

The Bioflavonoid Galangin Blocks Aryl Hydrocarbon Receptor Activation and Polycyclic Aromatic Hydrocarbon-Induced Pre-B Cell Apoptosis

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ABSTRACT

Bioflavonoids are plant compounds touted for their potential to treat or prevent several diseases including cancers induced by common environmental chemicals. Much of the biologic activity of one such class of pollutants, polycyclic aromatic hydrocarbons (PAH), is mediated by the aryl hydrocarbon receptor/transcription factor (AhR). For example, the AhR regulates PAH immunotoxicity that manifests as pre-B cell apoptosis in models of B cell development. Because bioflavonoids block PAH-induced cell transformation and are structurally similar to AhR ligands, it was postulated that some of them would suppress PAH-induced, AhR-dependent immunotoxicity, possibly through a direct AhR blockade. This hypothesis was tested using a model of B cell development in which pre-B cells are cultured with and are dependent on bone marrow stromal or hepatic parenchymal cell monolayers. Of seven bioflavonoids

screened, galangin (3,5,7-trihydroxyflavone) blocked PAH-induced but not C₂-ceramide- or H₂O₂-induced pre-B cell apoptosis. Because galangin blocked AhR-dependent reporter gene expression, AhR complex-DNA binding, and AhR nuclear translocation, inhibition of a relatively early step in AhR signaling was implicated. This hypothesis was supported by the ability of galangin to bind the AhR and stabilize AhR-90-kDa heat shock protein complexes in the presence of AhR agonists. These studies demonstrate the utility of pre-B cell culture systems in identifying compounds capable of blocking PAH immunotoxicity, define at least one mechanism of galangin activity (i.e., repression of AhR activation), and motivate the use of this and similar dietary bioflavonoids as relatively nontoxic inhibitors of AhR agonist activity and as pharmacologic agents with which to dissect AhR signaling pathways.

Bioflavonoids are naturally occurring polyphenolic plant products found in fruits, vegetables, tea, and wine. Biologically significant levels of these compounds (~1 g) are consumed daily by humans, particularly by those living in Western cultures (reviewed in Formica and Regelson, 1995). The use of these relatively nontoxic compounds for the prevention or treatment of a number of diseases has long been espoused (Formica and Regelson, 1995). Indeed, recent studies defining some of the intracellular effects of natural and synthetic flavonoids support this consideration. For example, the antioxidant activity of bioflavonoids suggests their use as anti-inflammatory drugs (Yochum et al., 1999), their ability to

block cell cycle through inhibition of cyclins and cyclin-dependent kinases suggests their potential to block cancer growth (Ahmad et al., 1998), and their putative ability to inhibit urokinase activity implies a bioflavonoid-based strategy for inhibition of tumor metastasis (Jankun et al., 1997). Accordingly, bioflavonoid analogs are currently under investigation as cancer therapeutics in clinical trials.

Notably, some bioflavonoids and their synthetic analogs inhibit malignant transformation induced with environmental chemicals, including polycyclic aromatic hydrocarbons (PAH) (Formica and Regelson, 1995; So et al., 1996). Some of these anticancer effects are probably mediated by inhibition of enzymes involved in the bioactivation of xenobiotics to mutagenic intermediates (Formica and Regelson, 1995; Obermeier et al., 1995, and references therein) as well as by inhibition of growth factor signaling (Kuo, 1997; Ahmad et

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; AhR, aryl hydrocarbon receptor; hsp90, 90-kDa heat shock protein; ARNT, aryl hydrocarbon receptor nuclear translocator; NF- κ B, nuclear factor- κ B; B[a]P, benzo[a]pyrene; DMBA, 7,12-dimethylbenzo[a]anthracene; galangin, 3,5,7-trihydroxyflavone; FCS, fetal calf serum; IP, immunoprecipitation; PI, propidium iodide; TE, Tris/EDTA; AhRE, aryl hydrocarbon receptor response element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; EMSA, electromobility shift assay; PMSF, phenylmethylsulfonyl fluoride; TCDF, 2,3,7,8-tetrachlorodibenzofuran; ANF, α -naphthoflavone; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; RT, reverse transcription.

al., 1998). Furthermore, some of the anticancer activity may be caused by direct blockade of receptors such as the aryl hydrocarbon receptor (AhR) and/or to inhibition of receptor signaling (Reiners et al., 1998; Ciolino and Yeh, 1999).

The AhR is a cytosolic protein that is associated with the 90-kDa heat shock protein (hsp90) and is bound and activated by PAH, dioxins, and planar polychlorinated biphenyls (Denison et al., 1988; Hankinson, 1995; Schimdt and Bradfield, 1996). On ligand binding, the AhR translocates to the nucleus, dimerizes with at least one nuclear binding partner, the AhR nuclear translocator (ARNT) (Pollenz et al., 1994), engages AhR-specific DNA response elements (Denison et al., 1988), and induces transcription of several genes, including those encoding cytochrome P-450 monooxygenases. These "phase 1" enzymes initiate catabolism of AhR ligands (Hankinson, 1995; Schimdt and Bradfield, 1996; Nebert et al., 2000). AhR-dependent gene transactivation is likely modulated by transcription coactivators (Kumar et al., 1999). Additional AhR signaling may be transduced by an AhR-associated immunophilin-like molecule (Carver and Bradfield, 1997) or effected through AhR dimerization with other transcription factors, such as Rb and nuclear factor- κ B (NF- κ B) (reviewed in Nebert et al., 2000).

An accumulating body of information supports the hypothesis that the AhR plays a role in PAH- and dioxin-induced immunosuppression as well as in malignant transformation (Nebert et al., 1990; Ladics et al., 1991; Safe and Krishan, 1995; Schimdt and Bradfield, 1996; Yamaguchi et al., 1997a,b). Using a model of B cell maturation, it was previously demonstrated that the developing immune system is exquisitely sensitive to prototypic PAH such as benzo[*a*]pyrene (B[a]P) and 7,12-dimethylbenz[*a*]anthracene (DMBA) (Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). In a series of mechanistic studies, it was shown that PAH induce pre-B cell apoptosis, prematurely activating a cell death program critical to deletion of autoantigen-reactive lymphocyte clones. Furthermore, at low PAH doses, pre-B cell apoptosis is dependent on AhR activity in bone marrow stromal cells representative of the bone marrow hematopoietic microenvironment and required for pre-B cell growth and development (Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). Because bioflavonoids inhibit AhR ligand-induced malignant transformation and structurally resemble AhR ligands, it was postulated that some of them would similarly suppress PAH-induced, AhR-dependent immunotoxicity, possibly through a direct AhR blockade. The identification of such compounds would suggest their use in maintaining competent immune responses after AhR ligand exposure.

Using murine bone marrow cultures as a rapid and sensitive screening system, several bioflavonoids were tested for their ability to rescue pre-B cells from PAH-induced apoptosis. One such compound, galangin (3,5,7-trihydroxyflavone), completely inhibited DMBA-induced pre-B cell death. Galangin is relatively abundant in India root and as much as 13.5 mg per gram of propolis (a plant resin) has been shown (So et al., 1996; Park et al., 1998). Here, a combination of molecular and biochemical techniques was used to assess the mechanism of galangin-dependent inhibition of PAH-induced pre-B cell apoptosis. The results have implications for the use of

this and similar dietary compounds as inhibitors of environmental chemical (i.e., AhR ligand)-mediated toxicity.

Materials and Methods

Cell Culture, Apoptosis Induction, and Quantitation. A murine B cell line, BU-11, was derived from long term C57BL/6 bone marrow cultures as described previously (Yamaguchi et al., 1997b). Cells of this line express a rearranged Ig heavy chain and surface CD43 and B220/CD45 antigens; it therefore represents B cells at the pro/pre-B cell stage in B lymphocyte development. For convenience, they are referred to as pre-B cells. The BU-11 cell line was maintained on an AhR⁺ bone marrow stromal cell line, BMS2 (Pietrangeli et al., 1988), or on an AhR⁺ hepatic parenchymal line, Hepa-1c1c7 (Hepa-1) (Near et al., 1999). Hepa-1 represents a good model for AhR signaling and has been widely used for AhR-mediated signal transduction studies. BU-11/BMS2 or BU-11/Hepa-1 cultures were grown at 37°C in 10% CO₂ in a 1:1 ratio of RPMI-1640 and DMEM (Gibco/BRL, Inc., Grand Island, NY) supplemented with 5% fetal calf serum (FCS) (Gibco/BRL), 2 mM L-glutamine (Gibco/BRL), 0.05 mM β -mercaptoethanol (Mallinckrodt, Paris, KY), and 50 U/ml penicillin-streptomycin (Gibco/BRL). Cell lines were fed every 3 days and BU-11 cells split 1:10 every 5 days to maintain logarithmic growth.

For apoptosis assays, BU-11/BMS2, BU-11/Hepa-1 cultures, or BU-11 cells grown in the presence of 2 ng/ml rIL-7 (Peprotech, Rocky Hill, NJ) were treated with vehicle (acetone, 0.1%; final concentration), DMBA, B[a]P, C₂-ceramide, or H₂O₂ in duplicate wells (all chemicals from Sigma Chemical Co., St. Louis, MO). Putative inhibitors of apoptosis were added to cultures at the same time as apoptosis inducers. Twenty-four hours later, duplicate wells were pooled and assayed for apoptosis. Apigenin and galangin were obtained from Aldrich Chemical Co. (Milwaukee, WI). Epicatechin, morin, myricetin, quercetin, and taxifolin were obtained from Sigma Chemical Co.

