

Carnosol, a Constituent of Zyflamend, Inhibits Aryl Hydrocarbon Receptor–Mediated Activation of *CYP1A1* and *CYP1B1* Transcription and Mutagenesis

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Abstract

The aryl hydrocarbon receptor (AhR), a ligand-activated member of the basic helix-loop-helix family of transcription factors, plays a significant role in polycyclic aromatic hydrocarbon (PAH)-induced carcinogenesis. In the upper aerodigestive tract of humans, tobacco smoke, a source of PAHs, activates the AhR leading to increased expression of *CYP1A1* and *CYP1B1*, which encode proteins that convert PAHs to genotoxic metabolites. Inhibitors of Hsp90 ATPase cause a rapid decrease in levels of AhR, an Hsp90 client protein, and thereby block PAH-mediated induction of *CYP1A1* and *CYP1B1*. The main objective of this study was to determine whether Zyflamend, a polyherbal preparation, suppressed PAH-mediated induction of *CYP1A1* and *CYP1B1* and inhibited DNA adduct formation and mutagenesis. We also investigated whether carnosol, one of multiple phenolic antioxidants in Zyflamend, had similar inhibitory effects. Treatment of cell lines derived from oral leukoplakia (MSK-Leuk1) and skin (HaCaT) with benzo[a]pyrene (B[a]P), a prototypic PAH, induced *CYP1A1* and *CYP1B1* transcription, resulting in enhanced levels of message and protein. Both Zyflamend and carnosol suppressed these effects of B[a]P. Notably, both Zyflamend and carnosol inhibited Hsp90 ATPase activity and caused a rapid reduction in AhR levels. The formation of B[a]P-induced DNA adducts and mutagenesis was also inhibited by Zyflamend and carnosol. Collectively, these results show that Zyflamend and carnosol inhibit Hsp90 ATPase leading to reduced levels of AhR, suppression of B[a]P-mediated induction of *CYP1A1* and *CYP1B1*, and inhibition of mutagenesis. Carnosol-mediated inhibition of Hsp90 ATPase activity can help explain the chemopreventive activity of herbs such as Rosemary, which contain this phenolic antioxidant. *Cancer Prev Res*; 5(4); 593–602. ©2012 AACR.

Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated member of the basic helix-loop-helix family of transcription factors (1). It binds with high affinity to environmental toxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin; ref. 2) and polycyclic aromatic hydrocarbons (PAH), which are found in tobacco smoke, automobile exhaust, and charbroiled food. The AhR mediates most of the biochemical and toxicologic responses that occur following exposure to polychlorinated dioxins and PAH (3). Studies in genetically engineered mice have suggested a significant role for

the AhR in carcinogenesis. AhR-deficient mice were protected against PAH-induced skin tumors (4). Constitutive activation of the AhR led to mice that were more susceptible to chemical carcinogens (5).

In the absence of ligand, the AhR exists in the cytosol in a complex containing a dimer of the chaperone Hsp90, p23, and XAP2 (6, 7). Following ligand binding, the AhR translocates into the nucleus and forms a heterodimer with the AhR nuclear transporter (ARNT; refs. 8–10). The AhR-ARNT heterodimer binds to the upstream regulatory region of genes containing xenobiotic responsive elements (XRE), resulting in the transcriptional activation of a network of genes including *CYP1A1* and *CYP1B1*. Consistent with this mechanism, levels of *CYP1A1* and *CYP1B1* are increased in the oral and bronchial mucosa of smokers (11–13). The induction of xenobiotic metabolism by AhR ligands can provide a first line of defense against environmental carcinogens (14). Alternatively, the induction of xenobiotic metabolizing enzymes by ligand-activated AhR can increase the formation of carcinogenic metabolites, thus creating a link between the AhR and chemical carcinogenesis (15–17). Activation of the AhR can suppress antitumor immune responses and promote tumor cell survival (18). The connection between activation of AhR signaling and

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carcinogenesis has stimulated a search for chemopreventive agents that antagonize the AhR (19).

Zyflamend, a widely used polyherbal formulation produced from the extracts of 10 common herbs (rosemary, turmeric, ginger, holy basil, green tea, hu zhang, Chinese goldthread, barberry, oregano, and Baikal skullcap), possesses anticancer properties in preclinical models and is being investigated for its potential to inhibit prostate carcinogenesis in men (20–23). Several of the phytochemicals in Zyflamend including resveratrol, curcumin, epigallocatechin gallate, and carnosol have been reported to antagonize AhR-dependent gene expression and inhibit chemical carcinogenesis (24–29). In this study, we had 3 main objectives. First, we investigated whether Zyflamend inhibited PAH-mediated induction of CYP1A1 and CYP1B1 and blocked mutagenesis. Second, we investigated whether the effects of Zyflamend were mimicked by carnosol, a prototypic phenolic antioxidant, in Zyflamend. Third, we determined the mechanism by which both Zyflamend and carnosol suppressed AhR-dependent gene expression. Taken together, our results suggest that Zyflamend and carnosol inhibit Hsp90 ATPase activity leading, in turn, to reduced levels of AhR, inhibition of PAH-mediated induction of CYP1A1 and CYP1B1 and suppression of mutagenesis. The ability of carnosol to inhibit Hsp90 ATPase, a widely recognized pharmacologic target, is likely to be important for understanding the chemopreventive activity of herbs such as Rosemary, which contain high concentrations of this phenolic antioxidant.

