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p53 Modulates Hsp90 ATPase Activity and Regulates Aryl Hydrocarbon Receptor Signaling

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Abstract

The aryl hydrocarbon receptor (AhR), a client protein of heat shock protein 90 (Hsp90), is a ligand activated transcription factor that plays a role in polycyclic aromatic hydrocarbon (PAH) induced carcinogenesis. Tobacco smoke activates AhR signaling leading to increased transcription of *CYP1A1* and *CYP1B1*, which encode proteins that convert PAHs to mutagens. Recently, p53 was found to regulate Hsp90 ATPase activity via effects on activator of Hsp90 ATPase (Aha1). It's possible, therefore, that AhR-dependent expression of *CYP1A1* and *CYP1B1* might be affected by p53 status. The main objective of this study was to determine if p53 modulated AhR-dependent gene expression and PAH metabolism. Here we show that silencing p53 led to elevated Aha1 levels, increased Hsp90 ATPase activity and enhanced *CYP1A1* and *CYP1B1* expression. Overexpression of wild type p53 suppressed levels of *CYP1A1* and *CYP1B1*. The significance of Aha1 in mediating these p53-dependent effects was determined. Silencing of Aha1 led to reduced Hsp90 ATPase activity and down regulation of *CYP1A1* and *CYP1B1*. In contrast, overexpressing Aha1 was associated with increased Hsp90 ATPase activity and elevated levels of *CYP1A1* and *CYP1B1*. Using p53 heterozygous mutant epithelial cells from Li-Fraumeni Syndrome patients, we show that monoallelic mutation of p53 was associated with elevated levels of *CYP1A1* and *CYP1B1* under both basal conditions and following treatment with benzo[a]pyrene. Treatment with CP-31398, a p53 rescue compound, suppressed benzo[a]pyrene-mediated induction of *CYP1A1* and *CYP1B1* and the formation of DNA adducts. Collectively, our results suggest that p53 affects AhR-dependent gene expression, PAH metabolism and possibly carcinogenesis.

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Conflicts of Interest

The authors have no conflicts of interest to declare

Keywords

p53; Hsp90; aryl hydrocarbon receptor; CYP1A1; CYP1B1

Introduction

The aryl hydrocarbon receptor (AhR) is a member of the basic-helix-loop-helix family of transcription factors. It binds with high affinity to polycyclic aromatic hydrocarbons (PAH) which are found in tobacco smoke, diesel engine emissions and charbroiled food (1). Studies in genetically engineered mice suggest a role for the AhR in carcinogenesis (2, 3). Mice engineered to be AhR-deficient were protected against PAH induced skin tumors (2). In transgenic mice, a constitutively active AhR rendered mice more susceptible to carcinogens (3).

In the absence of ligand, the AhR is present in the cytosol in a complex that includes a dimer of the chaperone Hsp90. Once a PAH such as benzo[a]pyrene (B[a]P) binds to the AhR, it translocates to the nucleus and forms a heterodimer with the AhR nuclear transporter (ARNT). The activated AhR/ARNT complex binds to the upstream regulatory regions of genes containing xenobiotic response elements (XRE), resulting in the induction 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 (4–6). CYP1A1 and CYP1B1 can convert B[a]P to a toxic metabolite, anti-7, 8-dihydroxy-9, 10-epoxy-7, 8, 9, 10-tetrahydrobenzo[a]pyrene (BPDE; (7)), which covalently binds to DNA, forming bulky DNA adducts that induce mutations (8). In this regard, metabolically activated PAHs including BPDE have been reported to form adducts within the *p53* tumor suppressor gene that correspond to *p53* mutational hot spots in lung cancer (9, 10).

The *p53* tumor suppressor gene encodes a homo-tetrameric transcription factor which is activated in response to a variety of cell stressors (11–14). Under these conditions, the *p53* protein is stabilized, initiating a transcriptional program that may result in apoptosis, senescence, DNA repair or cell cycle arrest (13). More than 50% of cancers contain mutant *p53* (15). *p53* mutations have also been found in premalignant lesions (16–18). Recently, we showed that *p53* regulated Hsp90 ATPase activity via effects on Aha1 raising the possibility that Hsp90 client proteins such as AhR might be affected (19). Although PAH metabolites such as BPDE have been suggested to cause *p53* mutations (9, 10), little is known about the potential of *p53* to modulate the Aha1-Hsp90-AhR signaling axis resulting in changes in levels of CYP1A1 and CYP1B1 and PAH metabolism. Hence, this study had several objectives. First, we investigated whether *p53* regulated AhR-dependent expression of CYP1A1 and CYP1B1 in cell lines derived from the aerodigestive tract. Second, we used *p53* heterozygous mutant epithelial cell lines from Li-Fraumeni Syndrome (LFS) patients to determine if a similar mechanism was operative. Finally, we investigated whether CP-31398, a *p53* rescue agent (20, 21), suppressed B[a]P-mediated induction of CYP1A1 and CYP1B1 and DNA adduct formation. Taken together, this study provides new insights into mechanisms by which *p53* regulates AhR signaling and ultimately PAH metabolism,

which may be important for both understanding chemical carcinogenesis and developing chemopreventive strategies.