Quantitation of apoptosis was performed by DNA staining with propidium iodide (PI) and flow cytometry as described previously (Yamaguchi et al., 1997a,b). Cells undergoing DNA fragmentation and apoptosis were weaker in PI fluorescence than those in the typical G₀/G₁ cell cycle peak (Yamaguchi et al., 1997a,b). In all experiments, an increase in the percentage of cells staining weakly with PI correlated with the percentage undergoing morphologic changes (i.e., a decrease in cell size as assayed by flow cytometry) characteristic of apoptosis.

DNA Fragmentation Assays. BU-11/BMS2 and BU-11/Hepa-1 cultures were treated as described above and assayed for DNA fragmentation according to Yamaguchi et al. (1997b). Briefly, BU-11 cells (10⁶) were washed with PBS and resuspended in cold 10 mM Tris/1 mM EDTA buffer, pH 8.0 (TE), containing 0.2% Triton X-100. Debris was pelleted and supernatant transferred to a fresh tube. After addition of 35 μ l of 3 M sodium acetate, DNA was extracted with phenol-chloroform. Fragmented DNA in the supernatant was precipitated with ethanol, pelleted, washed, dried, and resuspended in TE buffer. Loading buffer (6 μ l) consisting of 40% sucrose in TE, 1% SDS (Sigma), 0.05% bromphenol blue, and 2.5 μ g/ml RNase (Gibco/BRL) was added and samples were incubated at 37°C for 10 min. Samples were loaded into a 2% agarose gel and electrophoresed at 50 V for 2 h. DNA was visualized by staining the gel with ethidium bromide.

Luciferase Reporter Gene Assay. Mouse hepatoma cells (HIL1.c2) transfected with an AhR response element (AhRE)-driven firefly luciferase gene (provided by Dr. M. Denison, University of California, Davis, CA) were used for reporter gene transcription assays. Cells were treated for 5 h at 37°C with vehicle, DMBA, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Ultra Scientific, North Kings Town, RI), and/or galangin and lysed with Promega cell lysis buffer (Promega, Madison, WI). The samples were centrifuged for 10 min at 6000 rpm at 4°C and supernatants analyzed by the Luciferase Assay System (Promega) for luciferase activity according to the man-

ufacturer's protocol. Results were normalized by the respective protein concentrations of lysates.

Electromobility Shift Assay(s) (EMSA). Nuclear extracts for EMSA were prepared from Hepa-1 cells in HENG buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, pH 8.0, 430 mM NaCl, 1.5 mM MgCl₂, and 25% glycerol) containing 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml aprotinin, leupeptin, and sodium orthovanadate (Sigma). Nuclear proteins (2 µg) were incubated at room temperature for 30 min with [γ -³²P]ATP-labeled oligonucleotide probe corresponding to multiple AhRE in the murine *CYP1A1* gene promoter (Denison et al., 1988). To confirm the presence of the AhR in AhRE-binding complexes, 5 µg of control mouse immunoglobulins or mouse polyclonal anti-AhR antibody (Affinity Bioreagents, Golden, CO) was included with the nuclear extract/AhRE mixtures. Samples were run on nondenaturing 4% polyacrylamide gels in 0.5 × 45 mM Tris, 45 mM boric acid, 1 mM EDTA. Gels were dried and AhR complex-AhRE binding visualized by autoradiography.

AhR Immunoblotting. Western immunoblotting with whole cell or nuclear protein extracts were performed exactly as described (Yamaguchi et al., 1997b). Filters were probed with a 1:500 dilution of polyclonal anti-AhR antibody (BioMol Inc., Plymouth Meeting, PA) at room temperature. After 1 h, membranes were washed and incubated with a 1:3000 dilution of horseradish peroxidase-goat anti-rabbit IgG (Sigma) for an additional hour at room temperature. Membranes were washed with Tris-buffered saline/Tween 20 and developed with chemiluminescence for AhR detection (Yamaguchi et al., 1997b).

Nuclear and Cytosolic Protein Isolation. Subconfluent BMS2 and Hepa-1 cell monolayers were lifted from flasks with a cell scraper, washed in cold PBS, and resuspended in 0.5 ml P₁₀EG buffer (8.4 M KH₂PO₄·3H₂O, 10 mM EDTA, pH 7.4, 10% glycerol) supplemented with 20 mM Na₂Mo₄ and 1% IGEPAL CA-630 detergent (Sigma). Cells were gently pipetted and nuclei centrifuged for 5 min at 5000g at 4°C. Supernatant was collected and designated as the cytosolic fraction. The pellet was washed twice with P₁₀EG and the quality of the nuclear preparation monitored by phase contrast microscopy. Nuclei were then lysed with protein lysis buffer (0.1% Triton X-100, 150 mM NaCl, 25 mM Tris-HCl, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 50 mM NaF, 1 mM EDTA, 1 mM sodium orthovanadate, and 1 mM PMSF) on ice for 10 min. Protein concentrations were determined as described earlier (Yamaguchi et al., 1997b).

AhR Ligand Binding Analysis. Specific binding of radioactive TCDD to the AhR was determined by sucrose density gradient centrifugation as described earlier (Mann et al., 1999). Hepa-1 cells were washed once with cold PBS containing 1 mM EDTA and EGTA and once with "AhR buffer" [25 mM MOPS, pH 7.5, 1 mM EDTA, 5 mM EGTA, 0.02% NaN₃, 20 mM Na₂MoO₄, 10% glycerol, and 1 mM dithiothreitol] containing protease inhibitors (20 mM TLCK, 5 µg/ml leupeptin, 13 µg/ml aprotinin, 7 µg/ml pepstatin A, and 0.1 mM PMSF). Cells were scraped from the flask and transferred to a 1-ml glass centrifuge tube on ice. Cells were sonicated on ice with a Virsonic 475 Ultrasonic Cell Disruptor (Virtis Co, Gardiner, NY) for several 5-s bursts. Samples were transferred to a 7-ml Dounce homogenizer and, after adding 0.1 mM PMSF, homogenized for 50 strokes on ice. Samples were centrifuged for 10 min at 750g and then for 10 min at 12,000g. Supernatants were removed and centrifuged at 100,000g for 70 min. After removal of the top lipid layer, supernatant (cytosol) was removed and frozen in liquid nitrogen until further use.

Hepa-1 cytosolic AhR was analyzed by velocity sedimentation in sucrose gradients in a vertical tube rotor. Cytosol (1 mg of protein) was incubated with [³H]TCDD (1 nM) with or without 100 nM 2,3,7,8-tetrachlorodibenzofuran (TCDF), 10 µM galangin, or 1 µM α -naphthoflavone (ANF) for 2 h at 4°C. [³H]TCDD concentrations were verified by sampling each tube for total DPM. Unbound [³H]TCDD was removed with charcoal-dextran (1 mg of charcoal/mg of protein). After incubation, 300 µl of each cytosol sample (0.6 mg)

was layered onto a 10–30% linear sucrose density gradient. [¹⁴C]Catalase (11.3 S) and [¹⁴C]ovalbumin (3.6 S) were added as internal sedimentation markers. Gradients were spun for 140 min at 60,000 rpm at 4°C in a VTi 65.2 rotor. Fractions (150 µl) were collected and disintegrations per minute per fraction were determined in a Beckman LS5000TD scintillation counter.

Synthesis of an AhR Expression Construct and Plasmid Transfection of Hepa-1 Cells. Full-length *AhR* cDNA was polymerase chain reaction (PCR) amplified using the *pμ-AhR* plasmid (kindly provided by Dr. C. Bradfield, University of Wisconsin, Madison, WI) as template with the following primers carrying *Xba*I restriction sites: sense 5'-CTA GTC TAG ACC ATG AGC AGC GGC GCC AAC-3' and antisense 5'-CTA GTC TAG AAA GCT TAG TAT CGA ATT-3'. *AhR* cDNA was amplified with Turbo *Pfu* DNA polymerase (Stratagene, La Jolla, CA) in 30 cycles with a 5-min hot start and the following cycle conditions: denaturation at 95°C for 1 min, annealing at 63°C for 40 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR product was gel-purified and subcloned into the *Xba*I site of the *T7-pCDNA3* plasmid encoding the T7 major capsid protein (Invitrogen, Carlsbad, CA). *T7-pCDNA3* was constructed by linking the DNA sequence, digested out from the pTOPE pET translation vector (Novagen Inc., Madison, WI), coding for an 11-amino-acid leader peptide of the T7 major capsid protein to the *Bam*HI site of *pCDNA3* by blunt-end cloning. The proper (sense) orientation of *AhR* cDNA linked to *T7-pCDNA3* was confirmed by restriction analyses and DNA sequencing.