Materials and Methods

Materials

Keratinocyte growth media (KGM) and keratinocyte basal media (KBM) were obtained from Lonza. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and Lipofectamine 2000 were from Invitrogen. Antibody to β -actin, Lowry protein assay kits, Lactate dehydrogenase (LDH) release assay kit, benzo[a]pyrene (B[a]P), α -naphthoflavone (α NF), and ATP were obtained from Sigma Chemical. Antibodies to CYP1A1, AhR, XAP2, and Hsp90 were from Santa Cruz Biotechnology, and antibody to p23 was obtained from Affinity Bioreagents. Antiserum to CYP1B1 was a generous gift of Dr. Craig B. Marcus (Oregon State University, Corvallis, OR). Western Lighting Plus ECL was purchased from Perkin Elmer. Nitrocellulose membranes were from Schleicher and Schuell. RNA was prepared with a kit from QIAGEN. PCR primers were synthesized by Sigma-Genosys. Murine leukemia virus reverse transcriptase and Taq polymerase were purchased from Applied Biosystems. Reagents for the luciferase assay were from Analytical Luminescence. Carnosol was obtained from Kalsec. NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were from Boehringer Mannheim. 17-allylamino-17-demethoxygeldanamycin (17-AAG) was from Cayman Chemicals. pSV β gal and plasmid DNA isolation kits were from Promega Corp. The XRE-luciferase construct was a gift from Dr. Michael S. Denison (University of California, Davis). A BPDE (anti) standard was obtained from the National

Cancer Institute Carcinogen Repository at the Midwest Research Institute. DNA extraction for mutagenesis was carried out with a Recoverase kit (Stratagene). For mutagenesis assays, a home made phage packaging extract prepared from *E coli* NM759 and BHB2688 (bacterial strains supplied by Dr. Peter Glazer, Yale University School of Medicine, New Haven, CT) was employed. Zyflamend, olive oil, and Rosemary extract were gifts from New Chapter, Inc. (Brattleboro, VT). Zyflamend is derived from extracts of 10 different herbs (w/w): holy basil (12.8%), ginger (12.8%), turmeric (14.1%), rosemary (19.2%), green tea (12.8%), hu zhang (10.2%), barberry (5.1%), oregano (5.1%), baikal skullcap (2.5%), and Chinese goldthread (5.1%).

Cell culture

MSK-Leuk1 cells were established from a premalignant dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue (30). Cells were routinely maintained in KGM, grown to 70% confluency, and trypsinized with 0.125% trypsin-2 mmol/L EDTA solution. HaCaT cells are spontaneously immortalized human epithelial keratinocytes (31). These cells were routinely maintained in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin, grown to 70% confluence and trypsinized with 0.125% trypsin-2 mmol/L EDTA solution. In all experiments, cells were grown in basal medium for 12 hours before treatment. Treatments were carried out in growth factor-free basal medium containing Zyflamend, Rosemary extract, or carnosol reconstituted in dimethyl sulfoxide. Cell toxicity was assessed by measurements of trypan blue exclusion and LDH release. There was no evidence of cell toxicity under the conditions used.

For mutagenesis studies, the BB rat cell line (Agilent Technologies) was employed. Cells were grown in DMEM containing 10% fetal calf serum (Hyclone) to about 30% confluence. Glutamine, G418-sulfate, and penicillin-streptomycin (Mediatech) were added to concentrations of 0.29 mg/mL, 50 IU/mL, 50 μ g/mL, and 0.22 mg/mL, respectively. The medium was changed to 1% serum 16 hours before the addition of inhibitor, and cells were treated with Zyflamend or carnosol overnight before addition of B[a]P. Two hours later, the medium was brought to 10% serum, and cells were grown until confluent and then harvested. Separate experiments were not done to confirm the authenticity of the cell lines used in our experiments.

Western blot analysis

Cell lysates were prepared by treating cells with lysis buffer as described previously (32). Lysates were sonicated for 8 minutes on ice and centrifuged at $14,000 \times g$ for 10 minutes at 4°C to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry (33). SDS-PAGE was conducted under reducing conditions on 10% polyacrylamide gels. The resolved proteins were transferred onto nitrocellulose sheets and then incubated with antisera to CYP1A1, CYP1B1, AhR, Hsp90, XAP2, p23, and β -actin. Secondary antibody to immunoglobulin G conjugated to horseradish

peroxidase was used. The blots were then reacted with the ECL Western blot detection system, according to the manufacturer's instructions.

Real-time quantitative PCR

Total RNA from cell lysates was isolated with the RNeasy Mini Kit (QIAGEN). RNA quantification and quality assessment was done by a NanoDrop 2000c (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies). RNA (1 μ g) was reverse transcribed with murine leukemia virus reverse transcriptase and oligo d(T)16 primer. The resulting cDNA was then used for amplification. Each PCR reaction volume was 20 μ L and contained 5 μ L cDNA, 2 \times SYBR Green PCR master mix, and forward and reverse primers. Primers used were: CYP1A1, forward 5'-CCTGCT-AGGGTTAGGAGGTC-3', reverse 5'-GCTCAGCCTAGTTC-AGCAG-3' and CYP1B1, forward 5'-ACGTACCGGCCAC-TATCACT-3', reverse 5'-CTCGAGTCTGCACATCAGGA-3'. Experiments were carried out with a 7500 real time PCR system (Applied Biosystems). β -Actin served as an endogenous normalization control. Relative expression was determined by ddCT (relative quantification) analysis.

Transient transfection

MSK-Leuk1 cells were seeded at a density of 8×10^5 cells per well in 6-well dishes and grown to 70% confluence. In each well, cells were cotransfected with 1.8 μ g of the XRE luciferase plasmid pGudLuc6.1 and 0.2 μ g of pSV β gal using 2 μ g Lipofectamine 2000 per the manufacturer's instructions. After 6 hours of incubation, the medium was replaced with basal medium, and the cells were allowed to grow for 12 hours before the experiments were carried out. The activities of luciferase and β -galactosidase were measured in cellular extract.

Hsp90 activity

The ATPase assay was based on a regenerating coupled enzyme assay (34). Hsp90 was immunoprecipitated from cell lysates and the pellet was resuspended and used for assay. Reaction was conducted in a 1 mL assay containing 100 mmol/L Tris-HCl pH 7.4, 20 mmol/L KCl, 6 mmol/L MgCl₂, 0.8 mol/L ATP, 0.1 mmol/L NADH, 2 mmol/L phosphoenolpyruvate, 0.2 mg pyruvate kinase, 0.05 mg L-LDH, and Hsp90 immunoprecipitated from cell lysates. Equal amounts of Hsp90 protein were used in each treatment group. Sufficient NADH was added to give an initial absorbance of 0.3 at 340 nm before addition of Hsp90 and activity was detected as a decrease in absorbance. Hsp90 ATPase activity is expressed as pmol/min/mg protein.