Materials and Methods

Materials

Keratinocyte growth media (KGM) and keratinocyte basal media (KBM) were obtained from Lonza. RPMI, fetal bovine serum (FBS), and Lipofectamine 2000 were from Invitrogen. Antibody to β -actin, Lowry protein assay kits, lactate dehydrogenase (LDH) release assay kit, B[a]P, α -naphthoflavone (α NF), and ATP were from Sigma Chemical. Antibodies to CYP1A1, AhR, p53 and Hsp90 were from Santa Cruz Biotechnology. Antibody to Aha1 was from Abcam. Antiserum to CYP1B1 was a generous gift of Dr. Craig B. Marcus (Oregon State University, Corvallis, OR). CP-31398 and PU-H71 were obtained from Tocris Bioscience. 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. NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were from Boehringer Mannheim. 17-allylamino-17-demethoxygeldanamycin (17-AAG) was from Cayman Chemicals. Reagents for the luciferase assay, 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). Aha1 expression vector was obtained from GeneCopoeia. p53 luciferase plasmid was from Panomics. A BPDE (anti) standard was obtained from the National Cancer Institute Carcinogen Repository at the Midwest Research Institute.

Cell culture

MSK-Leuk1 cells were established from a premalignant dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue (22). Sequencing indicated a GC to AT transition in exon 8 (position 14525) in one allele of p53, resulting in a glu to lys mutation in codon 286 (data not shown). Cells were routinely maintained in KGM, grown to 70% confluence, and trypsinized with 0.125% trypsin-2 mmol/L EDTA solution. BEAS2B, NCI-H1770 and NCI-H1299 cell lines were obtained from the American Type Culture Collection (ATCC) and maintained according to ATCC instructions. The human colon carcinoma cell line EB-1 was kindly provided by Dr. Arnold J. Levine (Princeton University) (23, 24). This cell line carries wild-type p53 under the control of an inducible metallothionein promoter that exhibits little or no detectable basal activity but sustains high levels of p53 expression following stimulation with zinc chloride. These cells were maintained in RPMI media with 10% FBS, and supplemented with 0.5g/L geneticin (G418). The HME32, HME50 and IUSM/LFS/HME cells have been described previously and were provided by Dr. Brittney-Shea Herbert (Indiana University School of Medicine) (25, 26). Cell toxicity was assessed by measurements of trypan blue exclusion and LDH release. There was no evidence of cell toxicity under the conditions used. Separate experiments were not done to confirm the authenticity of the cell lines used in our experiments.

Immunoprecipitation

Immunoprecipitation was carried out with a kit from Upstate Biotechnology according to the manufacturer's instructions. The immunoprecipitates were then subjected to Western blot analysis.

Western blot analysis

Cell lysates were prepared by treating cells with lysis buffer as described previously (27). 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 (28). 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, p53, AhR, Hsp90, Aha1 and β -actin. Secondary antibody to immunoglobulin G conjugated to horseradish peroxidase was used. The blots were then incubated 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 μg) 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 μL and contained 5 μL cDNA, $2\times$ SYBR Green PCR master mix, and forward and reverse primers. Primers used were: CYP1A1, forward 5'-CCTGCTAGGGTTAGGAGGTC-3', reverse 5'-GCTCAGCCTAGTTCAAGCAG-3' and CYP1B1, forward 5'-ACGTACCGGCCACTATCACT-3', reverse 5'-CTCGAGTCTGCACATCAGGA-3'. Primers for Aha1 and β -actin, an endogenous normalization control, were purchased from Qiagen. Experiments were carried out with a 7500 real time PCR system (Applied Biosystems). Relative expression was determined by ddCT (relative quantification) analysis.

Transfections

Cells were grown to 60–70% confluence in 6-well dishes and then transfected using Lipofectamine 2000 for 24 hours. Subsequently, the medium was replaced with serum free medium for another 24 hours. Luciferase and β -galactosidase were measured in cellular extracts. MSK-Leuk1 cells stably expressing vector alone or Aha1 were selected by growing the cells in 500 $\mu\text{g}/\text{mL}$ hygromycin.