Hepa-1 cells (5 × 10⁶) were transfected in log phase growth with 20 µg of column- (Qiagen, Valencia, CA) purified *AhR*-*T7-pCDNA3* or vector alone by electroporation in a Bio-Rad Gene Pulsar (Bio-Rad, Richmond, CA) with settings of 960 µF and 180 V. Cells were incubated 10 min on ice and resuspended in 25 ml of 37°C Dulbecco's modified essential medium (DMEM) + 10% FCS (Gibco/BRL) and gently aliquoted into two 48-well culture plates. Cells were incubated at 37°C in a 10% CO₂ incubator. After 48 h, medium was replaced with DMEM + 10% FCS containing 1 mg/ml Geneticin (Gibco/BRL) and cells were incubated for 5 days to select for transfectants. Geneticin-resistant lines were expanded and tested for fusion protein expression using T7 epitope-specific antibody (Novagen). Stable lines expressing high levels of the T7-AhR fusion protein were grown for further studies.

Immunoprecipitations. Hepa-1 cells expressing T7-AhR fusion protein (Hepa-T7-AhR) were grown in 100-mm culture plates and treated with vehicle (0.1% acetone) or DMBA in the presence or absence of galangin or ANF for 1.5 h at 37°C. Cells were rinsed with cold PBS and lysed on ice for 20 min in 1 ml of immunoprecipitation (IP) lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% IGEPAL) containing 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 0.1 mM PMSF (final concentration). Lysates were collected and centrifuged at 12,000g for 15 min at 4°C. Supernatants containing 100 µg of protein in 500 µl of IP lysis buffer were incubated with either hsp90-specific antibodies (Stressgen, Victoria, BC, Canada), ARNT-specific (Affinity Bio-Reagents Inc., Golden, CO) antibodies, or control IgG for 1 h at 4°C. Protein A-Sepharose slurry (40 µl) (Sigma) was added to the antibody-treated lysates and incubated for 1 h at 4°C. Protein A-Sepharose beads were collected and washed 5 times with IP lysis buffer. Protein was eluted from the beads in 30 µl of 2× SDS gel loading buffer by heating at 90°C for 10 min, electrophoresed through 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Bio-Rad). Protein transfer was monitored by staining membranes with 0.1% ponceau S. After washing with Tris-buffered saline/Tween 20, membranes were treated with T7 epitope-specific, horseradish peroxidase-labeled antibody (Novagen) at a 1:5,000 dilution for 30 min at room temperature and developed for the detection of T7-AhR fusion protein by chemiluminescence.

Reverse Transcription (RT)-PCR for *CYP1A1* mRNA. *CYP1A1*-specific RT-PCR was carried out as described (Mann et al., 1999) with some modifications. Briefly, RNA was extracted from Hepa-1 cells using RNeasy mini kit (Qiagen) according to the man-

ufacturer's suggestions. Two micrograms of total RNA was combined with 50 ng of random hexamer primer, dNTP, and SuperScript II reverse transcriptase (160 units; Life Technologies) and reverse transcribed. cDNA amplification was performed by PCR using *CYP1A1*-specific primers (sense 5'-TCTT ggA gAC CTT CCg gCA TT-3'; antisense 5'-CCg ATg CAC TTT CgC TTg CC-3'). The 260-base-pair *CYP1A1* product was separated by 3% agarose gel electrophoresis and visualized with ethidium bromide. Equal sample loading was confirmed by monitoring β -actin (primers: sense 5'-gTC gTC gAC AAC ggC TCC ggC Atg Tg-3'; antisense 5'-CAT TgT AgA Agg TgT ggT gCC AgA TC-3'; product size, 256 base pairs) signals.

Results

Galangin Blocks PAH-Induced Pre-B Cell Apoptosis.

Using an in vitro model for B lymphopoiesis, it has been shown previously that pre-B cells maintained in the microenvironment provided by bone marrow stromal cells (Yamaguchi et al., 1997a,b; Mann et al., 1999) or hepatic parenchymal cells (Near et al., 1999) rapidly undergo apoptosis on exposure to low PAH doses. Pre-B cell apoptosis is mediated by a death signal delivered by the bone marrow stromal/hepatic parenchymal elements and is dependent on AhR activation within these "feeder" cells (Yamaguchi et al., 1997a,b; Near et al., 1999). These systems were used as platforms to rapidly screen compounds for their ability to inhibit PAH-induced apoptosis.

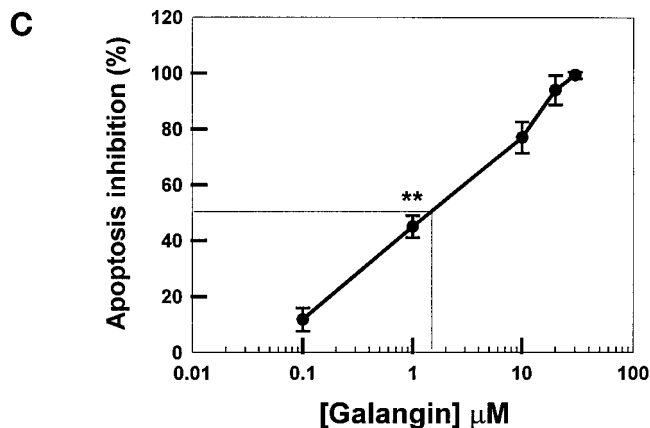
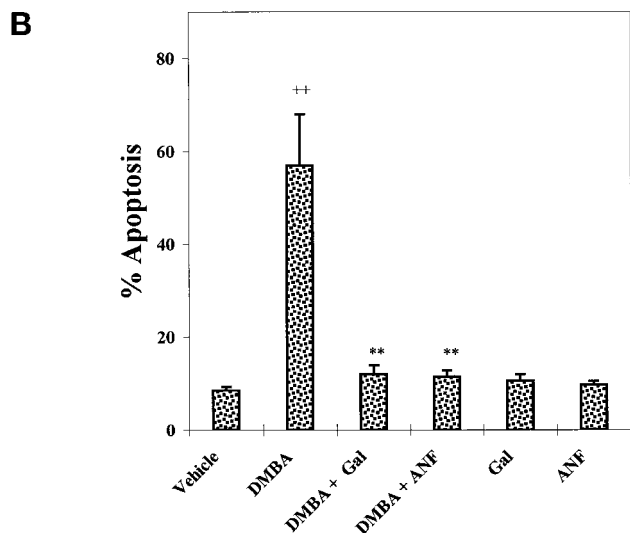
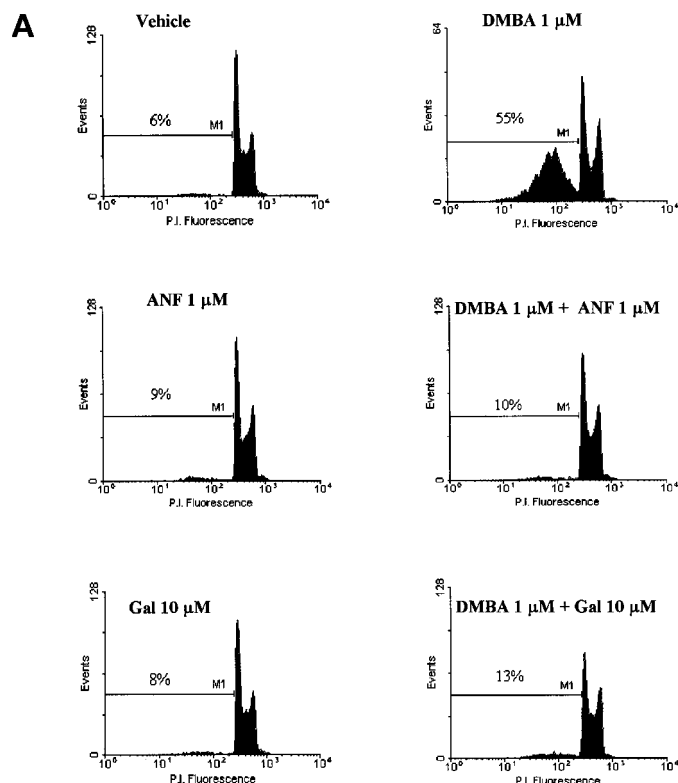
Bioflavonoids were chosen for initial studies because of their ability to inhibit PAH-induced carcinogenesis and/or their structural similarity to AhR ligands (Formica and Regelson, 1995; Gasiewicz et al., 1996; Lu et al., 1996; Jankun et al., 1997; Henry et al., 1999). Initial experiments were performed with galangin, a naturally occurring bioflavonoid found in India root, a spice used in herbal medicines. Specifically, addition of 1 μ M DMBA to cultures of pre-B (BU-11) cells maintained on cloned bone marrow stromal (BMS2) cells induced 50 to 60% of the pre-B cells to undergo apoptosis within 24 h, as quantitated by PI staining and flow cytometry (Fig. 1, A and B). This statistically significant level of apoptosis can be compared with the background level observed in vehicle-treated or untreated cultures (i.e., 5 to 10% apoptosis). As shown previously (Yamaguchi et al., 1997a,b; Near et al., 1999), addition of 1 μ M ANF, a synthetic flavonoid that is an AhR antagonist and inhibitor of *CYP1A1* monooxygenase activity (Blank et al., 1987; Lu et al., 1996; Reinert et al., 1998; Mann et al., 1999), significantly blocked DMBA-induced pre-B cell death. Similarly, inclusion of 10 μ M galangin in DMBA-treated cultures reduced the percentage of pre-B cells undergoing apoptosis essentially to background levels (Fig. 1, A and B). Neither ANF nor galangin affected the background level of apoptosis observed in the absence of DMBA (Fig. 1, A and B). Titration of the amount of galangin added to DMBA-treated cultures demonstrated an IC_{50} value of 1.27 μ M with \sim 80% inhibition at 10 μ M (Fig. 1C). That DMBA-induced pre-B cell death was caused by apoptosis and that galangin could block this apoptosis was confirmed by morphologic changes (nuclear condensation, cell shrinkage) observed visually and by flow cytometry after DMBA exposure but not after DMBA + galangin exposure (not shown) and by digestion of DNA into oligonucleosomal (i.e., multiples of 200 base pairs) fragments as visualized in agarose gels (Fig. 2). Interestingly, six other bioflavonoids (taxifolin, morin, epicatechin, myricetin, apigenin, and quer-

cetin) did not significantly block DMBA-induced pre-B cell apoptosis in BU-11/BMS2 cell culture (Fig. 3).