DNA adducts and B[a]P-tetrols

MSK-Leuk1 cells were grown to 70% confluence and placed in 10 mL KBM. Cells were pretreated with vehicle, Zylamend, carnosol, or α NF for 2 hours. Subsequently, MSK-Leuk1 cells received 1 μ mol/L B[a]P for 8 hours before cell harvest. Media (1 mL) was collected from each sample before cell harvest for the measurement of B[a]P-tetrols in media.

DNA was isolated and then hydrolyzed in 0.1 mol/L HCl at 90°C for 2 hours. This treatment releases B[a]P-tetrols from the N2-BPDE adducts (35, 36). After acid hydrolysis, the samples were cooled to room temperature and applied to a Restek Pinnacle II, 3 μ mol/L, 150 \times 2.1 mm C18 HPLC column. Aliquots of the hydrolysate were eluted in a mobile phase of 33% acetonitrile containing 10 mmol/L ammonium acetate, pH 6.0, at a flow rate of 0.2 mL/min. The eluate was analyzed with the fluorescence detector set at 344 nm excitation and 400 nm emission. A Shimadzu HPLC system consisting of an LC-20AD solvent delivery system, an SIL-10Ai autoinjector, an SPD-20AV UV-VIS detector, and an RF-10AxL fluorescence detector was used for analysis. Quantitation of the adducts was achieved by comparison with standards of the B[a]P-tetrol isomers. These were generated by incubating anti-BPDE in water at room temperature for 30 minutes (37). The major adduct designated BPDE tetrol I-1 (36) was produced in the cultured cells (35). Only trace amounts of the minor adduct, BPDE tetrol I-2, were detected. B[a]P-tetrols in the media result from the spontaneous decomposition of BPDE. Tetrols in the media were quantified by a similar method comparing with the standard of BPDE tetrols.

Mutagenesis

After treatment of the cells, DNA was extracted with a Recoverase kit per manufacturer's instructions, which involves cell lysis, isolation of nuclei, digestion with proteinase K and RNase, and dialysis on a membrane. Phage packaging was carried out with a phage packaging extract according to published methods (38).

The *cII* mutagenesis assay was then employed (39). The BB rat cell line contains a lambda shuttle vector that includes the bacterial *lacI* locus and also the *cII* gene, which is the target for the mutagenesis studies. This system also obviates the potential for *ex vivo* mutations that could complicate results. This assay detects mutations at the *cII* locus and possibly the regulator *cI* locus (39–42). The *cII* protein is a positive regulator of gene transcription that controls the decision between lytic or lysogenic development pathways in phage-infected cells. In appropriate *E. coli* (*E. coli* 1250) host cells, under specified conditions (25°C) only mutants give rise to phage plaques, whereas at 37°C all infected cells give rise to plaques, providing a phage titer (40–42). The mutant fraction (MF) is the ratio of mutant to nonmutant plaques and is the measure of mutagenesis.

Statistics

Comparisons between groups were made by Student *t* test. A difference between groups of $P < 0.05$ was considered significant.

Results

Zylamend suppresses B[a]P-mediated induction of CYP1A1 and CYP1B1 transcription in human epithelial cells

Initially, we evaluated the effect of Zylamend on B[a]P-mediated induction of CYP1A1 and CYP1B1. Western blot

analysis was carried out on lysates prepared from MSK-Leuk1 cells, a cell line that was established from an oral leukoplakia lesion. As shown in Fig. 1A, Zyflamend caused dose-dependent inhibition of B[a]P-mediated induction of CYP1A1 and CYP1B1. To confirm that these effects were not unique to MSK-Leuk1 cells, similar experiments were carried out in HaCaT cells, an immortalized human keratinocyte cell line. Once again, Zyflamend caused dose-dependent inhibition of B[a]P-mediated induction of CYP1A1 and CYP1B1 (Fig. 1B). To determine whether these suppressive effects of Zyflamend were pretranslational, levels of mRNA were measured by RT-qPCR. Consistent with the Western blot results, Zyflamend caused dose-dependent inhibition of B[a]P-mediated induction of CYP1A1 and CYP1B1 mRNA in MSK-Leuk1 and HaCaT cells (Fig. 2A and B).

Experiments were next carried out to determine whether Zyflamend blocked the induction of CYP1A1 and CYP1B1 mRNA by suppressing transcription. Transient transfections were carried out by an XRE-luciferase promoter construct. This promoter construct was selected because ligand-activated AhR binds to the XREs in the promoters of *CYP1A1* and *CYP1B1*, leading to increased transcription. Treatment with B[a]P led to a marked increase in XRE-luciferase activity in both MSK-Leuk1 and HaCaT cells, an effect that was

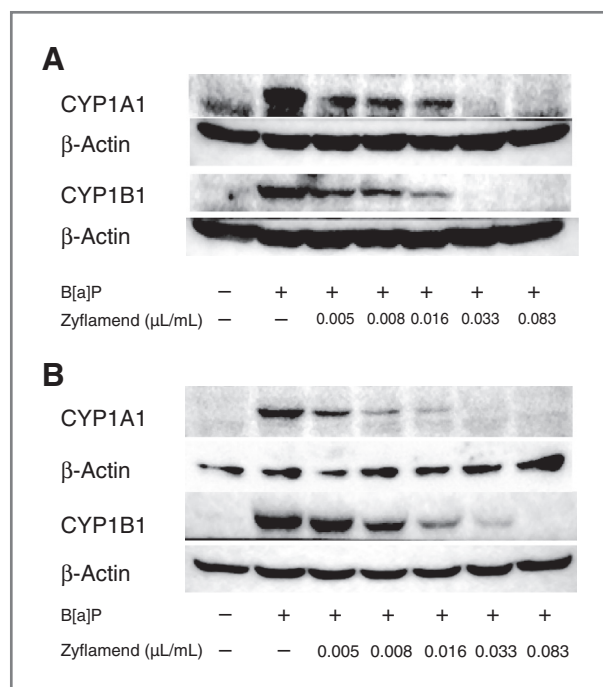


Figure 1. Zyflamend suppresses B[a]P-mediated induction of CYP1A1 and CYP1B1 proteins. MSK-Leuk1 (A) and HaCaT (B) cells were treated with vehicle or the indicated concentrations of Zyflamend for 2 hours. Cells were then treated with vehicle or 1 $\mu\text{mol/L}$ B[a]P. Five hours later, cells were harvested for Western blot analysis. Cellular lysate protein (100 $\mu\text{g/lane}$) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibodies specific for CYP1A1, CYP1B1, and β -actin.

suppressed by Zyflamend in a dose-dependent manner (Fig. 2C).