RNA Interference

MSK-Leuk1 cells were seeded in KGM for 24 hours before transfection. Two μg of siRNA oligonucleotides were transfected using DharmaFECT 4 transfection reagent according to the manufacturer's instructions.

ChIP assay

ChIP assays were performed with a kit (Upstate Biotechnology) according to the manufacturer's instructions. The cells (2×10^6) were cross-linked in a 1% formaldehyde solution for 10 minutes at 37°C. The cells were then lysed in 200 µL of SDS buffer and sonicated to generate 200–1000-bp DNA fragments. After centrifugation, the cleared supernatant was diluted 10-fold with ChIP buffer and incubated with 1.5 µg of the indicated antibody at 4°C. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65°C for 4 hours, and the DNA fragments were purified and dissolved in 50 µL of water. Ten µL of each sample were used as a template for PCR amplification. The forward and reverse primers used for amplifying the *CYP1A1* promoter are: 5'-ACCCGCCACCCTTCGACAGTTC-3' and 5'-TGCCCAGGCGTTGCGTGAGAAG-3'. Forward and reverse primers used to amplify the *CYP1B1* promoter are: 5'-GTTCCCTTATAAAGGGAG-3' and 5'-CTGCGATGGAAGCCGTTG-3' (27). PCR was performed at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds for 30 cycles. The PCR products generated from the ChIP template were sequenced, and the identity of the *CYP1A1* and *CYP1B1* promoters was confirmed.

Hsp90 activity

The ATPase assay was based on a regenerating coupled enzyme assay (29). 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, CP-31398, or αNF for 2 hours. Subsequently, cells received 1 µmol/L B[a]P for 12 hours before cell harvest. Medium (1 mL) was collected from each sample before cell harvest for the measurement of B[a]P-tetrols. 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 (30, 31). After acid hydrolysis, the samples were cooled to room temperature and applied to a Restek Pinnacle II, 3 µ, 150 × 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 0.12 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. 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 (32). The major adduct designated BPDE tetrol I-1 (31) was produced in the cultured cells (30). 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 released into the media were quantified by a similar method of comparison with the standard BPDE tetrols.

Statistics

Comparisons between groups were made by Student's *t* test. A difference between groups of $P < 0.05$ was considered significant.

Results

p53 regulates AhR-dependent gene expression

Here we evaluated the potential role of p53 as a determinant of AhR signaling. Initially, we used EB-1 cells, a cell line that expresses Zn⁺⁺ inducible wild type p53 (23, 24). EB-1 cells were transfected with a p53-luciferase reporter construct. Treatment with ZnCl₂ caused a dose-dependent increase in p53 expression and a corresponding increase in p53 luciferase activity (Fig. 1A). Next, in order to determine if p53 regulated AhR-mediated gene expression, we carried out transient transfections using an XRE-luciferase construct. This reporter construct was selected because the AhR binds to XREs in the promoters of *CYP1A1* and *CYP1B1*, leading to altered transcription. The same concentration range of ZnCl₂ that activated p53 luciferase caused a dose-dependent decrease in XRE-luciferase activity (Fig. 1B), and a reduction in amounts of CYP1A1 and CYP1B1 mRNA and protein in the EB-1 cells (Fig. 1, C and D).

B[a]P, a PAH that binds to the AhR, is a prototypic inducer of CYP1A1 and CYP1B1. As shown in Fig. 1E, levels of CYP1A1 and CYP1B1 were lower in B[a]P-treated EB-1 cells in which ZnCl₂ was used to overexpress p53. Because PAHs are believed to contribute to smoking-related epithelial malignancies, we next evaluated the importance of p53 in regulating CYP1A1 and CYP1B1 expression in cell lines derived from the upper aerodigestive tract. MSK-Leuk1 cells were derived from an oral premalignant lesion and express one mutant allele of p53 (22). Silencing of p53 in MSK-Leuk1 cells led to higher levels of CYP1A1 and CYP1B1 under basal conditions and following treatment with B[a]P (Fig. 1F). Three lung cell lines that vary in p53 status (BEAS2B, wild type; NCI-H1770, mutant; NCI-H1299, null) were transfected with either p53-luciferase or XRE-luciferase constructs (Fig. 1, G and H). Consistent with the findings in EB-1 cells, an inverse relationship was observed between p53 activity and XRE-luciferase activity. Here too, higher levels of CYP1A1 and CYP1B1 were found in cells that were null for p53 or expressed mutant p53 under both basal conditions and following treatment with B[a]P (Fig. 1, I and J).

