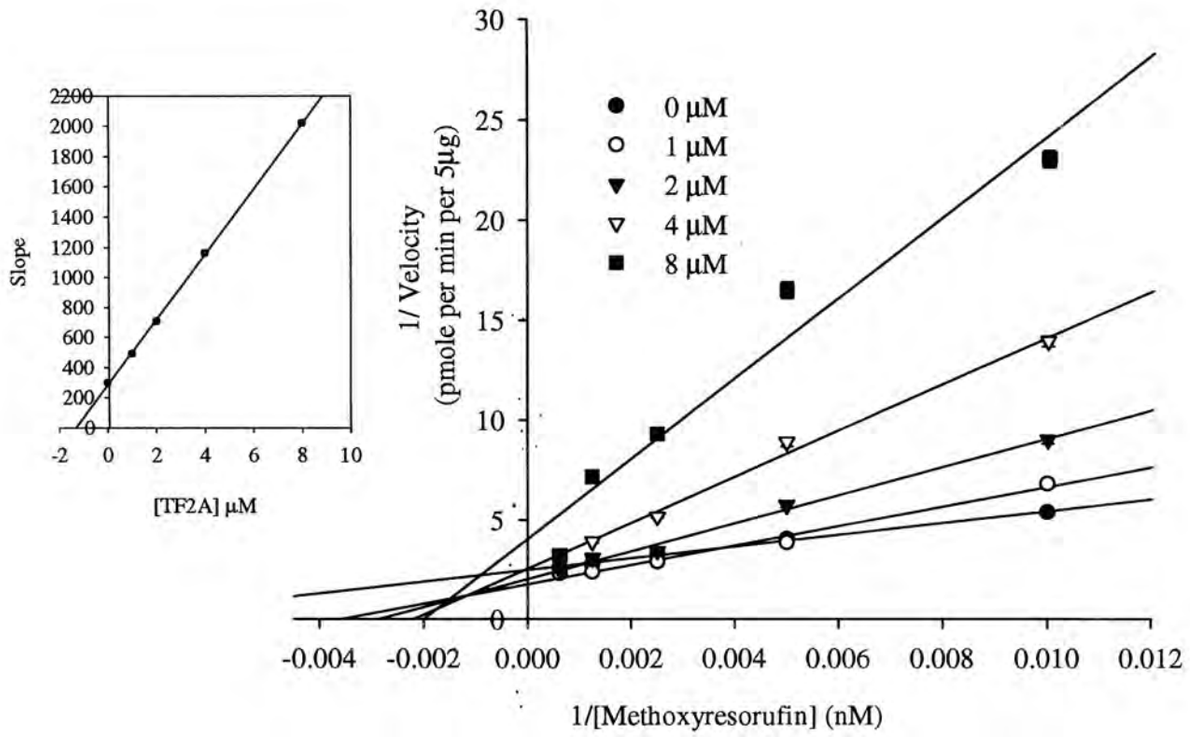


Figure 6.8 Lineweaver-Burk plot of TF2A on the MROD inhibition.

Lines shown here were determined by linear regression of the reciprocal data as described in figure 6.7. Ki value was equal to 1.3 µM.

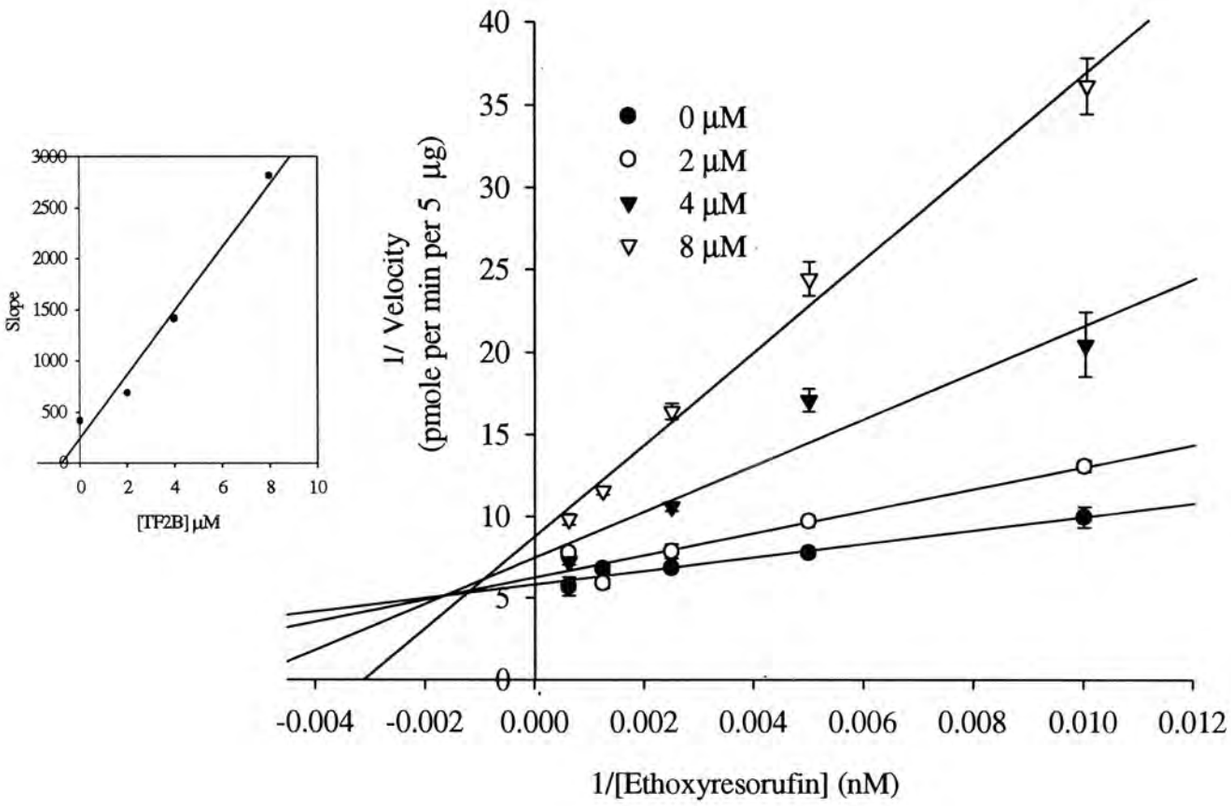


Figure 6.9 Lineweaver-Burk plot of TF2B on the EROD inhibition.

EROD activity in 5 µg of microsomes was measured in the presence of various concentrations of TF2B and 100-1600 nM ER and the double reciprocal plot was generated. The values are means ±SE, n=3. *Inset*, replot of the slopes obtained by linear regression of the data from the Lineweaver-Burk plot, with derivation of Ki, which was equal to 0.8 µM.