To investigate the mechanism by which Zyflamend blocked B[a]P-mediated activation of gene expression, levels of proteins known to be involved in AhR signaling were determined. Treatment with a concentration of Zyflamend that blocked the induction of CYP1A1 and CYP1B1 led to a significant decrease in the amount of AhR within two hours in both MSK-Leuk1 and HaCaT cell lines (Fig. 3A and B). In contrast, Zyflamend did not affect the amount of other proteins that are important for AhR signaling, including Hsp90, XAP2 and p23 (Fig. 3A and B). Because inhibitors of Hsp90 ATPase activity including 17-AAG are known to reduce AhR protein levels and suppress PAH-mediated induction of CYP1A1 and CYP1B1 (32, 43), we next determined whether Zyflamend inhibited Hsp90 ATPase activity. Interestingly, Zyflamend caused dose-dependent inhibition of Hsp90 ATPase activity over the same concentration range that blocked B[a]P-mediated induction of *CYP1A1* and *CYP1B1* transcription (Fig. 4A).

Carnosol suppresses B[a]P-mediated induction of CYP1A1 and CYP1B1

Because Zyflamend is a complex mixture of extracts from 10 common herbs, we were interested in determining whether any particular class of pure phytochemical possessed the same properties as the complex mixture. A two-step strategy was employed. First, we evaluated whether Rosemary, the most abundant component of Zyflamend, inhibited B[a]P-mediated induction of CYP1A1 and CYP1B1. Consistent with the findings for Zyflamend, Rosemary extract caused dose-dependent inhibition of B[a]P-mediated induction of CYP1A1 and CYP1B1 (Fig. 5A). Rosemary like other constituents of Zyflamend is enriched in polyphenolic antioxidants such as carnosol, which possess anticancer properties (28). Therefore, our second step was to focus on carnosol, a prototypic polyphenol, to determine whether its effects mimicked Zyflamend. Carnosol like Zyflamend and Rosemary caused dose-dependent suppression of B[a]P-mediated induction of CYP1A1 and CYP1B1 protein in both MSK-Leuk1 and HaCaT cells (Fig. 5B and C). Consistent with the findings for Zyflamend, carnosol also blocked B[a]P-mediated induction of CYP1A1 and CYP1B1 mRNA and XRE-luciferase activity (Fig. 5D and E). Like Zyflamend, treatment with carnosol also led to a rapid decrease in the amount of AhR protein without modulating levels of Hsp90, p23, and XAP2 (Fig. 3C). Carnosol also inhibited Hsp90 ATPase activity (Fig. 4B) over the same dose range that suppressed B[a]P-mediated activation of *CYP1A1* and *CYP1B1* transcription.

B[a]P-induced DNA adduct formation and mutagenesis are suppressed by Zyflamend and carnosol

Both the CYP1A1 and CYP1B1 mixed function oxidase systems can metabolize PAHs including B[a]P into DNA-reactive species that form adducts (44, 45). More specifically, B[a]P is converted to a toxic metabolite, anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydroB[a]P (BPDE),