We next investigated the effects of CP-31398, a p53 rescue compound, on AhR-dependent gene expression. Because CP-31398 has the potential to have off-target effects(33), it was important to establish the concentration range that activated p53. Transient transfections were carried out to determine the concentration range of CP-31398 that activated p53 in

MSK-Leuk1 cells. As shown in Fig. 2A, CP-31398 caused dose-dependent activation of p53. Notably, B[a]P-mediated induction of CYP1A1 and CYP1B1 was suppressed by the same concentrations of CP-31398 that activated p53 (Fig. 2B). Similarly, CP-31398 activated p53 and blocked B[a]P-mediated induction of CYP1A1 and CYP1B1 in NCI-H1770 cells (Fig. 2, C and D).

The Aha1-Hsp90 axis mediates the effects of p53 on AhR-dependent gene expression

Previously, we reported that loss of p53 function led to HSF-1-mediated induction of Aha1, activator of Hsp90 ATPase (19, 33, 34). Because AhR is a client protein of Hsp90, we determined whether the p53/Aha1/Hsp90 axis contributed to the regulation of CYP1A1 and CYP1B1 expression. Silencing of p53 in MSK-Leuk1 cells led to increased levels of Aha1 mRNA (data not shown) and protein (Fig. 3A), increased interactions between Aha1 and Hsp90 (Fig. 3B), increased Hsp90 ATPase activity (Fig. 3C) and elevated levels of CYP1A1 and CYP1B1 (Fig. 1F). Silencing of Aha1 led to reduced Hsp90 ATPase activity and decreased levels of CYP1A1 and CYP1B1 (Fig. 3, D and E). In contrast, overexpression of Aha1 in MSK-Leuk1 cells led to increased levels of Hsp90 ATPase activity and enhanced expression of CYP1A1 and CYP1B1 under both basal conditions and following treatment with B[a]P (Fig. 3, F and G). Next, experiments were performed to confirm that the observed changes in Hsp90 ATPase activity were important for regulating the expression of CYP1A1 and CYP1B1. Thus, treatment with either 17-AAG or PU-H71, prototypic inhibitors of Hsp90 ATPase activity, suppressed levels of CYP1A1 and CYP1B1 (Fig. 3H).

p53 regulates the intracellular localization of AhR in MSK-Leuk1 cells

Given the importance of AhR in regulating the transcription of *CYP1A1* and *CYP1B1*, we next determined the effects of p53 on the intracellular localization of AhR. Because silencing of p53 led to increased levels of CYP1A1 and CYP1B1 (Fig. 1F), we initially evaluated whether silencing p53 affected the localization of AhR. As shown in Fig. 4A, silencing p53 caused a shift in the intracellular localization of AhR from cytosol to nucleus. ChIP assays were performed to determine whether the increased nuclear localization of AhR in p53 deficient cells was associated with increased recruitment of AhR to the *CYP1A1* and *CYP1B1* promoters. In fact, silencing of p53 led to about a doubling in the recruitment of AhR to the promoters of both genes (Fig. 4B). Since CP-31398, the p53 rescue agent, suppressed B[a]P-mediated induction of CYP1A1 and CYP1B1 (Fig. 2, B and D), we evaluated its effects on B[a]P-mediated shuttling of AhR from the cytosol to the nucleus. As shown in Fig. 4C, B[a]P stimulated the translocation of AhR from the cytosol to the nucleus, an effect that was inhibited by CP-31398. Consistent with this finding, CP-31398 also inhibited B[a]P-mediated stimulation of AhR recruitment to the *CYP1A1* and *CYP1B1* promoters (Fig. 4D).

Levels of CYP1A1 and CYP1B1 are increased in epithelial cells from Li Fraumeni Syndrome p53 mutation carriers

Levels of Aha1 and Hsp90 ATPase activity are increased in LFS cells (19). Here we investigated whether these effects were also associated with changes in AhR-dependent gene expression. In fact, levels of CYP1A1 and CYP1B1 were increased in LFS cells compared

to wild-type p53 HME32 cells under both basal conditions (Fig. 5A) and following treatment with B[a]P (Fig. 5, B–E). The increased levels of CYP1A1 and CYP1B1 were associated with a marked increase in amounts of AhR in the nucleus compared to the cytosol in LFS cells (Fig. 5F).