As reported previously (Near et al., 1999), BU-11 cells also undergo apoptosis when cultured with AhR⁺ hepatic parenchymal cells (Hepa-1) and treated with B[a]P (Fig. 4, A and B). As observed in DMBA-treated BU-11/BMS2 cultures, ANF and galangin significantly blocked B[a]P-induced BU-11 cell apoptosis in BU-11/Hepa-1 cultures as quantitated by PI staining (Fig. 4A) and as visualized in DNA gels (Fig. 4B). Therefore, galangin-dependent inhibition of PAH immunotoxicity is not unique to pre-B cell apoptosis induced by DMBA in the presence of bone marrow stromal cells.

Galangin Is Not a General Apoptosis Inhibitor. To determine whether galangin acts as a general inhibitor of apoptosis, its ability to block pre-B cell death induced by C_2 -ceramide or H_2O_2 , two well-characterized inducers of lymphocyte apoptosis (Obeid et al., 1993; Dumont et al., 1999), was tested. BU-11 cells cocultured with BMS2 cells or maintained in rIL-7 alone were treated with 20 μ M C_2 -ceramide or 0.25–5.0 mM H_2O_2 and apoptosis was quantitated 12 h later. (In preliminary experiments, these doses were shown to be limiting for the induction of BU-11 cell apoptosis). Consistent with results obtained with mature lymphocytes, C_2 -ceramide induced significant apoptosis in BU-11 cells supported either by stromal cells or rIL-7 (Fig. 5), indicating that the complex intracellular signals induced by C_2 -ceramide and leading to cell death (Cifone et al., 1999, and references therein) are intact at this early stage in B cell development. Similarly, H_2O_2 induced significant levels of BU-11 cell apoptosis (Fig. 5). Notably, neither galangin nor ANF significantly affected C_2 -ceramide- or H_2O_2 -induced apoptosis. These results indicate that galangin and ANF do not inhibit all molecular mechanisms of pre-B cell apoptosis.

Galangin Inhibits TCDD- and DMBA-Induced, AhR-Driven Reporter Gene Expression. Pre-B cell apoptosis induced in vitro with limiting PAH doses (i.e., 10^{-8} to 10^{-6} M) is regulated by the AhR (Mann et al., 1999; Near et al., 1999). Because of its structural similarity to AhR antagonists and partial agonists (Gasiewicz et al., 1996; Lu et al., 1996), it was postulated that galangin inhibits DMBA-induced apoptosis by blocking AhR signaling. To test this hypothesis, a relatively distal event in AhR activation (i.e., AhR ligand-induced, AhRE-driven gene transcription) was assayed using AhR⁺ mouse hepatoma cells (H1L1.1c2) transfected with a cDNA plasmid containing a luciferase gene driven by multiple AhREs (Garrison et al., 1996). Both TCDD (10 nM) and DMBA (1 μ M) induced significant (approximately 100-fold) increases in luciferase activity within 5 h (Fig. 6). Luciferase activity induced with either TCDD or DMBA was significantly inhibited by 5 to 10 μ M galangin ($P < .05$). There was a small but statistically insignificant increase in luciferase activity after exposure to 10 μ M galangin alone ($P = .16$). Although theoretically possible, it is unlikely that this apparent increase represents a biologically significant level of AhR activation because 10 μ M galangin did not induce detectable levels of AhR nuclear translocation, *CYP1A1* mRNA induction, AhR-AhRE binding, or dissociation from hsp90 (below). These data indicate that, under these conditions, galangin blocks rather than induces AhR signaling, at least as defined as transcriptional regulation of an AhRE-controlled gene product.



Galangin Inhibits AhR Complex-DNA Binding. Galangin-dependent inhibition of luciferase activity could be caused by direct inhibition of gene transactivation and/or inhibition of some element in AhR signaling more proximal than gene transcription. Therefore, EMSAs were used to determine whether galangin inhibits activated AhR complex binding to AhRE sequences. Hepa-1 cells, which express high AhR levels, were treated with TCDD or DMBA \pm galangin for 1 h and nuclear protein extracts assayed by EMSA for AhRE-binding complexes. Treatment of Hepa-1 cells with 10 nM TCDD (Fig. 7, lane 2) or 1 μM DMBA (lane 4) resulted in the appearance of a prominent complex (marked "AhRC"). Formation of this band was completely inhibited with unlabeled AhRE oligonucleotide (not shown). That the AhR was present in this complex was confirmed by the ability of AhR-specific antibody, but not control IgG, to eliminate formation of the putative AhR-AhRE band (lanes 6 and 5, respectively). Formation of this AhR-containing complex was completely inhibited by addition of 10 μM galangin at the time of TCDD (lane 3) or DMBA (lane 7) challenge. Galangin alone did not induce a DNA-binding complex (lane 8) and had no effect on the formation of a nonspecific band (labeled "NS", lanes 1–8). From these data, it is concluded that galangin-mediated inhibition of AhR activity is effected near or at the point of AhR complex-DNA binding.

Galangin Blocks AhR Nuclear Translocation. Immediately before AhRE binding, the AhR complex undergoes a conformational change that triggers its nuclear translocation. To determine whether 10 μM galangin interferes with this process, BMS2 cells were treated with TCDD or DMBA \pm galangin and nuclear AhR levels assayed 1.5 h later by Western immunoblotting. Nuclear AhR was not detected in vehicle-treated cells (Fig. 8A, lane 2). Addition of 1 nM TCDD, 10 μM DMBA, or 1 μM DMBA induced AhR nuclear translocation (lanes 3 to 5, respectively). However, addition of 10 μM galangin at the time of TCDD or DMBA challenge completely inhibited AhR nuclear translocation (lanes 6 to 8). Addition of this dose of galangin alone did not induce detectable levels of nuclear AhR (lane 9). Similarly, exposure of Hepa-1 cells to 10 μM B[a]P for 1.5 h resulted in significant AhR nuclear translocation (Fig. 8B, lane 2) that was completely blocked by inclusion of 10 μM galangin (Fig. 8B, lane 3). Galangin alone did not induce AhR nuclear translocation in Hepa-1 cells (Fig. 8B, lane 4). These results are consistent with the hypothesis that galangin not only fails to activate the AhR but also that it inhibits a relatively early step in AhR activation.

Fig. 1. Galangin blocks DMBA-induced BU-11 cell apoptosis. A, BU-11/BMS2 cell cultures were treated in duplicate wells with vehicle, DMBA \pm ANF or galangin, ANF alone, or galangin alone, as indicated. BU-11 cells were harvested 24 h later and the percentage of apoptotic cells was assessed by PI staining and flow cytometry. Representative data from four independent experiments are presented. B, BU-11/BMS2 cell cultures were treated as above and the percentage of apoptotic cells assessed by propidium iodide staining and flow cytometry. Data are presented as the means \pm S.E. from four independent experiments. ++, significant apoptosis induced with 1 μM DMBA, $P < .003$. **, significant inhibition of apoptosis with 10 μM galangin (Gal) or 1 μM ANF, $P < .003$. C, BU-11/BMS2 cultures were treated in duplicate wells with 1 μM DMBA and the concentrations of galangin indicated. BU-11 cells were harvested 18 h later and assayed for the percentage of apoptotic cells as above. Data are presented as the means \pm S.E. from three independent experiments. In this series of experiments, 1 μM DMBA induced apoptosis in $52 \pm 9\%$ of BU-11 cells. The IC_{50} was reached at 1.27 μM galangin. **, significant apoptosis inhibition, $P < .005$.