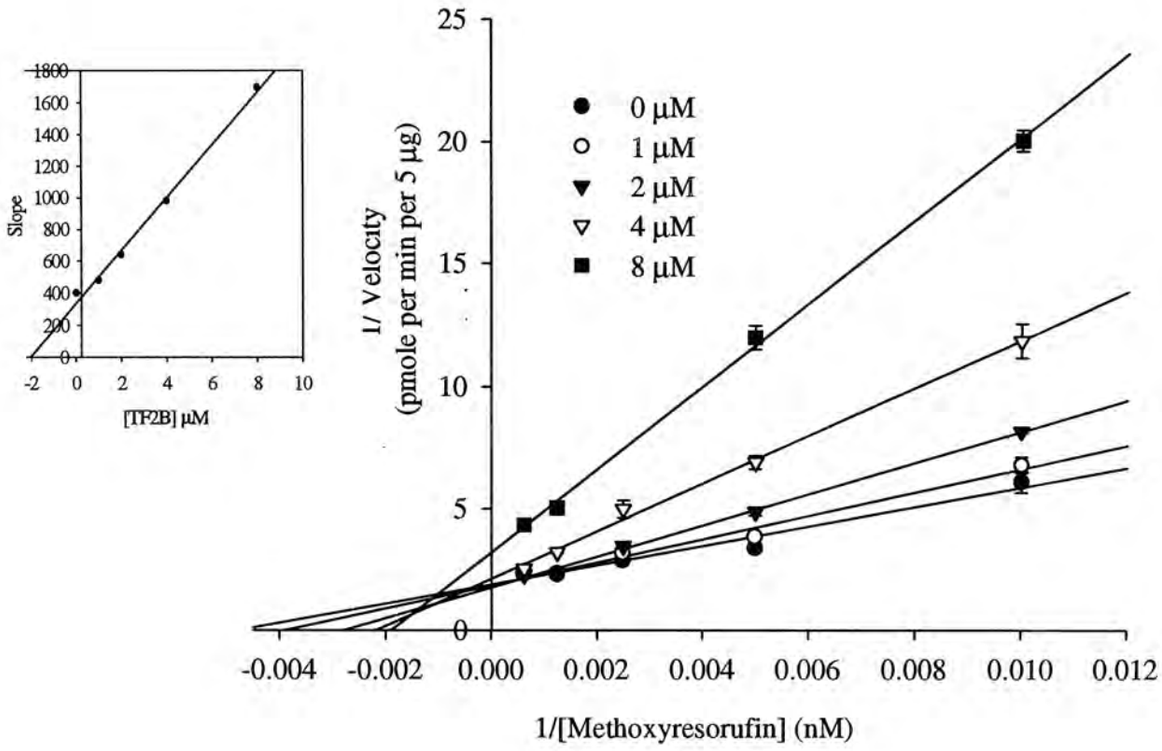


Figure 6.10 Lineweaver-Burk plot of TF2B on the MROD inhibition.

Lines shown here were determined by linear regression of the reciprocal data as described in figure 6.9. Ki value was equal to 2 µM.

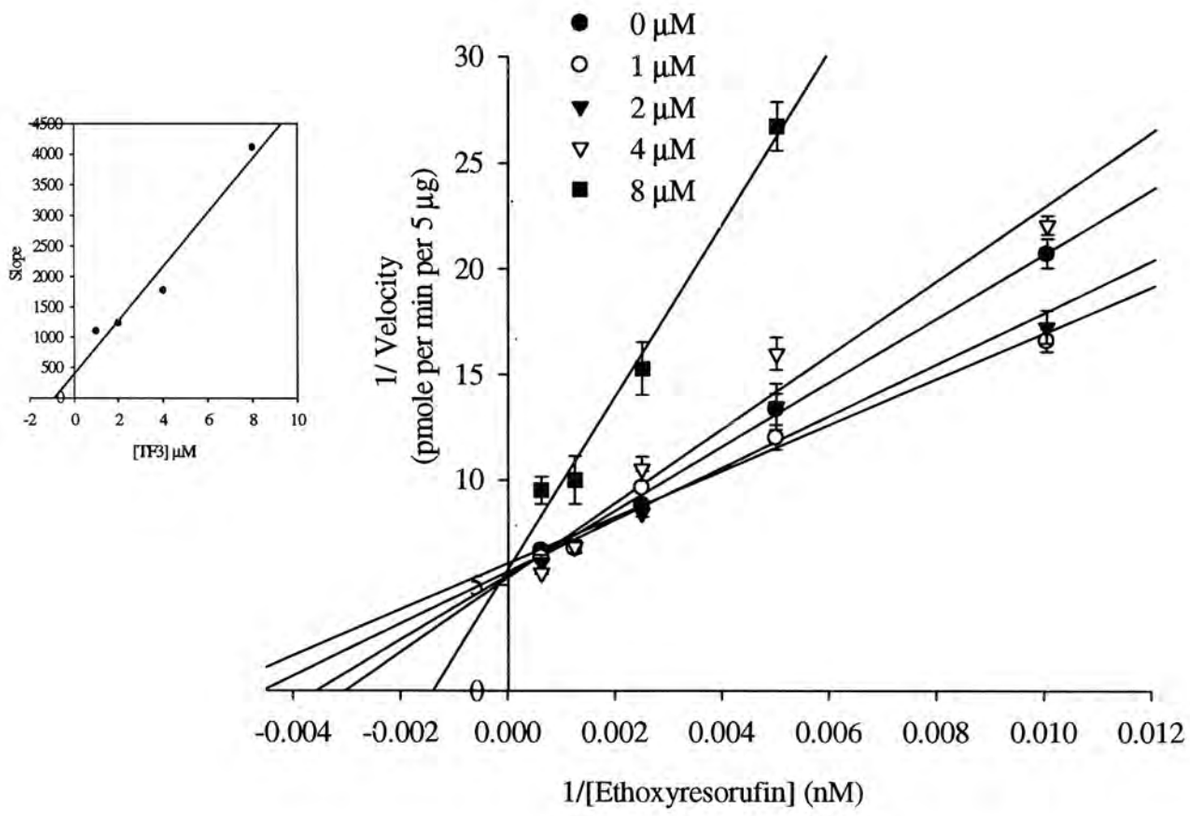


Figure 6.11 Lineweaver-Burk plot of TF3 on the EROD inhibition.

EROD activity in 5 µg of microsomes was measured in the presence of various concentrations of TF3 and 100-1600 nM ER and the double reciprocal plot was generated. The values are means ±SE, n=3. *Inset*, replot of the slopes obtained by linear regression of the data form the Lineweaver-Burk plot, with derivation of Ki, which was equal to 0.8 µM.

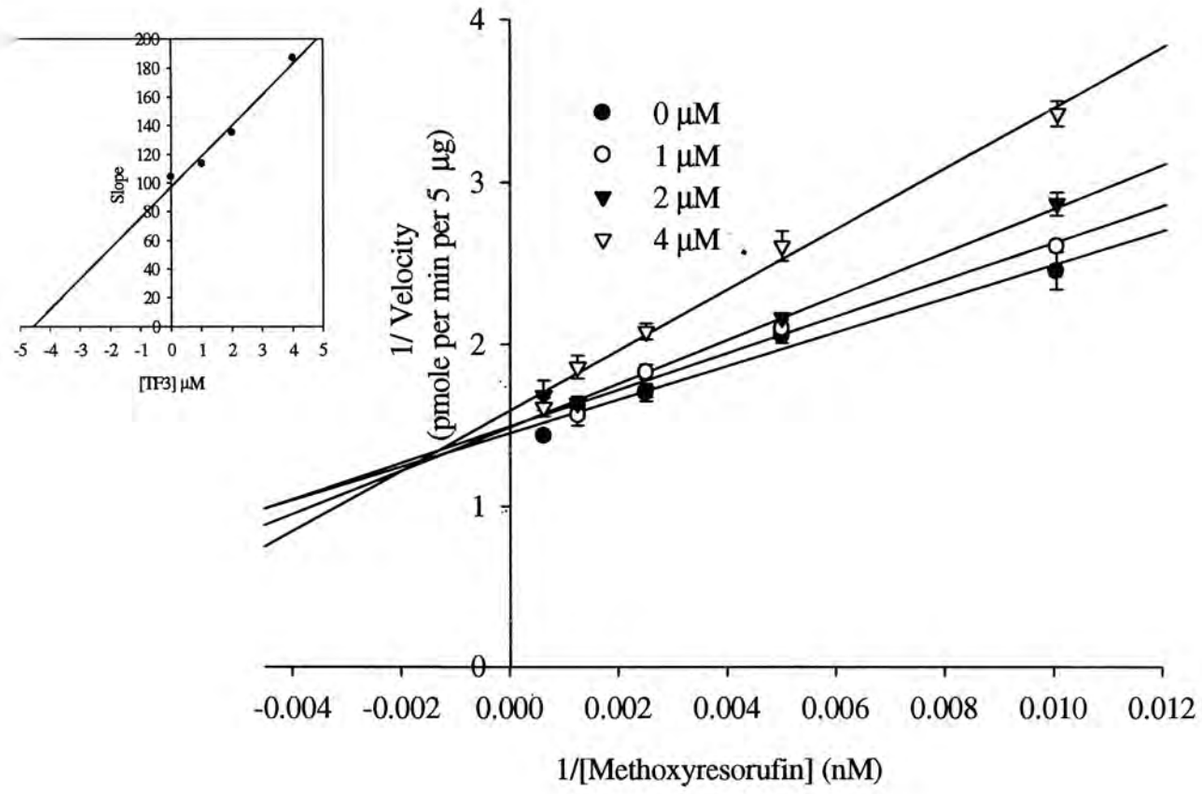


Figure 6.12 Lineweaver-Burk plot of TF3 on the MROD inhibition.