B[a]P-induced DNA adduct formation is suppressed by CP-31398

Both CYP1A1 and CYP1B1 can metabolize PAHs such as B[a]P into reactive species that form DNA adducts (35, 36). Because CP-31398 suppressed B[a]P-mediated induction of CYP1A1 and CYP1B1 (Fig. 2, B and D), we postulated that it might suppress B[a]P metabolism to BPDE and the formation of DNA adducts. Treatment with B[a]P led to a marked increase in DNA adducts, an effect that was attenuated by treatment with CP-31398 (Fig. 6A). α -Naphthoflavone, a known AhR antagonist that inhibits B[a]P metabolism (37), served as a control, and inhibited DNA adduct formation by >90%. The major pathway by which B[a]P is metabolized to a genotoxic metabolite involves its oxidation to BPDE. The epoxidation reactions in this pathway are catalyzed by the cytochrome P450 mixed function oxidase system, with CYP1A1 and/or CYP1B1 playing a major role (38). BPDE is extremely reactive and undergoes spontaneous hydrolysis in aqueous medium to B[a]P-tetrols. To determine if the observed suppression of DNA adducts mediated by CP-31398 reflected changes in B[a]P metabolism, the levels of B[a]P-tetrols were measured in the cell medium. As shown in Fig. 6B, treatment with CP-31398 caused more than a 75% suppression of B[a]P-tetrol formation ($P < 0.05$). Taken together, reactivating p53 with CP-31398 appears to inhibit the metabolic activation of B[a]P resulting in the suppression of B[a]P-induced DNA adducts.

Discussion

p53 regulates Aha1 levels and thereby Hsp90 ATPase activity (19). Several studies have demonstrated that AhR is a client protein of Hsp90 (39, 40). Previously, we showed that inhibiting Hsp90 ATPase activity blocked PAH-mediated induction of *CYP1A1* and *CYP1B1* transcription and DNA adduct formation (41). Here we extend upon these observations by demonstrating that p53 is a determinant of CYP1A1 and CYP1B1 levels under both basal conditions and following stimulation with B[a]P. Several lines of evidence support these points. Levels of CYP1A1 and CYP1B1 were increased in LFS-derived p53 heterozygous vs. normal epithelial cells. Silencing of p53 led to increased Aha1 levels, elevated Hsp90 ATPase activity and increased levels of CYP1A1 and CYP1B1. In contrast to the effects of silencing p53, expression of wild type p53 in EB-1 cells, a p53 null cell line, caused a gene dose-dependent suppression of CYP1A1 and CYP1B1 mRNA and protein. Moreover, overexpression of wild type p53 or treatment with CP-31398, the p53 rescue compound, blocked B[a]P-mediated induction of CYP1A1 and CYP1B1. Because p53 can regulate Hsp90 ATPase activity, the significance of Hsp90 in regulating AhR signaling was tested. We showed that 17-AAG and PU-H71, prototypic inhibitors of Hsp90 ATPase, down regulated the expression of CYP1A1 and CYP1B1. To further evaluate the functional significance of Aha1, activator of Hsp90 ATPase, in mediating p53-dependent changes in AhR-dependent gene expression, Aha1 was silenced. Silencing of Aha1 led to reduced Hsp90 ATPase activity and down regulated CYP1A1 and CYP1B1. Interestingly, stably

overexpressing Aha1 in MSK-Leuk1 cells was associated with increased Hsp90 ATPase activity and elevated levels of CYP1A1 and CYP1B1 both under basal conditions and following treatment with B[a]P. The above changes in levels of CYP1A1 and CYP1B1 reflected changes in gene transcription. This conclusion is supported by evidence that the AhR was primarily in the nucleus rather than the cytosol in LFS-derived p53 heterozygous vs. normal epithelial cells. Moreover, silencing of p53 led to a shift in the AhR from cytosol to nucleus in association with increased recruitment of the AhR to the promoters of *CYP1A1* and *CYP1B1*. Consistent with these findings, CP-31398 blocked B[a]P-mediated shuttling of AhR from cytosol to nucleus and the related recruitment of AhR to the promoters of *CYP1A1* and *CYP1B1*. Both endogenous and exogenous ligands can activate AhR-dependent gene expression (4, 42). It is possible, therefore, that p53 modulates the levels of an endogenous ligand leading to the observed effects. An alternate possibility is that these effects of p53 are ligand-independent. For example, p53 might have post-translational effects on Hsp90 that could impact on its chaperoning activity (27).

It is important to consider the potential implications of these mechanistic findings. Several studies have suggested that PAH metabolites may play a clinically relevant role in the pathogenesis of p53 mutations (9, 10). Possibly, PAH-mediated induction of CYP1A1 and CYP1B1 will contribute to p53 mutations resulting in further increases in xenobiotic metabolism, leading to increased DNA adduct formation and mutagenesis. It is also important to acknowledge that the AhR can suppress antitumor immune responses (43). p53 can influence innate immune responses as part of its tumor suppressor activities (44). Perhaps p53 mutations will contribute to a failure of immune surveillance by stimulating AhR. Whether these mechanisms contribute to the observed increased incidence of second primary tumors in head and neck squamous cell carcinoma patients who continue to smoke is unknown and warrants consideration (45).