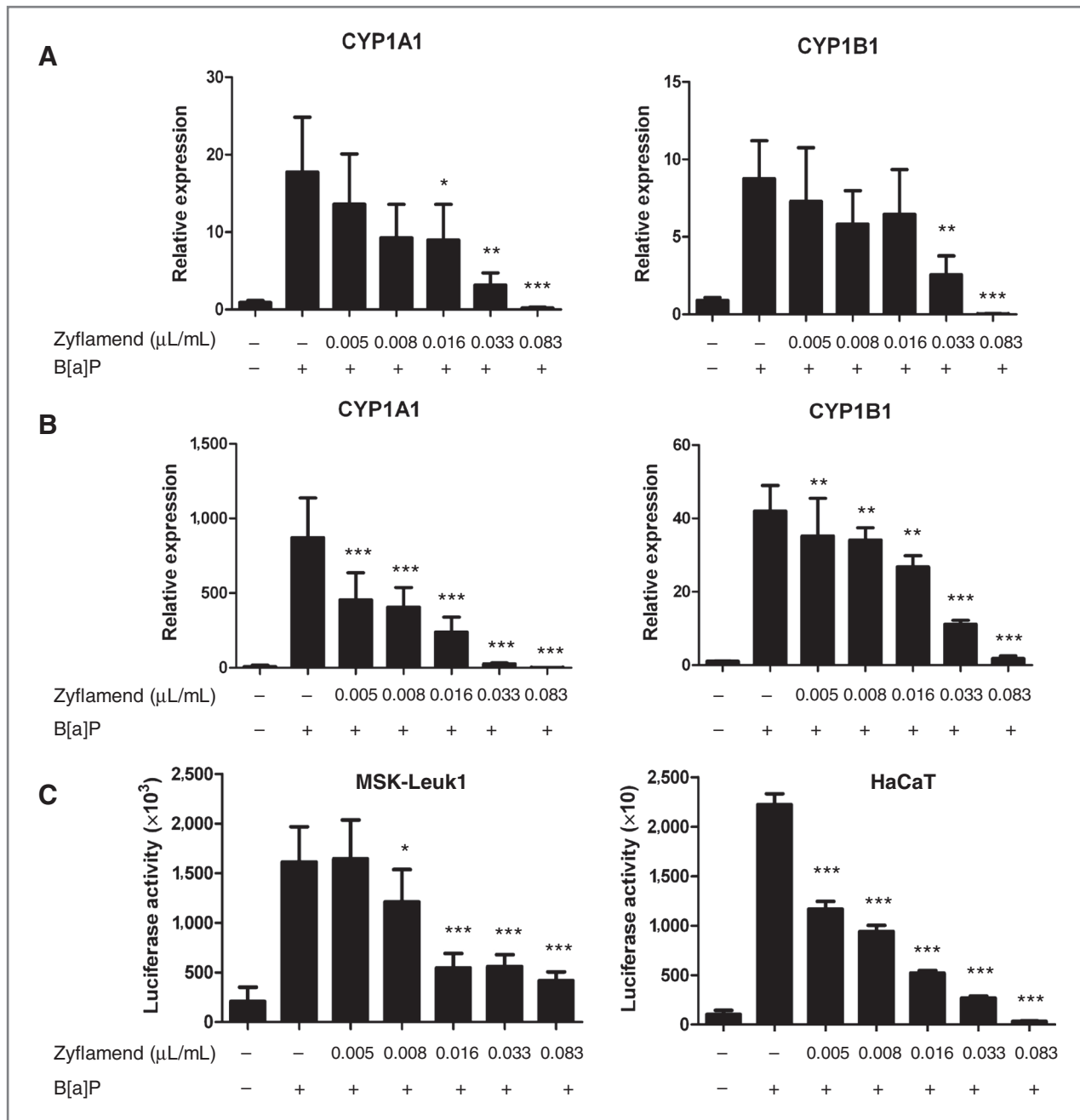


Figure 2. Zyflamend suppresses B[a]P-mediated induction of *CYP1A1* and *CYP1B1* transcription. MSK-Leuk1 (A) and HaCaT (B) cells were treated with vehicle or the indicated concentration of Zyflamend for 2 hours. Subsequently, cells received vehicle or 1 μmol/L B[a]P for 3 hours. Total RNA was then isolated and quantitative real-time PCR was conducted. Values for *CYP1A1* and *CYP1B1* were normalized to values obtained for β-actin. Columns, mean (*n* = 3); bars, SD. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. C, MSK-Leuk1 and HaCaT cells were transfected with 1.8 μg of pGudLuc6.1 (XRE-luciferase) and 0.2 μg of pSVβgal. After transfection, cells were treated with vehicle or the indicated concentration of Zyflamend for 2 hours, followed by treatment with vehicle or 1 μmol/L B[a]P. Reporter activities were measured in cellular extract 10 hours after treatment. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, mean (*n* = 6); bars, SD. *, *P* < 0.05; ***, *P* < 0.001.

which covalently binds to DNA, forming bulky adducts that induce mutations. Because Zyflamend blocked B[a]P-mediated induction of *CYP1A1* and *CYP1B1*, we postulated that it might inhibit both the formation of DNA adducts and mutagenesis. Zyflamend inhibited B[a]P-induced DNA

adduct formation by approximately 80% in MSK-Leuk1 cells (Fig. 6A). α-Naphthoflavone, a known AhR antagonist, served as a positive control, and inhibited DNA adduct formation by more than 90%. BPDE is extremely reactive and undergoes spontaneous hydrolysis in aqueous media to

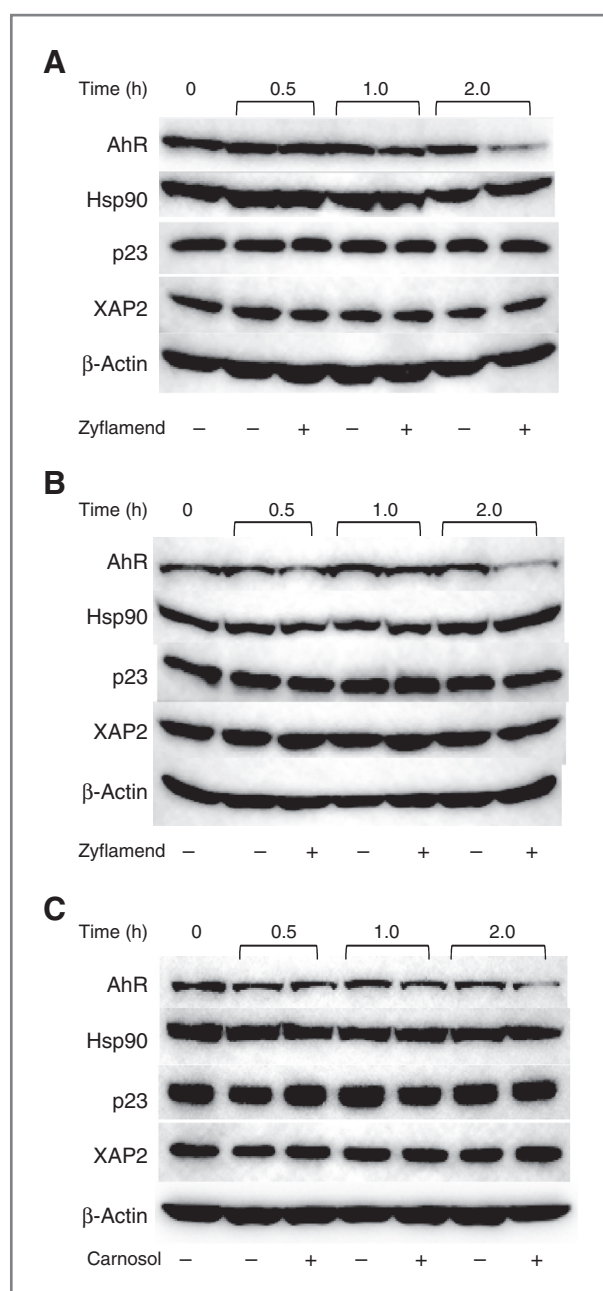


Figure 3. Zyflamend and carnosol downregulate levels of AhR protein in epithelial cells. MSK-Leuk1 (A) and HaCaT (B) cells were treated with vehicle or Zyflamend (0.033 μ L/mL) as indicated for 0 to 2.0 hours. Cells were then harvested for Western blot analysis. C, MSK-Leuk1 cells were treated with vehicle or 10 μ mol/L carnosol for 0 to 2.0 hours and were then harvested for Western blot analysis. A–C, cellular lysate protein was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibodies specific for AhR, Hsp90, p23, XAP2, and β -actin. Results are representative of a minimum of 3 independent experiments.