Lines shown here were determined by linear regression of the reciprocal data as described in figure 6.11. Ki value was equal to 4.5 µM.

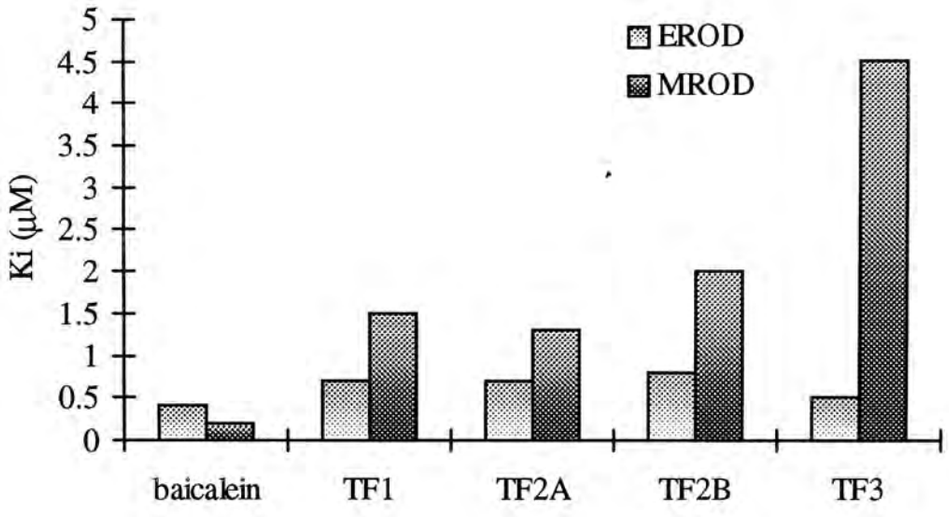


Figure 6.13 Competitive Ki value for baicalein and theaflavins on EROD and MROD inhibition.

This figure concluded the competitive Ki value for baicalein and theaflavins on EROD and MROD inhibition.

Discussion

The O-dealkylation of selected alkoxyresorufin is used in laboratory to determine the activity of isoform of cytochrome P450. In untreated rat liver microsomes, antibody inhibition studies showed that EROD is catalyzed mainly by CYP2C6 whereas MROD activity is catalysed mainly by constitutive CYP1A2 (Burke et al., 1994). Isoforms of cytochrome P450 metabolize both endogenous and exogenous compounds such as steroids, therapeutic drugs and chemical carcinogens. Modulation of cytochrome P450 by phytochemicals may render the activation/detoxification of theses substrates.

Baicalein and theaflavins are naturally occurring compounds that are suggested to be anticarcinogenic (Ikemoto et al., 2000; Isemura et al., 2000). Baicalein by itself induces both EROD and MROD activities in rats. Co-administration of baicalein and DMBA shows a reduction of CYP1 activities, which inhibits the mutagenicity of DMBA and possibly other PAHs in human breast cancer cells. In this study, baicalein exhibited the most potent competitive inhibition of EROD and MROD activities on CYP2C6 and CYP1A2 in a concentration-dependent manner.

Theaflavins are polyphenols in black tea that exert protective effect in DNA oxidative damage (Feng et al., 2002). They are reported to have antimutagenicity in rat liver S9 fraction and are suspected to inhibit cytochrome P450 enzymes (Apostolides et al., 1997). Our studies from chapter 4 showed that theaflavins inhibited CYP1 enzymes activities in vitro but not in vivo. They reduced DNA-damage in breast cancer cells by suppressing DMBA-DNA adduct formation. The present studies showed that the 4 theaflavins inhibited both EROD and MROD activities in rat microsomes. Among the 4 theaflavins, TF3 was the most potent CYP2C6 inhibitor while TF2A was the most potent CYP1A2 inhibitor. Furthermore,

all of them inhibited CYP2C6 to a greater extent than CYP1A2. TF3 displayed almost 5-fold selectivity for inhibiting CYP2C6 over CYP1A2. As mentioned above, CYP2C6 and CYP1A2 are responsible for procarcinogens metabolism whereas CYP1A2 also play an important role on drug metabolism. Accordingly, inhibition of CYP2C6 and CYP1A2 by baicalein and theaflavins may render their activities.

In summary, this study suggested that baicalein and theaflavins had various inhibitory effects upon CYP2C6 and CYP1A2 in vitro and therefore might affect the metabolism of their substrates.

CHAPTER 7 CONCLUSION

In this study, we investigated effects of baicalein, theaflavins, EGCG and isoflavones on CYP1 enzyme activities and DMBA-DNA adduct formation in a human breast cancer cell line MCF-7. The results indicated that all the above phytochemicals were capable to reduce DMBA genotoxicity in various extents. Baicalein, EGCG, biochanin A and genistein suppressed DMBA-induced CYP1 activities and expressions in MCF-7 cells, whereas theaflavins had no effects at this level.

CYP1 enzyme inhibition offers a possible basis for the inhibitory effects of certain phytochemicals against PAHs genotoxicity. However, Izzotti et al. (1999) suggest that the ideal detoxification agent should modulate both phase I and phase II pathways, resulting in the increased elimination of procarcinogens. Because of the complexity of the xenobiotic metabolizing system, thoughtful assessment for the activities of potential chemopreventive agents will definitely help characterizing their roles. On the other hand, it has been proposed that protection against one group of compounds may potentiate the toxic/ carcinogenic effects of another class of toxicants or *vice versa* (Sy et al., 2001). Thus, chemopreventive studies that research on agents manipulating xenobiotic metabolizing enzymes should be approached with caution.

In reality, it is difficult to evaluate the net effect of chemoprotective agents because exposure to many different environmental toxins and carcinogens occurs concurrently. Vakharia et al. (2001) show that metals could diminish PAH induced CYP1A1 activity in human hepatoma cells. In addition, it has been demonstrated that the metabolism of phytochemicals does play an important role on their chemopreventive bioavailability and bioactivity. Thus, one should never overlook the

importance of phytochemical metabolism. Actually, extensively studies have been carried out on the bioavailability of chemopreventive agents in different models (Fang et al., 2002; Peterson et al., 1998).

Our experiments herein evaluated the effects of baicalein, theaflavins, EGCG and isoflavones on PAH-induced CYP1 activities and DMBA genotoxicity in the human breast tumor cell line MCF-7. We showed that baicalein, EGCG, genistein and biochanin A reduced DMBA carcinogenicity by modulating CYP1 enzymes activities and DMBA-DNA adduct formation. Whether these compounds interact similarly with other xenobiotic metabolizing enzymes remain to be determined.