It is also relevant to consider our findings in the context of LFS. LFS is associated with an increased risk of several tumor types including breast cancer (46). Some studies have suggested an increased risk of non-small cell lung cancer in LFS families (47). Previously, using expression arrays, AhR signaling was suggested to be increased in phenotypically normal epithelial cells from LFS p53 mutation carriers (26). The current study extended upon these findings. We found a significant increase in the transcriptional activity of AhR in untreated, normal-appearing epithelial cells taken from LFS carriers leading to both elevated basal levels of CYP1A1 and CYP1B1 and increased expression following treatment with B[a]P. It's possible, therefore, that augmented xenobiotic metabolism, reduced immune surveillance or both may contribute to the increased cancer risk among LFS patients. Because CYP1B1 can catalyze the conversion of estrogens into DNA reactive mutagens (48), our data may be relevant for understanding the pathogenesis of tumors including breast cancer among LFS patients.

There was good concordance between the effects of CP-31398 on levels of B[a]P-tetrols in the medium and DNA adducts. This suggests that levels of B[a]P-tetrols in the medium serve as markers of cellular metabolism of B[a]P to BPDE. Our results suggest that CP-31398, the p53 rescue compound blocks B[a]P-mediated induction of CYP1A1 and CYP1B1, resulting in reduced B[a]P metabolism and DNA adduct formation. An alternative

explanation, such as induction of DNA repair activity by CP-31398, would not explain the significant reduction in levels of B[a]P-tetrols found in the medium following treatment with the p53 rescue agent. Based on our findings, it seems predictable that suppressing PAH-mediated induction of CYP1A1 and CYP1B1 should suppress the formation of DNA adducts and thereby inhibit carcinogenesis. However, this may not be the case *in vivo*. Studies with *CYP1A1* knockout mice have suggested that this enzyme is important in detoxification and protection against B[a]P toxicity (49). Moreover, some models of PAH induced carcinogenesis indicate that activation of AhR signaling may protect against chemical carcinogenesis (50). As mentioned above, the AhR plays a significant role in immune surveillance in addition to xenobiotic metabolism. Moreover, p53 rescue compounds should have multiple anti-tumor activities in addition to effects on AhR-dependent functions. Administering a p53 rescue compound topically as a rinse or lozenge or even in an aerosol might protect against tobacco smoke-mediated induction of AhR-dependent genes and possibly mutagenesis in the upper aerodigestive tract. Whether such agents will reduce the risk of smoking-related malignancies warrants consideration.

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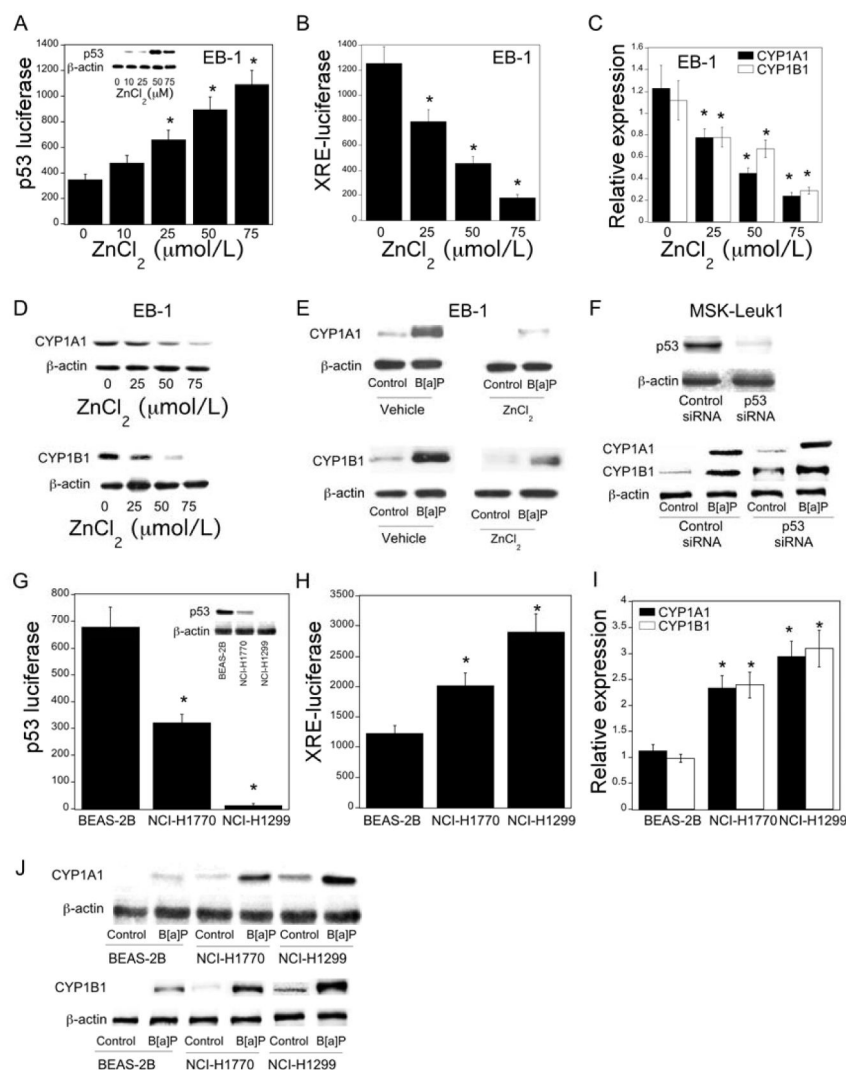