Galangin Inhibits Specific Binding of [3 H]TCDD in Hepa-1 Cytosol. To determine whether galangin is a ligand for the AhR, its ability to block [3 H]TCDD-AhR binding was determined. Hepa-1 cytosolic protein extracts, as an AhR source, were incubated for 2 h with 1.0 nM [3 H]TCDD in the presence or absence of 10 μ M galangin. Unbound [3 H]TCDD was removed and AhR-TCDD complexes detected by sucrose

density gradient centrifugation. Inhibition of [3 H]TCDD-AhR complexing with a known AhR antagonist, ANF, and an agonist, TCDF, was assessed as positive control samples. Incubation of [3 H]TCDD with Hepa-1 cytosolic protein resulted in an 9.1-S peak characteristic of ligand bound AhR

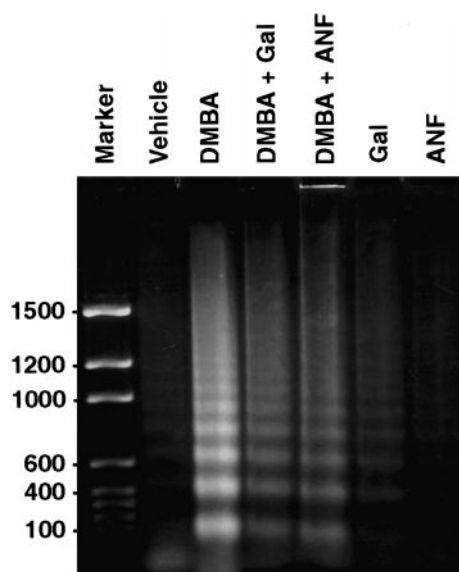


Fig. 2. Galangin blocks DMBA-induced DNA fragmentation. BU-11/BMS2 cell cultures were treated for 18 h with 1 μ M DMBA \pm 10 μ M galangin (Gal) or ANF, galangin alone, or ANF alone, as indicated. BU-11 cells were then harvested, DNA isolated and electrophoresed through 2% agarose gels to detect digestion into fragments characteristic of apoptosis.

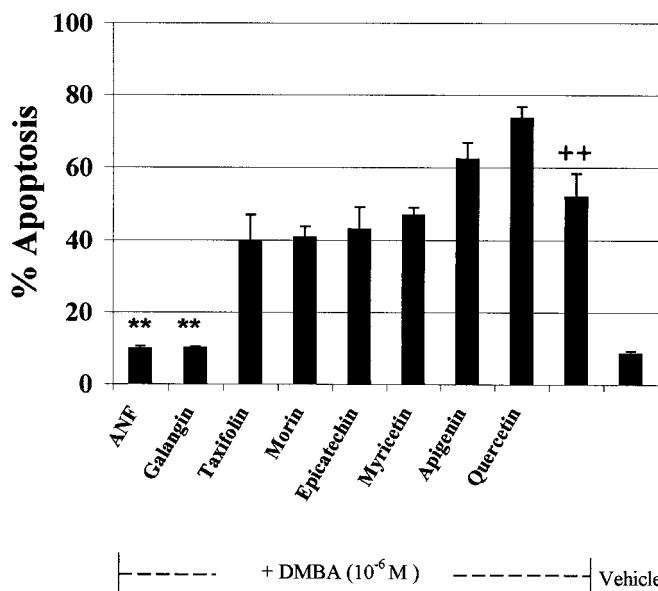


Fig. 3. Taxifolin, morin, epicatechin, myricetin, apigenin, and quercetin fail to block DMBA-induced BU-11 cell apoptosis. BU-11/BMS2 cell cultures were treated in duplicate wells with vehicle, 1 μ M DMBA alone, DMBA + 1 μ M ANF, or DMBA + 10 μ M the bioflavonoids galangin, taxifolin, morin, epicatechin, myricetin, apigenin, or quercetin. BU-11 cells were harvested 24 h later and the percentage of apoptotic cells assessed by PI staining and flow cytometry. Data represent the means \pm S.E. from 3 independent experiments. ++, statistically significant increase in apoptosis as compared with vehicle controls, $P < .01$. **, statistically significant inhibition of DMBA-induced apoptosis, $P < .01$.

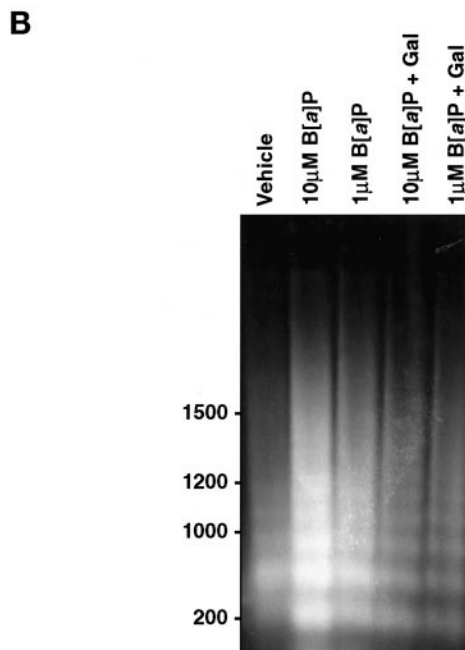
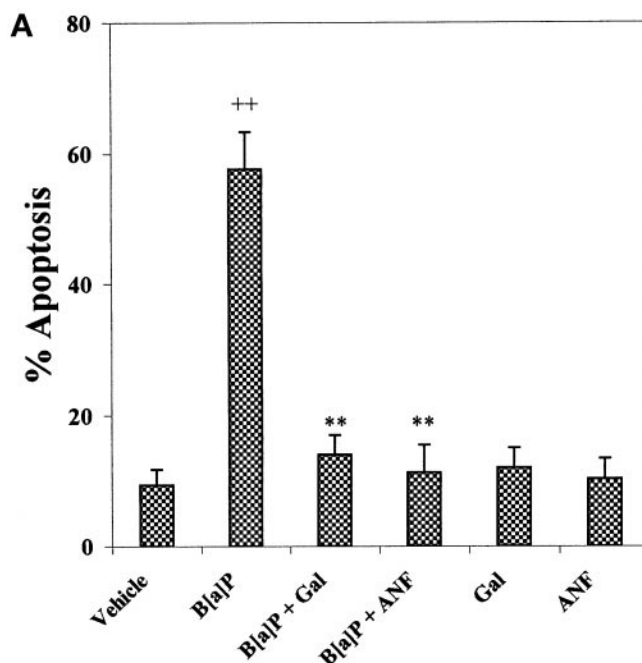


Fig. 4. Galangin blocks B[a]P-induced BU-11 cell apoptosis in BU-11/Hepa-1 cultures. A, BU-11/Hepa-1 cell cultures were treated with vehicle, 1 μ M B[a]P \pm 10 μ M galangin or 1 μ M ANF, galangin alone, or ANF alone as indicated. BU-11 cells were harvested 24 h later and the percentage of cells undergoing apoptosis assayed by PI staining and flow cytometry. Data represent the means \pm S.E. from three independent experiments. ++, significant apoptosis, $P < .002$. **, significant apoptosis inhibition, $P < .005$. B, BU-11/Hepa-1 cultures were treated with vehicle, 10 μ M B[a]P, 1 μ M B[a]P, 10 μ M B[a]P \pm 10 μ M galangin, or 1 μ M B[a]P + 10 μ M galangin as indicated. BU-11 cells were harvested 24 h later. DNA was isolated and electrophoresed through 2% agarose gels to detect digestion into fragments characteristic of apoptosis.

(Fig. 9). Radioactivity in that peak was significantly decreased by addition of 0.1 μ M TCDF, 1 μ M ANF, or 10 μ M galangin. The ability of galangin to inhibit binding of TCDD, a high-affinity AhR ligand, to the AhR demonstrates that galangin is able to bind to the AhR.

Galangin and ANF Block AhR Agonist-Induced hsp90-AhR Dissociation and AhR-ARNT Dimerization. Because AhR nuclear translocation and ARNT binding is probably caused by conformational changes marked by hsp90 dissociation, it was postulated that galangin, like other AhR antagonists, would inhibit the dissociation of AhR-hsp90 complexes in the presence of AhR agonists. This stabilization should result in coprecipitation of the AhR and hsp90 and a failure to coprecipitate AhR and ARNT. To test this hypothesis, Hepa-1 cells transfected with *pCDNA3-T7AhR*, a plasmid encoding a fusion protein easily detected with T7 epitope-specific antibody, were treated for 1.5 h with vehicle or 1 μ M DMBA \pm 10 μ M galangin, 10 μ M ANF, 10 μ M galangin, or 1 μ M ANF alone. Whole-cell protein extracts were then immunoprecipitated either with hsp90-specific (Fig. 10A) or ARNT-specific (Fig. 10B) antibody, and precipitates were probed in Western immunoblots with T7 epitope-specific antibody.

A significant level of AhR coprecipitated with hsp90 in vehicle-treated cells (Fig. 10A, lane 2). Consistent with previous reports demonstrating dissociation of hsp90 from AhR before or concomitant with nuclear translocation and AhR-ARNT dimerization (Henry et al., 1999), treatment with 1 μ M DMBA significantly reduced the amount of AhR-hsp90

complex (lane 3). The failure of 10 μ M galangin to significantly reduce the amount of hsp90 coprecipitating with the AhR (lane 4) supports the conclusion that, although capable of binding the AhR, galangin does not induce AhR nuclear translocation and is not an AhR agonist at the doses used. The slight decrease in band density observed in lane 4 of Fig. 10 was not observed in other experiments (Fig. 10C). However, galangin blocked DMBA-induced AhR-hsp90 dissociation (lane 5). Similarly, 1 μ M ANF alone did not affect AhR-hsp90 complexing but did inhibit DMBA-induced AhR-hsp90 dissociation (lanes 6 and 7, respectively). Data from 3 experiments in which hsp90 dissociation from the AhR was evaluated are summarized as relative band densities in Fig. 10C.