APPENDIX 1 PRIMER LISTS

Code	Sequence
CYP1A1 FOR	5'- TAGACACTGATCTGGCTGCAG -3'
CYP1A1 REV	5'- GGGAAGGCTCCATCAGCATC -3'
β-actin FOR	5'- GTGGGGCGCCCCAGGCACCA -3'
β-actin REV	5'- CTCCTTAATGTCACGCACGATTTC -3'
CYP1B1 FOR	5'- AACGTCATGAGTGCCGTGTGT -3'
CYP1B1 REV	5'- GGCCGGTACGTTCTCCAAATC -3'
XRE FOR	5'- CCGAGCATTGCACGAAACC -3'
XRE REV	5'- CATACTGAAGCAGGCGACAC -3'

APPENDIX 2 REAGENTS

Name	Components
10× dNTP solution	2 mM dATP, dCTP, dGTP, dTTP
10× PCR buffer	200 mM Tris-Cl pH8.4 at 23°C, 500 mM KCl, 25 mM $MgCl_2$
Cell lysis buffer (mammalian)	50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.2% v/v Nonidet P-40
LB medium	1 % w/v tryptone, 0.5 % w/v yeast extracts, 1 % w/v NaCl
LB plate	LB medium, 1.5 % w/v agar
LB^{amp} medium	LB medium, 100 µg/ ml ampicilin
LB^{amp} plate	LB medium, 1.5% w/v agar, 200 µg/ ml ampicilin
P1	50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 10% w/v RNaseA
P2	200 mM NaOH, 1% w/v SDS
P3	3 M KOAc, pH 5.5
PBS	0.8% w/v NaCl, 0.02% w/v KCl, 0.08% w/v $Na_2HPO_4 \bullet 2H_2O$, 0.02% w/v KH_2PO_4
Protease inhibitors	1 mM PMSF, 0.1% w/v leupeptin, 0.2% w/v aprotinin, 1% w/v soybean trypsin inhibitor, 1.5% w/v benzamidine, 1% w/v chymostatin, 1% w/v pepstatin
QBT	750 mM NaCl, 50 mM MOPS (pH 7.0), 15% v/v isopropanol, 0.15% v/v Triton X-100
QC	1 M NaCl, 50 mM MPOS (pH 7.0), 15% v/v isopropanol
QF	1.25 M NaCl, 50 mM Tris-Cl (pH 8.5), 15% v/v isopropanol
Sodium phosphate buffer	0.08 M monobasic sodium phosphate, 0.12 M dibasic sodium phosphate

TAE buffer	40 mM Tris-OAc (pH 8.0), 40 mM HOAc, 2 mM EDTA
TBE buffer	90 mM Tris-OAc (pH 8.0), 90 mM boric acid, 2 mM EDTA
TBST	10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.05% Tween-20
TE	10 mM Tris-Cl (pH 8.0), 1 mM EDTA

*The components of all homemade reagents are listed. For the commercially purchased reagents, please refer to the manufacturers' manual.

BIBLIOGRAPHY

Agarwal,R. Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents. *Biochem. Pharmacol.* **60**, 1051-1059 (2000).

Alexander,D.L., Eltom,S.E. & Jefcoate,C.R. Ah receptor regulation of CYP1B1 expression in primary mouse embryo-derived cells. *Cancer Res.* **57**, 4498-4506 (1997).

Apostolides,Z., Balentine,D.A., Harbowy,M.E., Hara,Y. & Weisburger,J.H. Inhibition of PhIP mutagenicity by catechins, and by theaflavins and gallate esters. *Mutat. Res.* **389**, 167-172 (1997).

Apostolides,Z., Balentine,D.A., Harbowy,M.E., Hara,Y. & Weisburger,J.H. Inhibition of PhIP mutagenicity by catechins, and by theaflavins and gallate esters. *Mutat. Res.* **389**, 167-172 (1997).

Appelt,L.C. & Reicks,M.M. Soy induces phase II enzymes but does not inhibit dimethylbenz[a]anthracene-induced carcinogenesis in female rats. *J. Nutr.* **129**, 1820-1826 (1999).

Backlund,M., Johansson,I., Mkrtchian,S. & Ingelman-Sundberg,M. Signal transduction-mediated activation of the aryl hydrocarbon receptor in rat hepatoma H4IIE cells. *J. Biol. Chem.* **272**, 31755-31763 (1997).

Barnes,S. Effect of genistein on in vitro and in vivo models of cancer. *J. Nutr.* **125**, 777S-783S (1995).

Birnbaum,L.S. Endocrine effects of prenatal exposure to PCBs, dioxins, and other xenobiotics: implications for policy and future research. *Environ. Health Perspect.* **102**, 676-679 (1994).

Birt,D.F., Hendrich,S. & Wang,W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol. Ther.* **90**, 157-177 (2001).

Boersma,B.J., Barnes,S., Kirk,M., Wang,C.C., Smith,M., Kim,H., Xu,J., Patel,R., Darley-Usmar,V.M. Soy isoflavonoids and cancer -- metabolism at the target site. *Mutat. Res.* **480-481**, 121-127 (2001).

Brosen,K. Drug interactions and the cytochrome P450 system. The role of cytochrome P450 1A2. *Clin. Pharmacokinet.* **29 Suppl 1**, 20-25 (1995).

Burke,M.D., Thompson,S., Weaver,R.J., Wolf,C.R. & Mayer,R.T. Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. *Biochem. Pharmacol.* **48**, 923-936 (1994).

Busby,M.G., Jeffcoat,A.R., Bloedon,L.T., Koch,M.A,. Black,T., Dix,K.J., Heizer,W.D., Thomas,B.F., Hill,J.M., Crowell,J.A., Zeisel,S.H. Clinical characteristics and pharmacokinetics of purified soy isoflavones: single-dose administration to healthy men. *Am. J. Clin. Nutr.* **75**, 126-136 (2002).

Buters,J.T., Sakai,S., Richter,T., Pineau,T., Alexander,D.L., Savas,U., Doehmer,J., Ward,J.M., Jefcoate,C.R., Gonzalez,F.J. Cytochrome P450 CYP1B1 determines susceptibility to 7, 12-dimethylbenz[a]anthracene-induced lymphomas. *Proc. Natl. Acad. Sci. U. S. A* **96**, 1977-1982 (1999).

Cassady,J.M., Zennie,T.M., Chae,Y.H., Ferin,M.A., Portuondo,N.E., Baird,W.M. Use of a mammalian cell culture benzo(a)pyrene metabolism assay for the detection of potential anticarcinogens from natural products: inhibition of metabolism by biochanin A, an isoflavone from Trifolium pratense L. *Cancer Res.* **48**, 6257-6261 (1988).

Chae,Y.H., Coffing,S.L., Cook,V.M., Ho,D.K., Cassady,J.M., Baird,W.M. Effects of biochanin A on metabolism, DNA binding and mutagenicity of benzo[a]pyrene in mammalian cell cultures. *Carcinogenesis* **12**, 2001-2006 (1991).

Chae,Y.H., Ho,D.K., Cassady,J.M., Cook,V.M., Marcus,C.B., Baird,W.M. Effects of synthetic and naturally occurring flavonoids on metabolic activation of benzo[a]pyrene in hamster embryo cell cultures. *Chem. Biol. Interact.* **82**, 181-193 (1992).

Chang,C.Y. & Puga,A. Constitutive activation of the aromatic hydrocarbon receptor. *Mol. Cell Biol.* **18**, 525-535 (1998).

Chen,S., Ruan,Q., Bedner,E., Deptala,A., Wang,X., Hsieh,T.C., Traganos,F., Darzynkiewicz,Z. Effects of the flavonoid baicalin and its metabolite baicalein on androgen receptor expression, cell cycle progression and apoptosis of prostate cancer cell lines. *Cell Prolif.* **34**, 293-304 (2001).