Figure 1. p53 regulates AhR signaling

A and B, EB-1 cells were transfected with 1.8 μg of p53 luciferase construct (A) or 1.8 μg of XRE-luciferase construct (B) and 0.2 μg of pSVβgal. 24 hours later, cells were treated with the indicated concentrations of ZnCl₂ for 12 hours and then cells were harvested. In A, cell lysates were also subjected to Western blotting and the blot was probed as indicated. C and D, cells were treated with the indicated concentrations of ZnCl₂ for 12 hours. C, RNA was isolated from cells and relative expression of CYP1A1 and CYP1B1 was quantified by real-time PCR. E, EB-1 cells were treated with vehicle or 75 μmol/L ZnCl₂ for 12 hours. Subsequently, cells were treated with DMSO (Control) or 1 μmol/L B[a]P for 12 hours. D and E, cell lysates were subjected to Western blotting and the blots were probed as indicated. F, MSK-Leuk1 cells were transfected with 2 μg of p53 siRNA or control siRNA. Cells were then treated with vehicle or 1 μmol/L B[a]P for 12 hours. Cell lysates were prepared and subjected to Western blotting and probed as indicated. G and H, cells were transfected with 1.8 μg of p53 luciferase construct (G) or 1.8 μg of XRE-luciferase (H) construct and 0.2 μg of pSVβgal for 24 hours. I, RNA was isolated from cells and relative expression of CYP1A1 and CYP1B1 mRNA was quantified by real-time PCR. J, cells were

treated with vehicle or 1 $\mu\text{mol/L}$ B[a]P for 12 hours. Cell lysates were subjected to Western blotting and probed as indicated. In A, B, G and H, luciferase and β -galactosidase activities were measured. Luciferase activity was normalized to β -galactosidase activity. In A–C, G–I, mean \pm SD are shown, n = 6. *, P < 0.01 compared with vehicle treated cells (A–C) or BEAS-2B cells (G–I).