B[a]P-tetrols. To determine whether the observed suppression of DNA adducts mediated by Zyflamend reflected changes in B[a]P metabolism, levels of B[a]P-tetrols were measured in cell media. As shown in Fig. 6B, both Zyfla-

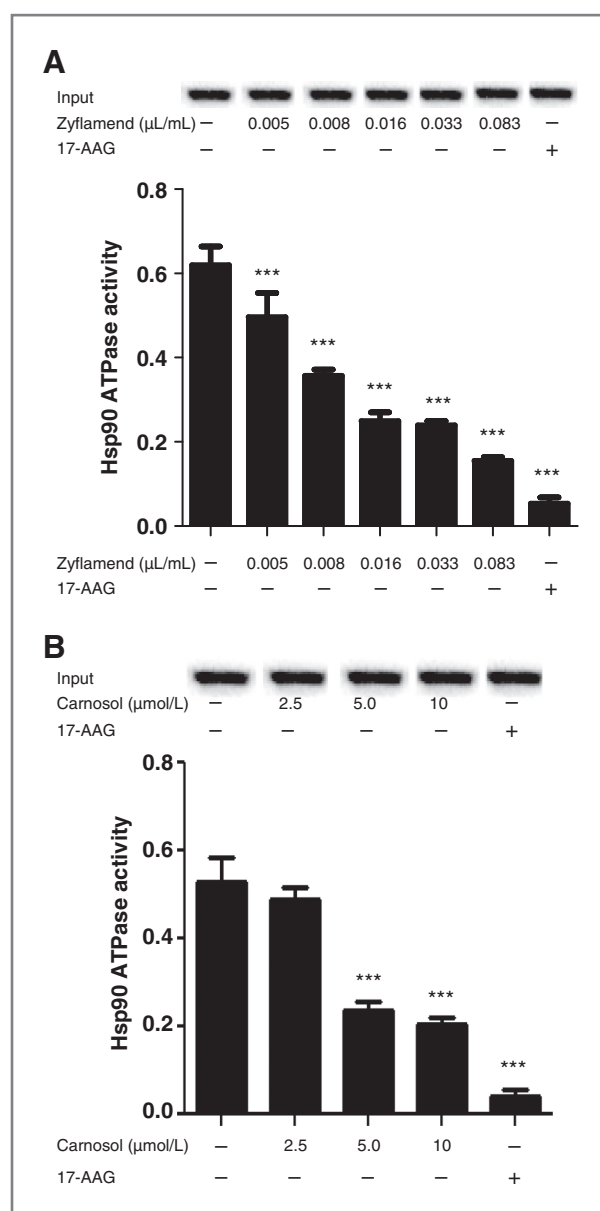


Figure 4. Zyflamend and carnosol inhibit Hsp90 ATPase activity. MSK-Leuk1 cells were treated with indicated concentrations of Zyflamend (A) or carnosol (B) for 30 minutes. Hsp90 was immunoprecipitated and Hsp90 ATPase activity was measured as described in Materials and Methods. The activity is presented as pmol/min/mg protein. The input indicates the amount of Hsp90 that was immunoprecipitated. Columns, mean ($n = 3$); bars, SD. ***, $P < 0.001$.

mend and α NF inhibited B[a]P-tetrol formation by approximately 90%. To extend these findings, we next investigated whether Zyflamend inhibited B[a]P-induced mutagenesis. As shown in Fig. 6C, treatment with Zyflamend led to dose-dependent suppression of B[a]P-induced mutations.

Because carnosol blocked B[a]P-mediated induction of CYP1A1 and CYP1B1, we postulated that it might also suppress the formation of DNA adducts and mutagenesis. Similar to results observed with Zyflamend, treatment with