Chou,F.P., Chu,Y.C., Hsu,J.D., Chiang,H.C. & Wang,C.J. Specific induction of glutathione S-transferase GSTM2 subunit expression by epigallocatechin gallate in rat liver. *Biochem. Pharmacol.* **60**, 643-650 (2000).

Ciolino,H.P. & Yeh,G.C. Inhibition of aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and CYP1A1 expression by resveratrol. *Mol. Pharmacol.* **56**, 760-767 (1999).

Ciolino,H.P. & Yeh,G.C. The flavonoid galangin is an inhibitor of CYP1A1 activity and an agonist/antagonist of the aryl hydrocarbon receptor. *Br. J. Cancer* **79**, 1340-1346 (1999).

Ciolino,H.P., Daschner,P.J. & Yeh,G.C. Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem. J.* **340 (Pt 3)**, 715-722 (1999).

Ciolino,H.P., Daschner,P.J., Wang,T.T. & Yeh,G.C. Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. *Biochem. Pharmacol.* **56**, 197-206 (1998).

Conney,A.H., Chang,R.L., Jerina,D.M. & Wei,S.J. Studies on the metabolism of benzo[a]pyrene and dose-dependent differences in the mutagenic profile of its ultimate carcinogenic metabolite. *Drug Metab Rev.* **26**, 125-163 (1994).

Corchero,J., Pimprale,S., Kimura,S. & Gonzalez,F.J. Organization of the CYP1A cluster on human chromosome 15: implications for gene regulation. *Pharmacogenetics* **11**, 1-6 (2001).

Couture,L.A., Abbott,B.D. & Birnbaum,L.S. A critical review of the developmental toxicity and teratogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin: recent advances toward understanding the mechanism. *Teratology* **42**, 619-627 (1990).

Cribb,A.E., Spielberg,S.P. & Griffin,G.P. N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. *Drug Metab Dispos.* **23**, 406-414 (1995).

Dashwood,R.H., Xu,M., Hernaez,J.F., Hasaniya,N., Youn,K., Razzuk,A. Cancer chemopreventive mechanisms of tea against heterocyclic amine mutagens from cooked meat. *Proc. Soc. Exp. Biol. Med.* **220**, 239-243 (1999).

Dialyna,I.A., Arvanitis,D.A. & Spandidos,D.A. Genetic polymorphisms and transcriptional pattern analysis of CYP1A1, AhR, GSTM1, GSTP1 and GSTT1 genes in breast cancer. *Int. J. Mol. Med.* **8**, 79-87 (2001).

Dipple, A. *et al.* In *Diet and Cancer; Markers, Prevention and Treatment*; Jacobs M.M, Ed.; Plenum Press: New York, 1994; p 101.

Dipple,A., Pigott,M.A., Bigger,C.A., Blake,D.M. 7,12-dimethylbenz[a] anthracene--DNA binding in mouse skin: response of different mouse strains and effects of various modifiers of carcinogenesis. *Carcinogenesis.* **5**, 1087-90 (1984).

Dohr,O., Vogel,C. & Abel,J. Different response of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-sensitive genes in human breast cancer MCF-7 and MDA-MB 231 cells. *Arch. Biochem. Biophys.* **321**, 405-412 (1995).

Dbreosti,I.E., Wargovich,M.J. & Yang,C.S. Inhibition of carcinogenesis by tea: the evidence from experimental studies. *Crit Rev. Food Sci. Nutr.* **37**, 761-770 (1997).

Environmental Protection Agency (1990) Aerometric Information Retrieval System (AIRS), Data for 1985-1990. Environmental Protection Agency, Research Triangle Park, NC.

Fang,N., Yu,S. & Badger,T.M. Characterization of isoflavones and their conjugates in female rat urine using LC/MS/MS. *J. Agric. Food Chem.* **50**, 2700-2707 (2002).

Feng,Q., Torii,Y., Uchida,K., Nakamura,Y., Hara,Y., Osawa,T. Black tea polyphenols, theaflavins, prevent cellular DNA damage by inhibiting oxidative stress and suppressing cytochrome P450 1A1 in cell cultures. *J. Agric. Food Chem.* **50**, 213-220 (2002).

Fritz,W.A., Coward,L., Wang,J. & Lamartiniere,C.A. Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. *Carcinogenesis* **19**, 2151-2158 (1998).

Giri,A.K. & Lu,L.J. Genetic damage and the inhibition of 7,12-dimethylbenz[a]anthracene-induced genetic damage by the phytoestrogens, genistein and daidzein, in female ICR mice. *Cancer Lett.* **95**, 125-133 (1995).

Goldwyn,S., Lazinsky,A. & Wei,H. Promotion of health by soy isoflavones: efficacy, benefit and safety concerns. *Drug Metabol. Drug Interact.* **17**, 261-289 (2000).

Gonzalez,F.J. & Gelboin,H.V. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab Rev.* **26**, 165-183 (1994).

Gradin,K., Whitelaw,M.L., Toftgard,R., Poellinger,L. & Berghard,A. A tyrosine kinase-dependent pathway regulates ligand-dependent activation of the dioxin receptor in human keratinocytes. *J. Biol. Chem.* **269**, 23800-23807 (1994).

Guengerich,F.P. & Shimada,T. Activation of procarcinogens by human cytochrome P450 enzymes. *Mutat. Res.* **400**, 201-213 (1998).

Guengerich,F.P. Reactions and significance of cytochrome P-450 enzymes. *J. Biol. Chem.* **266**, 10019-10022 (1991).

Hammons,G.J. *et al.* Effects of chemoprotective agents on the metabolic activation of the carcinogenic arylamines PhIP and 4-aminobiphenyl in human and rat liver microsomes. *Nutr. Cancer* **33**, 46-52 (1999).

Hanna,I.H., Dawling,S., Roodi,N., Guengerich,F.P. & Parl,F.F. Cytochrome P450 1B1 (CYP1B1) pharmacogenetics: association of polymorphisms with functional differences in estrogen hydroxylation activity. *Cancer Res.* **60**, 3440-3444 (2000).

Helsby,N.A., Chipman,J.K., Gescher,A. & Kerr,D. Inhibition of mouse and human CYP 1A- and 2E1-dependent substrate metabolism by the isoflavonoids genistein and equol. *Food Chem. Toxicol.* **36**, 375-382 (1998).

Hemminki,K., Koskinen,M., Rajaniemi,H. & Zhao,C. Dna adducts, mutations, and cancer 2000. *Regul. Toxicol. Pharmacol.* **32**, 264-275 (2000).

Huang,H.C., Wang,H.R. & Hsieh,L.M. Antiproliferative effect of baicalein, a flavonoid from a Chinese herb, on vascular smooth muscle cell. *Eur. J. Pharmacol.* **251**, 91-93 (1994).

Huang,H.C., Wang,H.R. & Hsieh,L.M. Antiproliferative effect of baicalein, a flavonoid from a Chinese herb, on vascular smooth muscle cell. *Eur. J. Pharmacol.* **251**, 91-93 (1994).

Ikemoto,S., Sugimura,K., Yoshida,N., Yasumoto,R., Wada,S., Yamamoto,K., Kishimoto,T. Antitumor effects of Scutellariae radix and its components baicalein, baicalin, and wogonin on bladder cancer cell lines. *Urology* **55**, 951-955 (2000).

International Agency for Research on Cancer (1983) Polynuclear aromatic compounds, part I, chemical, environmental and experimental data. In IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Human, Vol.32, IARC, Lyon, p1-453.

Isemura,M., Saeki,K., Kimura,T., Hayakawa,S., Minami,T., Sazuka,M. Tea catechins and related polyphenols as anti-cancer agents. *Biofactors* **13**, 81-85 (2000).