AhR-ARNT complexes were not detected in vehicle-treated cultures (Fig. 10B, lane 2). Treatment of cells with 1 μ M DMBA (lane 3) but not 10 μ M galangin (lane 4) resulted in significant levels of AhR coprecipitating with ARNT (Fig. 10B, lane 3). As would be expected from the ability of galangin to inhibit AhR nuclear translocation (Fig. 8), galangin blocked DMBA-induced AhR-ARNT complexing (lane 5). Similarly ANF failed to induce AhR-ARNT complexing (lane 6) and blocked DMBA-induced AhR-ARNT complexing (lane 7). Collectively, these experiments demonstrate that galangin inhibits AhR signaling at least in part by binding to the AhR without destabilizing AhR-hsp90 complexes. In the biologic system described herein, this inhibition of AhR signaling contributes to a reduction in PAH-induced immunotoxicity.

Galangin Is a Weak AhR Agonist at High Doses. Several investigators have concluded that bioflavonoids activate the AhR at relatively high doses (Gasiewicz et al., 1996; Ciolino and Yeh, 1999; Reiners et al., 1999). To determine whether high galangin doses similarly activate the AhR, Hepa-1 and BMS2 cells were treated with 10 to 90 μ M

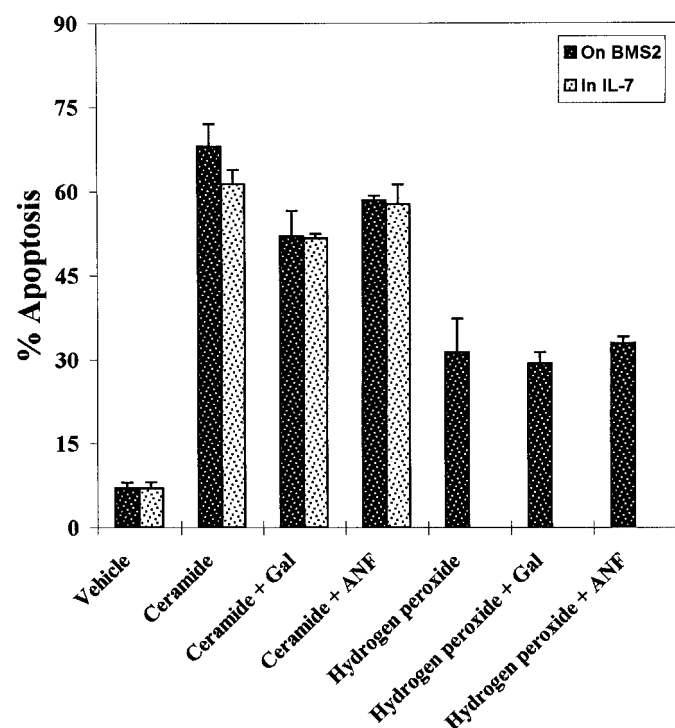


Fig. 5. Galangin does not inhibit C_2 -ceramide or H_2O_2 -induced apoptosis. BU-11/BMS2 cell cultures or cultures of BU-11 cells in rIL-7-containing media were treated in duplicate with 20 μ M C_2 -ceramide, 0.25–5.0 mM H_2O_2 \pm 10 μ M galangin, or 1 μ M ANF. BU-11 cells were harvested 12 h later and the percentage of cells undergoing apoptosis assayed by PI staining and flow cytometry. Data represent the means \pm S.E. from three independent experiments. C_2 -ceramide and H_2O_2 induced significant levels of apoptosis, $P < .005$ and $P < .01$, respectively.

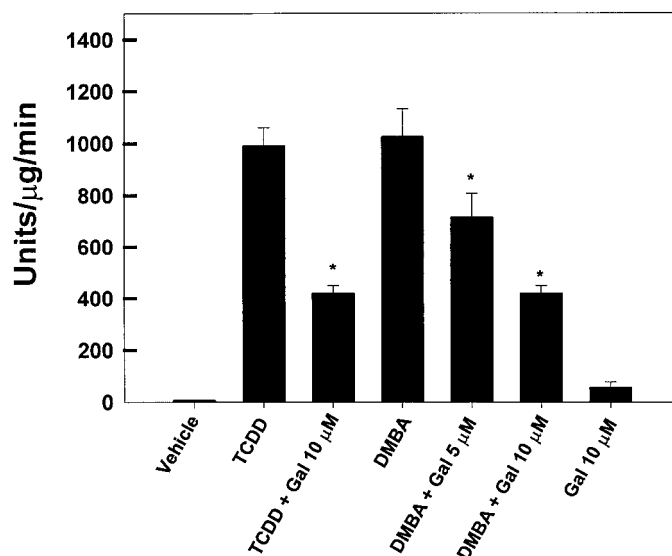


Fig. 6. Galangin inhibits TCDD- and DMBA-induced luciferase reporter gene expression. H1L1.c2 cells, transfected with an AhRE-driven firefly luciferase gene, were treated for 5 h with vehicle, 10 nM TCDD, or 1 μ M DMBA \pm 5 to 10 μ M galangin, or with 10 μ M galangin alone. Cells were then lysed and luciferase activity determined as described under *Materials and Methods*. Data are averaged from three independent experiments. Luciferase activity in the presence of galangin alone was not significantly different from in the presence of vehicle ($P = .16$). *, significant inhibition of luciferase activity, $P < .05$.

galangin. Total cell and nuclear protein was isolated 1.5 h later and analyzed by AhR-specific immunoblotting. As expected, nuclear AhR was not detected in either cell line after vehicle treatment (Fig. 11, A and B, lane 2). In contrast, TCDD induced significant AhR nuclear translocation (Fig. 11, A and B, lane 3), which was completely blocked by addition of 10 μ M galangin (Fig. 11, A and B, lane 4). At the doses used for apoptosis inhibition studies, i.e. 10 μ M and as previously shown (Fig. 8), galangin failed to induce AhR nuclear translocation in either cell line (Fig. 11, A and B, lane 5). In Hepa-1 cells, galangin doses as high as 30 μ M failed to induce AhR nuclear translocation (Fig. 11A, lane 6). However, 60 or 90 μ M galangin induced AhR nuclear translocation in both cell types (Fig. 11A, lanes 7, 8; Fig. 11B, lane 6).

To determine whether nuclear translocation of the AhR induced by high doses of galangin results in transcriptional activity, *CYP1A1* mRNA expression was assayed 14 h after treatment of Hepa-1 cells with galangin or DMBA. (BMS2 cells were not included in these studies because we had demonstrated previously that *CYP1A1* is not inducible in these cells with either DMBA or TCDD) (Mann et al., 1999). Although 10 μ M galangin did not induce detectable *CYP1A1* mRNA levels (Fig. 12, lane 2), a significantly higher dose, 60 μ M, induced low levels of *CYP1A1* mRNA (lane 3). The low level of *CYP1A1* mRNA induction seen after galangin exposure can be contrasted with the strong signal seen after DMBA exposure (lane 4). Collectively, these data are consistent with previous reports on bioflavonoids and demonstrate that some AhR ligands can act as AhR antagonists at low doses and weak agonists at high doses.

Discussion

Cocultures of bone marrow stromal cells and immature B lymphocytes have been used extensively to define factors

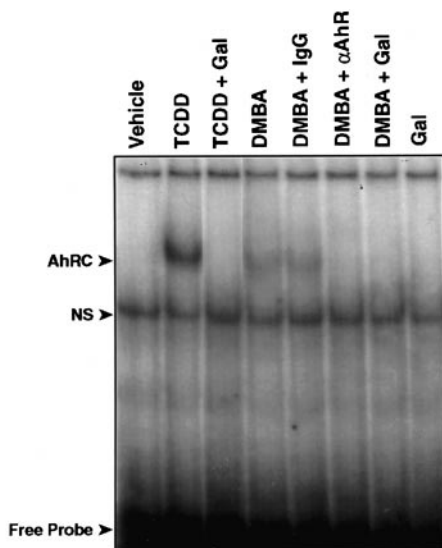


Fig. 7. Galangin inhibits TCDD- and DMBA-induced AhR complex-DNA binding. Hepa-1 cells were treated for 1 h with vehicle, 10 nM TCDD \pm 10 μ M galangin, 1 μ M DMBA \pm galangin, or galangin alone as indicated. Cells were harvested and nuclear extracts incubated with [32 P]-AhRE. A nonspecific band observed in all samples is labeled "NS". The band containing the putative AhR complex is labeled "AhRC". Formation of this band was inhibited with unlabeled AhRE oligonucleotide (not shown). Addition of 5 μ g AhR-specific antibody (lane 6) but not control mouse Ig (lane 5) to nuclear extracts from DMBA-treated cells eliminated the predominant "AhRC" band.

that influence B lymphopoiesis (Whitlock et al., 1984; Pietrangeli et al., 1988). Our laboratory has used this system, and one utilizing hepatic parenchymal cells in lieu of bone marrow stromal cells, to demonstrate the deleterious effects of low PAH doses on the developing immune system and to define mechanisms through which PAH-induced immunotoxicity is mediated (Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). For the studies described herein, we took advantage of the sensitivity and relative simplicity of these systems and our understanding of the effects of PAH on both the stromal cell and pre-B cell compartments to rapidly screen for naturally occurring bioflavonoids that block PAH immunotoxicity and to define their mechanism of action.