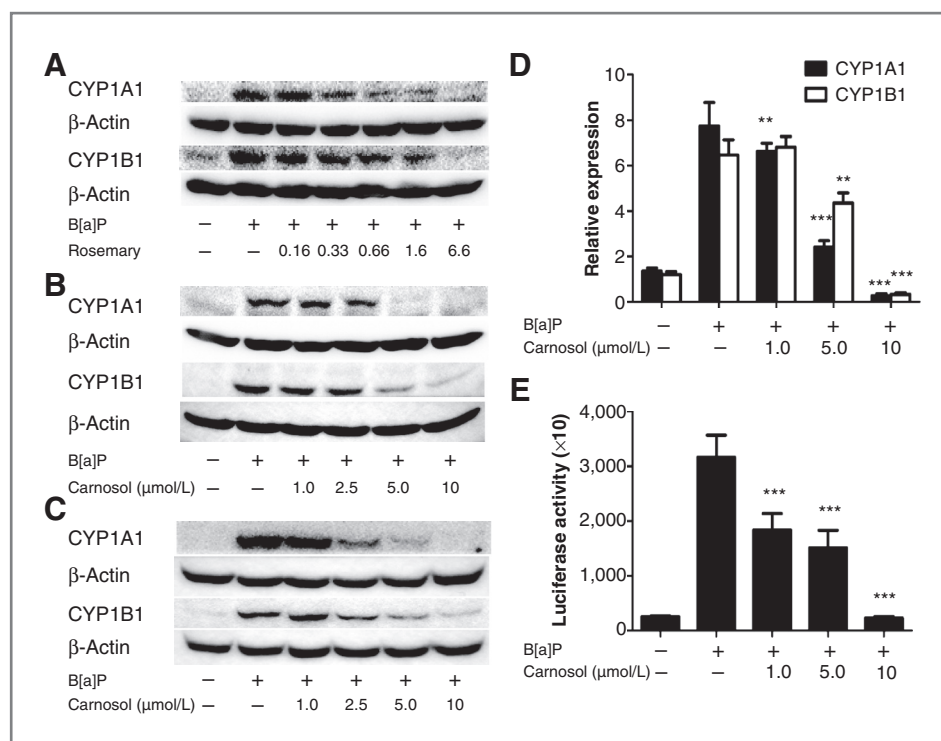


Figure 5. Carnosol, a component of Rosemary, suppresses B[a]P-mediated induction of CYP1A1 and CYP1B1. A and B, MSK-Leuk1 cells were treated with vehicle, the indicated concentrations of Rosemary extract (A) or carnosol (B) for 2 hours. Rosemary concentrations are presented as $\times 10^{-3}$ $\mu\text{L}/\text{mL}$ of media. Subsequently, cells received vehicle or 1 $\mu\text{mol}/\text{L}$ B[a]P for 5 hours before being harvested for Western blot analysis. C, HaCaT cells were treated with vehicle or the indicated concentrations of carnosol for 2 hours. Cells then received vehicle or 1 $\mu\text{mol}/\text{L}$ B[a]P for 5 hours before being harvested for Western blot analysis. A–C, immunoblots were probed with antibodies specific for CYP1A1, CYP1B1, and β -actin. D, MSK-Leuk1 cells were treated with vehicle or carnosol for 2 hours. Subsequently, cells received vehicle or 1 $\mu\text{mol}/\text{L}$ B[a]P for 3 hours. Total RNA was isolated and quantitative real-time PCR was carried out. Values for CYP1A1 and CYP1B1 were normalized to values obtained for β -actin. Columns, means ($n = 3$); bars, SD. **, $P < 0.01$; ***, $P < 0.001$. E, MSK-Leuk1 cells were transfected with 1.8 μg of pGudLuc6.1 (XRE-luciferase), and 0.2 μg of pSV β gal. After transfection, cells were treated with vehicle or the indicated concentration of carnosol for 2 hours, followed by treatment with vehicle or 1 $\mu\text{mol}/\text{L}$ B[a]P. Reported activities were measured in cellular extract 10 hours after treatment. Luciferase activity represents data that have been normalized to β -galactosidase activity. Columns, mean ($n = 6$); bars, SD. ***, $P < 0.001$.

carnosol markedly reduced B[a]P-mediated formation of DNA adducts (Fig. 6D). Moreover, the formation of B[a]P-tetrols (Fig. 6E) and mutagenesis (Fig. 6F) were suppressed.

Discussion

Approximately, 30% of the cancer burden in the United States can be attributed to tobacco smoke exposure. Despite multiple efforts to reduce tobacco use, cessation is not possible for many individuals. Consequently, alternate risk reduction strategies need to be considered. One such approach is chemoprevention. Hence, developing a practical approach to reduce the procarcinogenic effects of PAHs in tobacco smoke remains a significant goal. As detailed above, there is considerable evidence that targeting the AhR is a potential strategy to reduce PAH-induced cancer (19). In this study, we found that both Zyflamend and carnosol, a prototypic phenolic antioxidant in Zyflamend, blocked B[a]P-mediated activation of *CYP1A1* and *CYP1B1* transcription and reduced the formation of DNA adducts and mutagenesis in B[a]P-treated cells. These effects seemed to be a consequence of inhibition of Hsp90 ATPase activity.

Hsp90 is a molecular chaperone that modulates client protein folding and thereby prevents nonspecific aggregation of unfolded or misfolded proteins. In addition to AhR, Hsp90 is a chaperone for numerous other client proteins that have been implicated in cancer including ErbB2, androgen receptor (AR), Akt, Flt3, Bcr-Abl, and B-Raf (46, 47). Inhibitors of Hsp90 suppress levels of numerous client proteins and are being evaluated as potential anticancer agents (46, 48, 49). Here, we showed that both Zyflamend and carnosol caused dose-dependent inhibition of Hsp90 ATPase activity over the same concentration range that blocked the induction of AhR-dependent gene expression. Consistent with the known effects of 17-AAG, a prototypic Hsp90 inhibitor, both Zyflamend and carnosol caused a rapid decrease in AhR protein levels and blocked induction of AhR-dependent gene expression (32, 43). Because inhibitors of Hsp90 ATPase activity stimulate the degradation of client proteins, it is predictable that Zyflamend and carnosol should have additional effects. In support of this possibility, Zyflamend was recently found to destabilize AR protein levels in prostate cancer cells, thereby causing a rapid reduction in AR levels and reduced responsiveness to