Izzotti,A. Camoirano,A., Cartiglia,C., Grubbs,C.J., Lubet,R.A., Kelloff,G.J., De Flora,S. Patterns of DNA adduct formation in liver and mammary epithelial cells of rats treated with 7,12-dimethylbenz(a)anthracene, and selective effects of chemopreventive agents. *Cancer Res.* **59**, 4285-4290 (1999).

Jeffy,B.D. Schultz,E.U., Selmin,O., Gudas,J.M., Bowden,G.T., Romagnolo,D. Inhibition of BRCA-1 expression by benzo[a]pyrene and its diol epoxide. *Mol. Carcinog.* **26**, 100-118 (1999).

Karki,N.T., Pokela,R., Nuutinen,L. & Pelkonen,O. Aryl hydrocarbon hydroxylase in lymphocytes and lung tissue from lung cancer patients and controls. *Int. J. Cancer* **39**, 565-570 (1987).

Katiyar,S.K. & Mukhtar,H. Tea antioxidants in cancer chemoprevention. *J. Cell Biochem. Suppl* **27**, 59-67 (1997).

Katiyar,S.K., Agarwal,R., Wang,Z.Y., Bhatia,A.K. & Mukhtar,H. (-)-Epigallocatechin-3-gallate in Camellia sinensis leaves from Himalayan region of Sikkim: inhibitory effects against biochemical events and tumor initiation in Sencar mouse skin. *Nutr. Cancer* **18**, 73-83 (1992).

Katiyar,S.K., Agarwal,R., Zaim,M.T. & Mukhtar,H. Protection against N-nitrosodiethylamine and benzo[a]pyrene-induced forestomach and lung tumorigenesis in A/J mice by green tea. *Carcinogenesis* **14**, 849-855 (1993).

Kavanagh,K.T., Hafer,L.J., Kim,D.W., Mann,K.K., Sherr,D.H., Rogers,A.E., Sonenshein,G.E. Green tea extracts decrease carcinogen-induced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture. *J. Cell Biochem.* **82**, 387-398 (2001).

Kawajiri,K., Nakachi,K., Imai,K., Watanabe,J. & Hayashi,S. The CYP1A1 gene and cancer susceptibility. *Crit Rev. Oncol. Hematol.* **14**, 77-87 (1993).

Kikuchi,H. & Hossain,A. Signal transduction-mediated CYP1A1 induction by omeprazole in human HepG2 cells. *Exp. Toxicol. Pathol.* **51**, 342-346 (1999).

Kim,J.H., Stansbury,K.H., Walker,N.J., Trush,M.A., Strickland,P.T., Sutter,T.R. Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1. *Carcinogenesis* **19**, 1847-1853 (1998).

Kim,J.Y., Lee,S., Kim,D.H., Kim,B.R., Park,R., Lee,B.M. Effects of flavonoids isolated from Scutellariae radix on cytochrome P-450 activities in human liver microsomes. *J. Toxicol. Environ. Health A* **65**, 373-381 (2002).

Kishida,T., Nashiki,K., Izumi,T. & Ebihara,K. Soy isoflavonoid aglycons genistein and daidzein Do not increase the cytochrome P-450 content of the liver microsomes of mice. *J. Agric. Food Chem.* **48**, 3872-3875 (2000).

Kleiner,H.E., Vulimiri,S.V., Reed,M.J., Uberecken,A. & DiGiovanni,J. Role of cytochrome P450 1a1 and 1b1 in the metabolic activation of 7,12-dimethylbenz[a]anthracene and the effects of naturally occurring furanocoumarins on skin tumor initiation. *Chem. Res. Toxicol.* **15**, 226-235 (2002).

Kriek,E., Rojas,M., Alexandrov,K. & Bartsch,H. Polycyclic aromatic hydrocarbon-DNA adducts in humans: relevance as biomarkers for exposure and cancer risk. *Mutat. Res.* **400**, 215-231 (1998).

Kronenberg,S., Esser,C. & Carlberg,C. An aryl hydrocarbon receptor conformation acts as the functional core of nuclear dioxin signaling. *Nucleic Acids Res.* **28**, 2286-2291 (2000).

Kuntz,S., Wenzel,U. & Daniel,H. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. *Eur. J. Nutr.* **38**, 133-142 (1999).

Lamartiniere,C.A., Moore,J.B., Brown,N.M., Thompson,R., Hardin,M.J., Barnes,S. Genistein suppresses mammary cancer in rats. *Carcinogenesis* **16**, 2833-2840 (1995).

Landi,M.T., Sinha,R., Lang,N.P. & Kadlubar,F.F. Chapter 16. Human cytochrome P4501A2. *IARC Sci. Publ.* 173-195 (1999).

Larsen,M.C., Angus,W.G., Brake,P.B., Eltom,S.E., Sukow,K.A., Jefcoate,C.R. Characterization of CYP1B1 and CYP1A1 expression in human mammary epithelial cells: role of the aryl hydrocarbon receptor in polycyclic aromatic hydrocarbon metabolism. *Cancer Res.* **58**, 2366-2374 (1998).

Lee,B.M., Jang,J.J. & Kim,H.S. Benzo[a]pyrene diol-epoxide-I-DNA and oxidative DNA adducts associated with gastric adenocarcinoma. *Cancer Lett.* **125**, 61-68 (1998).

Lee,H., Wang,H.W., Su,H.Y. & Hao,N.J. The structure-activity relationships of flavonoids as inhibitors of cytochrome P-450 enzymes in rat liver microsomes and the mutagenicity of 2-amino-3-methyl-imidazo[4,5-f]quinoline. *Mutagenesis* **9**, 101-106 (1994).

Leung,L.K., Su,Y., Chen,R., Zhang,Z., Huang,Y., Chen,Z.Y. Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J. Nutr.* **131**, 2248-2251 (2001).

Levine,M., Law,E.Y., Bandiera,S.M., Chang,T.K. & Bellward,G.D. In vivo cimetidine inhibits hepatic CYP2C6 and CYP2C11 but not CYP1A1 in adult male rats. *J. Pharmacol. Exp. Ther.* **284**, 493-499 (1998).

Li,D., Firozi,P.F., Wang,L.E., Bosken,C.H., Spitz,M.R., Hong,W.K., Wei,Q. Sensitivity to DNA damage induced by benzo(a)pyrene diol epoxide and risk of lung cancer: a case-control analysis. *Cancer Res.* **61**, 1445-1450 (2001).

Li,D., Wang,M., Dhingra,K. & Hittelman,W.N. Aromatic DNA adducts in adjacent tissues of breast cancer patients: clues to breast cancer etiology. *Cancer Res.* **56**, 287-293 (1996).

Liang,Y.C., Lin-shiau,S.Y., Chen,C.F. & Lin,J.K. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J. Cell Biochem.* **67**, 55-65 (1997).

Lin,C.C. & Shieh,D.E. The anti-inflammatory activity of Scutellaria rivularis extracts and its active components, baicalin, baicalein and wogonin. *Am. J. Chin Med.* **24**, 31-36 (1996).

Lusska,A., Wu,L. & Whitlock,J.P., Jr. Superinduction of CYP1A1 transcription by cycloheximide. Role of the DNA binding site for the liganded Ah receptor. *J. Biol. Chem.* **267**, 15146-15151 (1992).

MacDonald,C.J., Ciolino,H.P. & Yeh,G.C. Dibenzoylmethane modulates aryl hydrocarbon receptor function and expression of cytochromes P50 1A1, 1A2, and 1B1. *Cancer Res.* **61**, 3919-3924 (2001).

Matsukawa,Y., Marui,N., Sakai,T., Satomi,Y., Yoshida,M., Matsumoto,K., Nishino,H., Aoike,A. Genistein arrests cell cycle progression at G2-M. *Cancer Res.* **53**, 1328-1331 (1993).