In the initial screen of seven bioflavonoids, galangin consistently blocked immunotoxicity induced by DMBA in BU-11/BMS2 cultures that manifested as pre-B cell apoptosis. Apoptosis was inhibited 50% at approximately equimolar concentrations of DMBA and galangin. Similarly, galangin inhibited pre-B cell apoptosis induced with B[a]P in BU-11/Hepa-1 cocultures, suggesting that this bioflavonoid may afford protection against PAH in hematopoietic tissue in general. In contrast, galangin did not affect pre-B cell apoptosis induced with limiting doses of C_2 -ceramide or H_2O_2 . C_2 -ceramide is a second messenger produced by sphingomy-

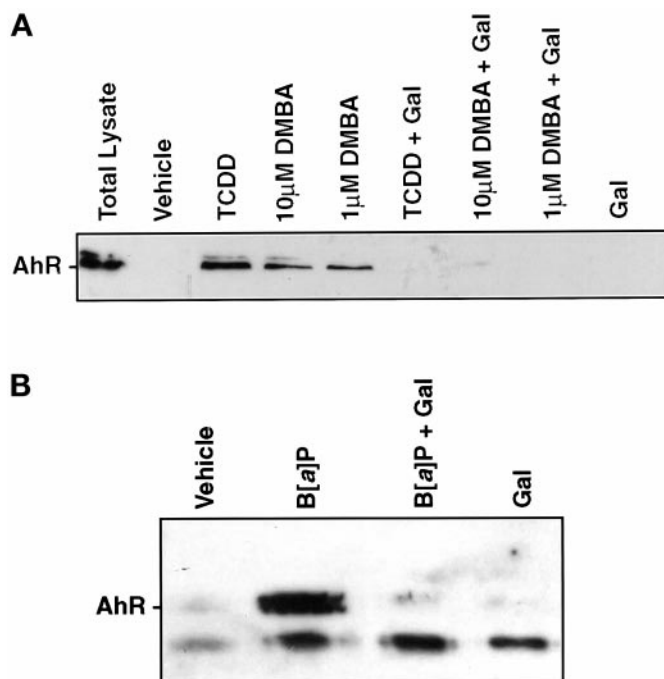


Fig. 8. Galangin blocks TCDD-, DMBA- and B[a]P-induced AhR nuclear translocation. A, proteins (50 μ g) from whole cell extracts of untreated BMS2 cells (lane 1) or nuclear proteins (100 μ g) from BMS2 cells treated for 1.5 h with vehicle (lane 2), 1 nM TCDD (lane 3), 10 μ M DMBA (lane 4), 1 μ M DMBA (lane 5), 1 nM TCDD + 10 μ M galangin (lane 6), 10 μ M DMBA + 10 μ M galangin (lane 7), 1 μ M DMBA + 10 μ M galangin (lane 8), or 10 μ M galangin alone (lane 9) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with AhR-specific antibody. Data from a representative experiment (three total) are presented. The 95-kDa AhR protein is indicated (B). Nuclear proteins (100 μ g) from Hepa-1 cells treated for 1.5 h with vehicle (lane 1), 10 μ M B[a]P (lane 2), B[a]P + 10 μ M galangin (lane 3), or galangin alone (lane 4) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with AhR-specific antibody. Data from a representative experiment (three total) are presented. The 95-kDa AhR protein is indicated.

elin hydrolysis and by ceramide synthase-dependent synthesis and is involved in lymphocyte apoptosis induced by cross-linking antigen-specific receptors (i.e., during clonal deletion) and by tumor necrosis factor, Fas ligand, ionizing radiation, and chemotherapeutics (Cifone et al., 1999, and references therein). The failure of galangin to block C_2 -ceramide-induced pre-B cell apoptosis indicates that signals distal to ceramide generation, such as caspase 8 and caspase 3 activation (Cifone et al., 1999), are not likely to be targeted by this bioflavonoid. Interestingly, a functional AhR may be essential for ceramide-induced apoptosis in Hepa-1 cell lines (Reiners and Clift, 1999). The interplay between the ceramide signaling pathway and the AhR has not yet been defined.

Similarly, H_2O_2 has been widely used as an inducer of oxidative stress and apoptosis (Dumont et al., 1999). In several systems, H_2O_2 -induced apoptosis can be blocked with a variety of antioxidants, radical oxygen scavengers, inhibitors of NF- κ B activation or caspase 3 inhibitors (Dumont et al., 1999). Although some bioflavonoids are antioxidants (Formica and Regelson, 1995; Kuo, 1997), the failure of galangin to block H_2O_2 -induced pre-B cell apoptosis suggests, but does not prove, that radical scavenging within pre-B cells by galangin does not contribute to apoptosis inhibition in this system.

In previous studies, we and others have shown that PAH-induced pre-B cell apoptosis is dependent on bone marrow stromal or hepatic parenchymal feeder cells (Yamaguchi et al., 1997a,b; Heidel et al., 1999; Mann et al., 1999; Near et al., 1999). This is the case for stromal cell-dependent primary pre-B cells (Yamaguchi et al., 1997a), pro/pre-B cell lines (e.g., BU-11) (Yamaguchi et al., 1997b), and for a stromal cell-independent pre-B cell line, 70Z/3 (Heidel et al., 1999). At limiting PAH doses (10^{-8} to 10^{-6} M DMBA), pre-B cell apoptosis is AhR-dependent (Yamaguchi et al., 1997b; Mann et al., 1999; Near et al., 1999). This AhR-dependence can be partially overcome with high PAH doses [e.g., 10^{-5} M DMBA (Heidel et al., 1999; Near et al., 1999)]. Because PAH-induced BU-11 cell apoptosis is regulated by the AhR, it was postulated that galangin would exert its inhibitory effect on PAH-induced apoptosis by modulating the AhR signal transduc-

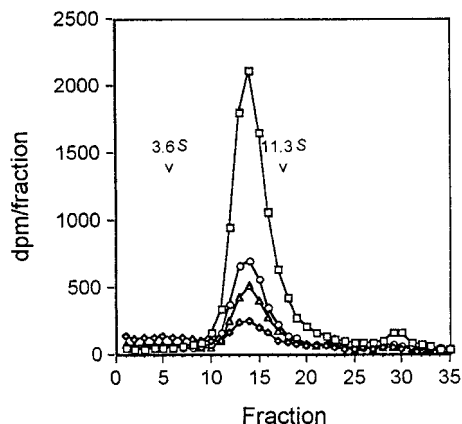


Fig. 9. Galangin is an AhR inhibitor: Hepa-1 cytosol was incubated with [3 H]TCDD (1 nM) in the presence or absence of 100 nM TCDF, 1 μ M ANF, or 10 μ M galangin for 2 h. Unbound [3 H]TCDD was removed by charcoal-dextran treatment and 0.6 mg of protein was applied to 10 to 30% sucrose gradients. Samples were then centrifuged, fractions collected, and dpm/fraction determined. \square , [3 H]TCDD; \diamond , +TCDF (0.1 μ M); \circ , +ANF (1 μ M); \triangle , +galangin (10 μ M).

tion pathway. Indeed, the ability of galangin to block AhRE-driven, TCDD- or DMBA-induced luciferase activity indicated that some step in the AhR signaling cascade is compromised by this bioflavonoid.

This result is in agreement with a recent report showing that galangin inhibits AhR activity in human breast tumor cells (Ciolino and Yeh, 1999). In these studies, it was suggested that galangin may be an AhR ligand. However, AhR binding studies were not performed. The ability of galangin to bind and activate the AhR was specifically addressed in the current studies by tracking an AhR signaling pathway backward from AhRE-driven gene induction to ligand binding and hsp90 dissociation. Interestingly, much of the activity of galangin in the breast tumor cell model (e.g., inhibition