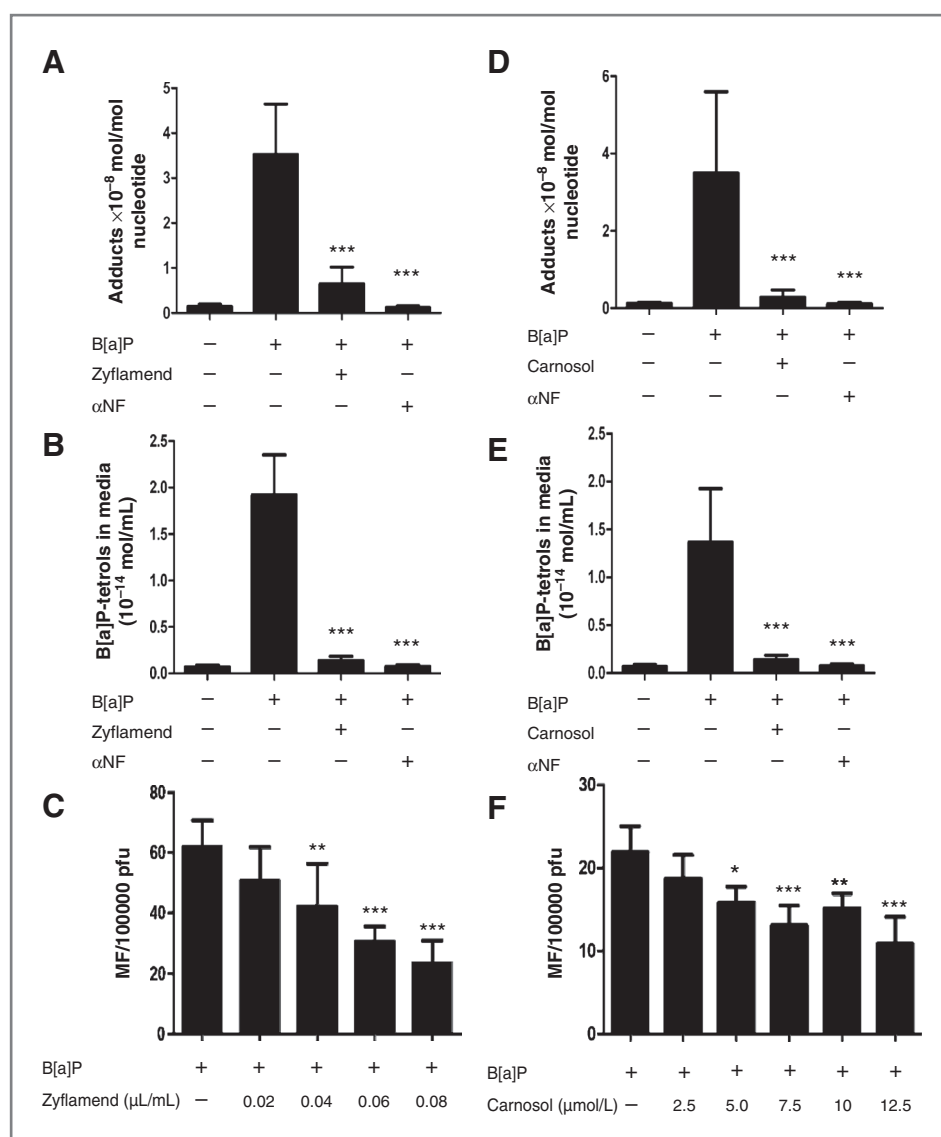


Figure 6. Zyflamend and carnosol inhibit B[a]P-induced DNA adduct formation and mutagenesis. A, B, D, and E, MSK-Leuk1 cells were pretreated with vehicle, Zyflamend (1 μ L/10 mL), carnosol (10 μ mol/L), or 1 μ mol/L α -naphthoflavone (α NF) as indicated for 2 hours. Cells were then treated with vehicle or 1 μ mol/L B[a]P for 8 hours. DNA was isolated for quantification of DNA adducts (A, D); B[a]P-tetrol formation was measured in the media (B, E). C and F, BB cells were pretreated with the indicated concentrations of Zyflamend or carnosol overnight and subsequently 2 μ mol/L B[a]P was added. Cells were then grown to confluence and DNA was isolated. Mutagenesis assays were then carried out with the isolated DNA. Columns, mean ($n = 6$); bars, SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

dihydrotestosterone, an AR agonist (23). We note that Zyflamend has been evaluated in a clinical trial involving men with high-grade prostatic intraepithelial neoplasia (22). Nearly half of the study subjects showed a 25–50% decrease in PSA, a gene regulated by the AR. The reduction in PSA levels may have reflected decreased AR signaling due, in part, to downregulation of AR protein levels. Based on the findings in our study, it will be worthwhile to determine whether treatment with Zyflamend or carnosol suppress levels of other Hsp90 client proteins.

It is important to put the carnosol findings into context. First, carnosol, a major constituent of Rosemary, was previously shown to inhibit the induction of AhR-dependent genes but the underlying mechanism was uncertain (29). As mentioned above, these results strongly suggest that carnosol-mediated inhibition of Hsp90 ATPase activity causes a rapid decrease in AhR protein levels thereby inhibiting the

induction of CYP1A1 and CYP1B1 by B[a]P. The fact that carnosol inhibits Hsp90 ATPase activity is likely to contribute to the chemopreventive activity of Rosemary against skin tumorigenesis in mice (28). Celastrol and gadunin, 2 other phenolic antioxidants that inhibit Hsp90 ATPase activity, also block the induction of AhR-dependent genes (32, 50). On the basis of this constellation of findings, we speculate that inhibition of Hsp90 ATPase activity will be a property shared by many phenolic antioxidants with known chemopreventive properties. Future studies are warranted to elucidate the mechanism by which these compounds inhibit Hsp90 ATPase activity. Moreover, the fact that multiple phenolic antioxidants inhibit Hsp90 ATPase activity is likely to help explain why Zyflamend, a polyherbal formulation, inhibits Hsp90 ATPase activity. Because Zyflamend is in clinical use, it will be worthwhile to determine whether standard doses inhibit Hsp90 ATPase activity

and thereby modulate client protein function in humans. It is possible, for example, that treatment with Zyflamend will inhibit Hsp90 ATPase activity and thereby downregulate the elevated levels of CYP1A1 and CYP1B1 found in the oral mucosa of smokers (11). Recent evidence that ligands of the AhR suppress antitumor immune responses and promote tumor cell survival suggests that Zyflamend may have a range of additional significant antitumor effects that can be explored (18). Another consideration is the optimal route of administration of Zyflamend. The results of the recent human prostate study suggest that orally administered Zyflamend may be sufficiently bioavailable to have systemic effects (22). Administering Zyflamend topically as a rinse or lozenge might protect against tobacco smoke-mediated induction of AhR-dependent genes in the oral mucosa. Taken together, this study shows that Zyflamend and carnosol inhibit Hsp90 ATPase activity leading to reduced levels of AhR, suppression of B[a]P-mediated induction of CYP1A1 and CYP1B1, and inhibition of DNA adduct formation and mutagenesis. Carnosol-mediated inhibition of Hsp90 ATPase activity is likely to contribute to the chemopreventive activity of herbs such as Rosemary, which contain this phenolic antioxidant.

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Disclosure of Potential Conflicts of Interest

A.J. Dannenberg is a member of the scientific advisory board of Tragara Pharmaceuticals, Inc.

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