Mosmann,T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63 (1983).

Motoo,Y. & Sawabu,N. Antitumor effects of saikosaponins, baicalin and baicalein on human hepatoma cell lines. *Cancer Lett.* **86**, 91-95 (1994).

Mukhtar,H. & Ahmad,N. Green tea in chemoprevention of cancer. *Toxicol. Sci.* **52**, 111-117 (1999).

Murray,G.I., Taylor,M.C., McFadyen,M.C., McKay,J.A., Greenlee,W.F., Burke,M.D., Melvin,W.T. Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Res.* **57**, 3026-3031 (1997).

Naik,H.R., Lehr,J.E. & Pienta,K.J. An in vitro and in vivo study of antitumor effects of genistein on hormone refractory prostate cancer. *Anticancer Res.* **14**, 2617-2619 (1994).

Nakahata,N., Kutsuwa,M., Kyo,R., Kubo,M., Hayashi,K., Ohizumi,Y. Analysis of inhibitory effects of scutellariae radix and baicalein on prostaglandin E2 production in rat C6 glioma cells. *Am. J. Chin Med.* **26**, 311-323 (1998).

Nebert,D.W., Puga,A. & Vasiliou,V. Role of the Ah receptor and the dioxin-inducible [Ah] gene battery in toxicity, cancer, and signal transduction. *Ann. N. Y. Acad. Sci.* **685**, 624-640 (1993).

Oetari,S., Sudibyo,M., Commandeur,J.N., Samhoedi,R. & Vermeulen,N.P. Effects of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver. *Biochem. Pharmacol.* **51**, 39-45 (1996).

Ohishi,T., Kishimoto,Y., Miura,N., Shiota,G., Kohri,T., Hara,Y., Hasegawa,J., Isemura,M. Synergistic effects of (-)-epigallocatechin gallate with sulindac against colon carcinogenesis of rats treated with azoxymethane. *Cancer Lett.* **177**, 49-56 (2002).

Peterson,T.G., Coward,L., Kirk,M., Falany,C.N., Barnes,S. The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A. *Carcinogenesis.* **17**, 1861-1869 (1996).

Peterson,T.G. Ji,G.P., Kirk,M., Coward,L., Falany,C.N., Barnes,S. Metabolism of the isoflavones genistein and biochanin A in human breast cancer cell lines. *Am. J. Clin. Nutr.* **68**, 1505S-1511S (1998).

Pollard,M. & Wolter,W. Prevention of spontaneous prostate-related cancer in Lobund-Wistar rats by a soy protein isolate/isoflavone diet. *Prostate* **45**, 101-105 (2000).

Ren,M.Q., Kuhn,G., Wegner,J. & Chen,J. Isoflavones, substances with multi-biological and clinical properties. *Eur. J. Nutr.* **40**, 135-146 (2001).

Roberts,E.A., Furuya,K.N., Tang,B.K. & Kalow,W. Caffeine biotransformation in human hepatocyte lines derived from normal liver tissue. *Biochem. Biophys. Res. Commun.* **201**, 559-566 (1994).

Rosenberg Zand,R.S., Jenkins,D.J., Brown,T.J. & Diamandis,E.P. Flavonoids can block PSA production by breast and prostate cancer cell lines. *Clin. Chim. Acta* **317**, 17-26 (2002).

Rundle,A., Tang,D., Hibshoosh,H., Estabrook,A., Schnabel,F., Cao,W., Grumet,S., Perera,F.P. The relationship between genetic damage from polycyclic aromatic hydrocarbons in breast tissue and breast cancer. *Carcinogenesis* **21**, 1281-1289 (2000).

Sazuka,M., Imazawa,H., Shoji,Y., Mita,T., Hara,Y., Isemura,M. Inhibition of collagenases from mouse lung carcinoma cells by green tea catechins and black tea theaflavins. *Biosci. Biotechnol. Biochem.* **61**, 1504-1506 (1997).

Schut,H.A. & Yao,R. Tea as a potential chemopreventive agent in PhIP carcinogenesis: effects of green tea and black tea on PhIP-DNA adduct formation in female F-344 rats. *Nutr. Cancer* **36**, 52-58 (2000).

Schwarz,D., Kisselev,P., Cascorbi,I., Schunck,W.H. & Roots,I. Differential metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-dihydrodiol by human CYP1A1 variants. *Carcinogenesis* **22**, 453-459 (2001).

Setchell,K.D., Borriello,S.P., Hulme,P., Kirk,D.N. & Axelson,M. Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. *Am. J. Clin. Nutr.* **40**, 569-578 (1984).

Shapiro,G.I., Koestner,D.A., Matranga,C.B. & Rollins,B.J. Flavopiridol induces cell cycle arrest and p53-independent apoptosis in non-small cell lung cancer cell lines. *Clin. Cancer Res.* **5**, 2925-2938 (1999).

Shertzer,H.G. *et al.* Inhibition of CYP1A1 enzyme activity in mouse hepatoma cell culture by soybean isoflavones. *Chem. Biol. Interact.* **123**, 31-49 (1999).

Shimada,T., Puga,A., Chang,C., Smith,P., Nebert,D.W., Setchell,K.D., Dalton,T.P. Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. *Cancer Res.* **56**, 2979-2984 (1996).

Shimizu,Y., Nakatsuru,Y., Ichinose,M., Takahashi,Y., Kume,H., Mimura,J., Fujii-Kuriyama,Y., Ishikawa,T. Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci. U. S. A* **97**, 779-782 (2000).

Shou,M., Gonzalez,F.J. & Gelboin,H.V. Stereoselective epoxidation and hydration at the K-region of polycyclic aromatic hydrocarbons by cDNA-expressed cytochromes P450 1A1, 1A2, and epoxide hydrolase. *Biochemistry* **35**, 15807-15813 (1996).

Smith TJ; Yang CS In *Food Phytochemicals for Cancer Prevention I Fruits and Vegetables;* Huang MT; Osawa T; Ho CT; Rosen RT, Ed.; American Chemical Society: Washington, DC, 1994; p 17.

Smith,L.E., Denissenko,M.F., Bennett,W.P., Li,H., Amin,S., Tang,M., Pfeifer,G.P. Targeting of lung cancer mutational hotspots by polycyclic aromatic hydrocarbons. *J. Natl. Cancer Inst.* **92**, 803-811 (2000).

Smith,W.A., Freeman,J.W. & Gupta,R.C. Effect of chemopreventive agents on DNA adduction induced by the potent mammary carcinogen dibenzo[a,l]pyrene in the human breast cells MCF-7. *Mutat. Res.* **480-481**, 97-108 (2001).

Spink,B.C., Fasco,M.J., Gierthy,J.F. & Spink,D.C. 12-O-tetradecanoylphorbol-13-acetate upregulates the Ah receptor and differentially alters CYP1B1 and CYP1A1 expression in MCF-7 breast cancer cells. *J. Cell Biochem.* **70**, 289-296 (1998).

Steele,V.E. Kelloff,G.J., Balentine,D., Boone,C.W., Mehta,R., Bagheri,D., Sigman,C.C., Zhu,S., Sharma,S. Comparative chemopreventive mechanisms of green tea, black tea and selected polyphenol extracts measured by in vitro bioassays. *Carcinogenesis* **21**, 63-67 (2000).

Stoner,G.D. & Mukhtar,H. Polyphenols as cancer chemopreventive agents. *J. Cell Biochem. Suppl* **22**, 169-180 (1995).

Suganuma,M., Okabe,S., Sueoka,N., Sueoka,E., Matsuyama,S., Imai,K., Nakachi,K., Fujiki,H. Green tea and cancer chemoprevention. *Mutat. Res.* **428**, 339-344 (1999).