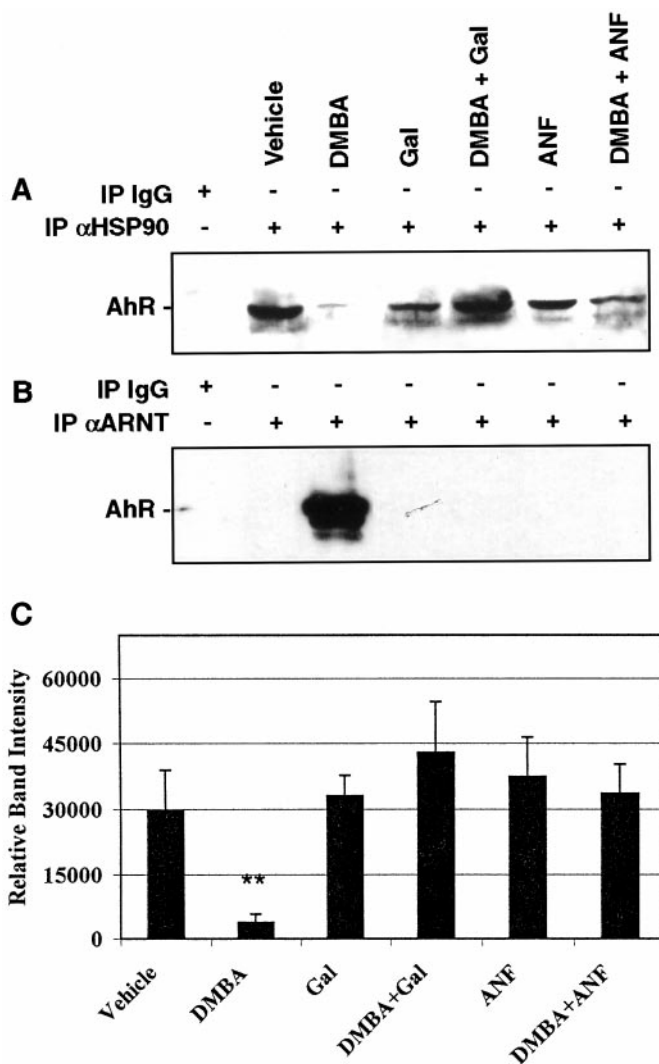


Fig. 10. Galangin and ANF block AhR agonist-induced hsp90-AhR dissociation and AhR-ARNT dimerization. Stable Hepa-1 *pCDNA3-T7AhR* transfectants were treated for 1.5 h with vehicle, 1 μ M DMBA \pm 10 μ M galangin or 1 μ M ANF, galangin alone, or ANF alone as indicated. Proteins (100 μ g) from whole cell lysates were immunoprecipitated with an IgG isotype control antibody (Panels A and B, lane 1), an hsp90-specific antibody (Panel A, lanes 2 to 7) or ARNT-specific antibody (Panel B, lanes 2 to 7) and 20 μ l of eluted protein (from a total of 30 μ l) electrophoresed and transferred to nitrocellulose membranes. Membranes were probed with T7-epitope-specific antibody. Data from a representative experiment (three total) are presented in panels A and B. The ~97 kDa T7-AhR fusion protein is indicated as "AhR". Average band densities \pm S.E. from three experiments are presented in C. ** P < .01.

of DMBA metabolism and DNA adduct formation), was attributed to its ability to block CYP1A1 enzymatic activity. The ability of galangin to block CYP1A1 activity is irrelevant to the present studies because CYP1A1 protein is undetectable in BMS2 cells, CYP1A1 mRNA is not inducible with DMBA or TCDD in BMS2 cells, and CYP1A1 enzymatic activity is not likely to contribute to induction of pre-B cell apoptosis (Mann et al., 1999). These observations, together with the demonstration that this naturally occurring bioflavonoid blocks PAH-mediated toxicity in a primary hemato-

poietic organ (i.e., the bone marrow), underscore the novelty and significance of the present study.

AhRE-specific EMSAs indicated that galangin blocks the ability of DMBA and TCDD to stimulate AhR complex binding to AhRE sequences, and Western blotting analyses of nuclear proteins demonstrated galangin inhibition of DMBA-, B[a]P-, and TCDD-induced AhR nuclear translocation. These results implicate galangin-mediated inhibition as a relatively early event in the AhR signaling pathway, although they do not rule out concomitant effects at the levels of nuclear translocation or DNA binding. The ability of galangin to block AhR binding by radiolabeled TCDD in a cell-free system and to prevent AhR-hsp90 dissociation in intact cells in the presence of AhR agonists indicates that galangin directly binds the AhR, thereby abrogating AhR signaling. These results are consistent with previous studies that demonstrate that ANF and other synthetic flavones bind the AhR without triggering hsp90 dissociation (Blank et al., 1987; Gasiewicz et al., 1996; Lu et al., 1996; Henry et al., 1999). Binding to the AhR distinguishes galangin from another naturally occurring plant compound, resveratrol, which blocks activated AhR from binding to AhRE sequences but does not affect ligand-AhR binding (Ciolino et al., 1998). Interestingly, a more recent screen of additional plant compounds in the BU-11/BMS2 culture system indicated that a related bioflavonoid, chrysene, similarly blocks DMBA-induced pre-B cell apoptosis. The mechanism of this inhibition of PAH toxicity is under investigation.

It is important to note that at doses as high as 30 μ M, galangin alone did not induce significant AhRE-driven transcriptional activity, AhR-DNA binding, AhR nuclear translocation, and/or hsp90-AhR dissociation in BMS2 or Hepa-1 cells. Therefore, galangin's ability to inhibit PAH-induced pre-B cell apoptosis probably represents an inhibition rather than a diversion of AhR signaling. This result may be contrasted with those obtained with other naturally occurring polyphenols (e.g., keampferol, quercetin, indole-3-carbinol) (Chen et al., 1996; Ciolino et al., 1999) or synthetic flavone-derived AhR ligands (Gasiewicz et al., 1996; Lu et al., 1996; Henry et al., 1999) that can activate the AhR as well as decrease expression of some of the downstream markers of environmental chemical-induced AhR activation. In one study, 5 μ M galangin induced CYP1A1 mRNA expression in a human mammary tumor cell line (Ciolino and Yeh, 1999). This apparent difference from the present studies could reflect differences between transcriptional regulation in a human malignant cell line (Ciolino and Yeh, 1999) and in a murine hepatoma line (Hepa-1) or a nontransformed bone marrow stromal cell line (BMS2).

Like galangin, ANF blocked B[a]P and DMBA-induced pre-B cell apoptosis in BU-11/Hepa-1 and BU-11/BMS2 co-cultures, respectively (Fig. 1) (Yamaguchi et al., 1997a,b; Mann et al., 1999; Near et al., 1999). Because ANF binds the AhR in vitro (Blank et al., 1987; Gasiewicz et al., 1996), it was proposed that this synthetic flavone suppresses PAH immunotoxicity by AhR antagonism. In the present study, we confirm that ANF binds the AhR in a cell-free system and extend previous studies by demonstrating that ANF prevents AhR-hsp90 destabilization and blocks AhR-ARNT association in intact cells. Furthermore, the failure of ANF to inhibit C₂-ceramide or H₂O₂-induced pre-B cell apoptosis suggests that its antiapoptotic activity may be restricted to apoptosis

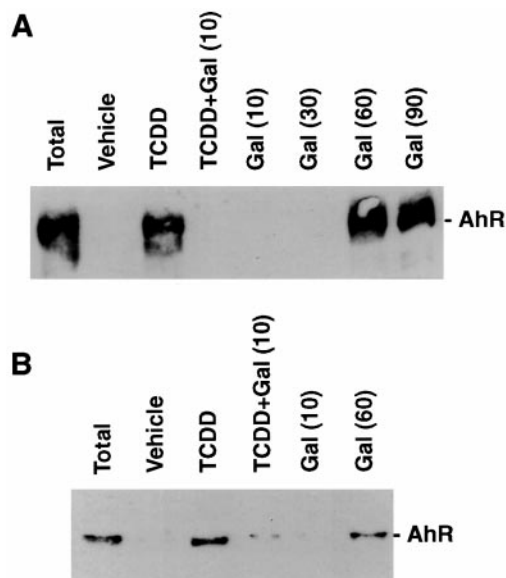


Fig. 11. High galangin doses (60–90 μ M) induce nuclear translocation of the AhR. Hepa-1 (A) or BMS2 cells (B) were treated with vehicle, 1 nM TCDD, TCDD + 10 μ M galangin, or 10, 60, or 90 μ M galangin, as indicated. Cells were harvested 1.5 h later and proteins extracted from whole cells and from nuclear preparations. Proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and membranes were probed with AhR-specific antibody. Data from two representative experiments are presented. The 95-kDa AhR protein is indicated.

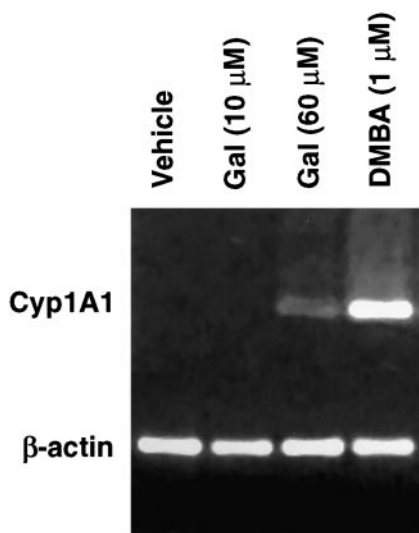


Fig. 12. High galangin doses (60 μ M) weakly induce CYP1A1 transcription in Hepa-1 cells. Hepa-1 cells were treated with vehicle, 10 μ M or 60 μ M galangin, or 1 μ M DMBA. Cells were harvested 14 h later, RNA was extracted and RNA assayed for CYP1A1 and β -actin mRNA expression by semiquantitative RT-PCR. A representative experiment is presented.

induced by PAH. These results are consistent with the hypothesis that at least some of the antiapoptotic activity of ANF is caused by AhR antagonism. However, these studies cannot rule out the possibility that ANF has additional activities, such as cytochrome P-450 inhibition, that uniquely affect PAH-induced apoptosis signaling distal to AhR activation.

Finally, one implication of the early block in AhR signaling demonstrated herein is the potential for galangin to moderate multiple AhR-dependent biologic responses. Since AhR activation has been implicated in malignant transformation (Kouri et al., 1982; Nebert et al., 1990; Safe and Krishan, 1995; Schindt and Bradfield, 1996), cytokine secretion, cell growth regulation, cell differentiation, and NF- κ B activation (reviewed in Nebert et al., 2000), these results suggest the use of this nontoxic bioflavonoid to inhibit multiple aberrant cellular responses to environmental AhR ligands like PAH, dioxins, and planar PCBs.

Acknowledgments

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