Suganuma,M., Okabe,S., Kai,Y., Sueoka,N., Sueoka,E., Fujiki,H. Synergistic effects of (--)-epigallocatechin gallate with (--)-epicatechin, sulindac, or tamoxifen on cancer-preventive activity in the human lung cancer cell line PC-9. *Cancer Res.* **59**, 44-47 (1999).

Sun,X.Y., Plouzek,C.A., Henry,J.P., Wang,T.T. & Phang,J.M. Increased UDP-glucuronosyltransferase activity and decreased prostate specific antigen production by biochanin A in prostate cancer cells. *Cancer Res.* **58**, 2379-2384 (1998).

Sutter,T.R., Tang,Y.M., Hayes,C.L., Wo,Y.Y., Jabs,E.W., Li,X., Yin,H., Cody,C.W., Greenlee,W.F. Complete cDNA sequence of a human dioxin-inducible mRNA identifies a new gene subfamily of cytochrome P450 that maps to chromosome 2. *J. Biol. Chem.* **269**, 13092-13099 (1994).

Sy,S.K., Tang,B.K., Pastrakuljic,A., Roberts,E.A. & Kalow,W. Detailed characterization of experimentally derived human hepatic CYP1A1 activity and expression using differential inhibition of ethoxyresorufin O-deethylation by fluvoxamine. *Eur. J. Clin. Pharmacol.* **57**, 377-386 (2001).

Tang,Y.M., Wo,Y.Y., Stewart,J., Hawkins,A.L., Griffin,C.A., Sutter,T.R., Greenlee,W.F. Isolation and characterization of the human cytochrome P450 CYP1B1 gene. *J. Biol. Chem.* **271**, 28324-28330 (1996).

Taylor,M.C., McKay,J.A., Murray,G.I., Greenlee,W.F., Marcus,C.B., Burke,M.D., Melvin,W.T. Cytochrome P450 1B1 expression in human malignant tumours. *Biochem. Soc. Trans.* **24**, 328S (1996).

Tian,Y., Ke,S., Denison,M.S., Rabson,A.B. & Gallo,M.A. Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. *J. Biol. Chem.* **274**, 510-515 (1999).

Trombino,A.F., Near,R.I., Matulka,R.A., Yang,S., Hafer,L.J., Toselli,P.A., Kim,D.W., Rogers,A.E., Sonenshein,G.E., Sherr,D.H. Expression of the aryl hydrocarbon receptor/transcription factor (AhR) and AhR-regulated CYP1 gene transcripts in a rat model of mammary tumorigenesis. *Breast Cancer Res. Treat.* **63**, 117-131 (2000).

Ueng,Y.F., Shyu,C.C., Lin,Y.L., Park,S.S., Liao,J.F., Chen,C.F. Effects of baicalein and wogonin on drug-metabolizing enzymes in C57BL/6J mice. *Life Sci.* **67**, 2189-2200 (2000).

Ueng,Y.F., Shyu,C.C., Liu,T.Y., Oda,Y., Lin,Y.L., Liao,J.F., Chen,C.F. Protective effects of baicalein and wogonin against benzo[a]pyrene- and aflatoxin B(1)-induced genotoxicities. *Biochem. Pharmacol.* **62**, 1653-1660 (2001).

Upadhyaya,P. & El Bayoumy,K. Effect of dietary soy protein isolate, genistein, and 1,4-phenylenebis(methylene)selenocyanate on DNA binding of 7,12-dimethylbenz[a]anthracene in mammary glands of CD rats. *Oncol. Rep.* **5**, 1541-1545 (1998).

Vadlamuri,S.V., Glover,D.D., Turner,T. & Sarkar,M.A. Regiospecific expression of cytochrome P4501A1 and 1B1 in human uterine tissue. *Cancer Lett.* **122**, 143-150 (1998).

Vakharia,D.D., Liu,N., Pause,R., Fasco,M., Bessette,E., Zhang,Q.Y., Kaminsky,L.S. Polycyclic aromatic hydrocarbon/metal mixtures: effect on PAH induction of CYP1A1 in human HEPG2 cells. *Drug Metab Dispos.* **29**, 999-1006 (2001).

Wang,T.T. & Phang,J.M. Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. *Cancer Res.* **55**, 2487-2489 (1995).

Wang,W., Liu,L.Q., Higuchi,C.M. & Chen,H. Induction of NADPH:quinone reductase by dietary phytoestrogens in colonic Colo205 cells. *Biochem. Pharmacol.* **56**, 189-195 (1998).

Wang,Z.Y., Khan,W.A., Bickers,D.R. & Mukhtar,H. Protection against polycyclic aromatic hydrocarbon-induced skin tumor initiation in mice by green tea polyphenols. *Carcinogenesis* **10**, 411-415 (1989).

Wei,H., Bowen,R., Zhang,X. & Lebwohl,M. Isoflavone genistein inhibits the initiation and promotion of two-stage skin carcinogenesis in mice. *Carcinogenesis* **19**, 1509-1514 (1998).

Weisburger,J.H., Rivenson,A., Reinhardt,J., Aliaga,C., Braley,J., Pittman,B., Zang,E. Effect of black tea on azoxymethane-induced colon cancer. *Carcinogenesis* **19**, 229-232 (1998).

Willey,J.C., Coy,E.L., Frampton,M.W., Torres,A., Apostolakos,M.J., Hoehn,G., Schuermann,W.H., Thilly,W.G., Olson,D.E., Hammersley,J.R., Crespi,C.L., Utell,M.J. Quantitative RT-PCR measurement of cytochromes p450 1A1, 1B1, and 2B7, microsomal epoxide hydrolase, and NADPH oxidoreductase expression in lung cells of smokers and nonsmokers. *Am. J. Respir. Cell Mol. Biol.* **17**, 114-124 (1997).

Williams,J.A. & Phillips,D.H. Mammary expression of xenobiotic metabolizing enzymes and their potential role in breast cancer. *Cancer Res.* **60**, 4667-4677 (2000).

Williams,J.A. *et al.* Determination of the enzymes responsible for activation of the heterocyclic amine 2-amino-3-methylimidazo[4,5-f]quinoline in the human breast. *Pharmacogenetics* **8**, 519-528 (1998).

Williams,S.N., Shih,H., Guenette,D.K., Brackney,W., Denison,M.S., Pickwell,G.V., Quattrochi,L.C. Comparative studies on the effects of green tea extracts and individual tea catechins on human CYP1A gene expression. *Chem. Biol. Interact.* **128**, 211-229 (2000).

Xu,M. & Dashwood,R.H. Chemoprevention studies of heterocyclic amine-induced colon carcinogenesis. *Cancer Lett.* **143**, 179-183 (1999).

Xu,Y., Ho,C.T., Amin,S.G., Han,C. & Chung,F.L. Inhibition of tobacco-specific nitrosamine-induced lung tumorigenesis in A/J mice by green tea and its major polyphenol as antioxidants. *Cancer Res.* **52** , 3875-3879 (1992).

Yang,C.S., Maliakal,P. & Meng,X. Inhibition of carcinogenesis by tea. *Annu. Rev. Pharmacol. Toxicol.* **42**, 25-54 (2002).

Yang,C.S., Prabhu,S. & Landau,J. Prevention of carcinogenesis by tea polyphenols. *Drug Metab Rev.* **33**, 237-253 (2001).

Zhou,J.R., Mukherjee,P., Gugger,E.T., Tanaka,T., Blackburn,G.L., Clinton,S.K. Inhibition of murine bladder tumorigenesis by soy isoflavones via alterations in the cell cycle, apoptosis, and angiogenesis. *Cancer Res.* **58**, 5231-5238