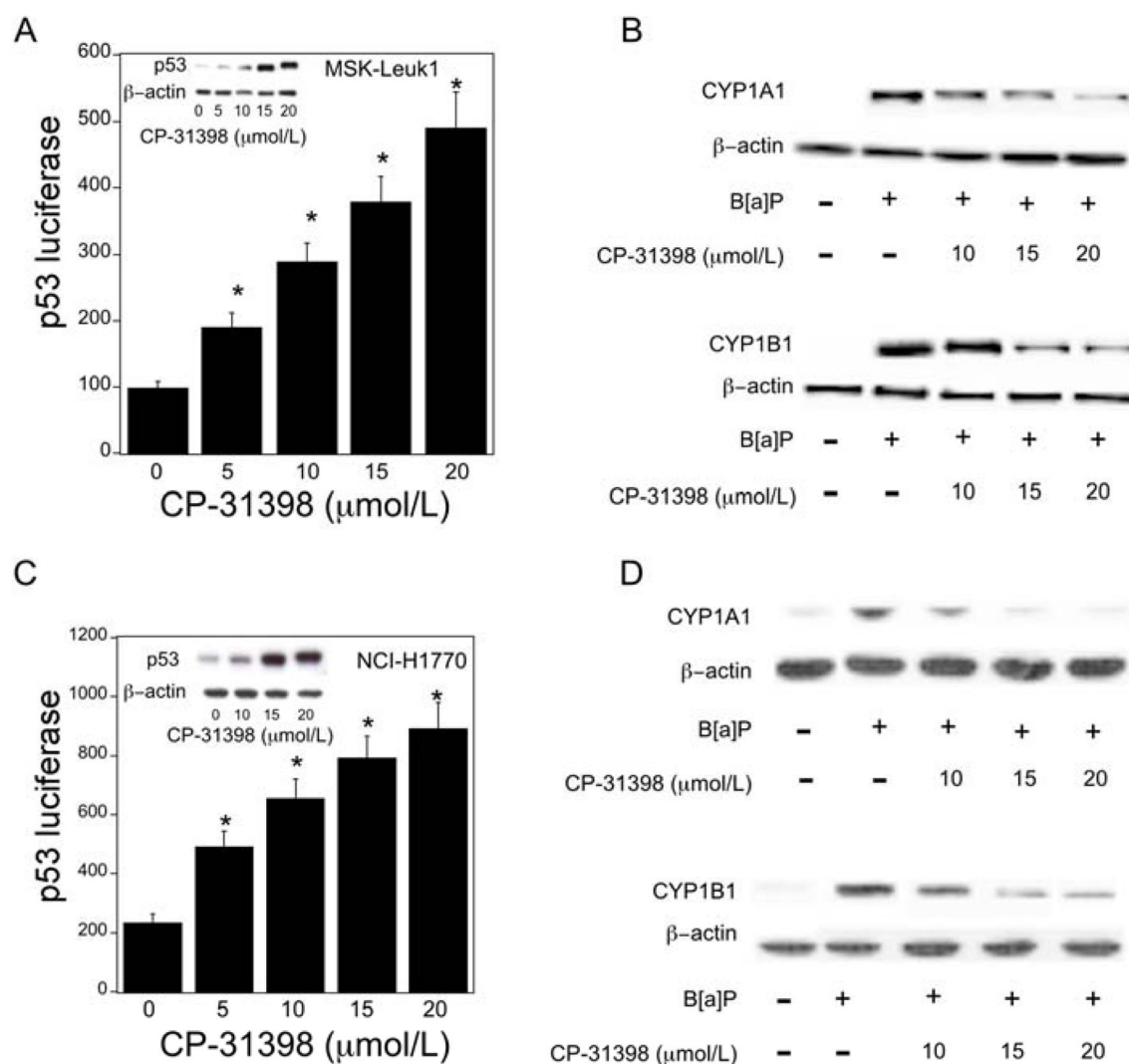


Figure 2. CP-31398, a p53 rescue compound, inhibits B[a]P-mediated induction of CYP1A1 and CYP1B1

A and C, cells were transfected with 1.8 μ g of p53 luciferase construct and 0.2 μ g of pSV β gal. Subsequently, the cells were treated with the indicated concentrations of CP-31398 for 24 hours and then luciferase and β -galactosidase activities were measured. Luciferase activity was normalized to β -galactosidase activity. A and C, mean \pm SD are shown, n = 6. *, P < 0.01 compared with vehicle-treated cells. B and D, cells were treated with vehicle or the indicated concentrations of CP-31398 for 2 hours. Subsequently, cells were treated with vehicle or 1 μ mol/L B[a]P. Twelve hours later cells were harvested for Western blot analysis. The blots were probed as indicated.

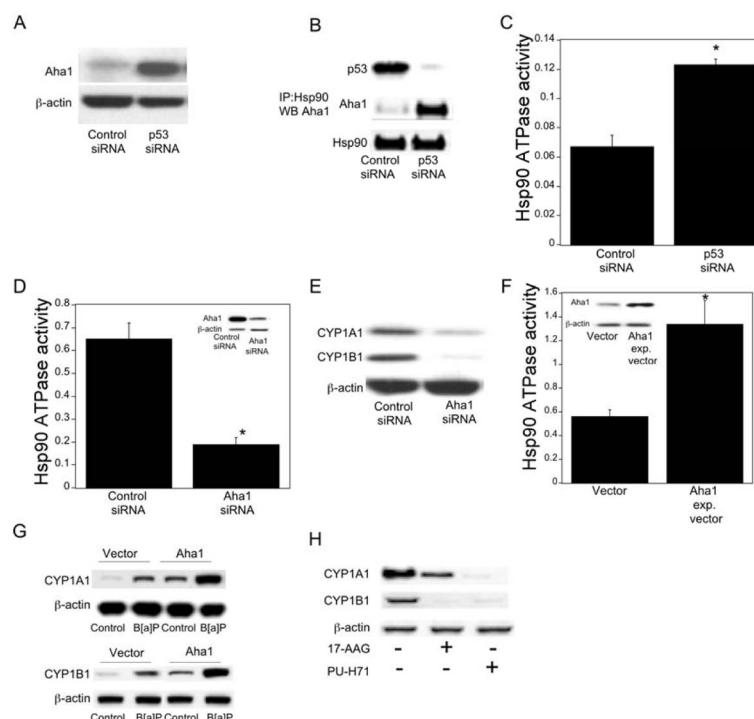


Figure 3. Aha1 and Hsp90 are important for p53-mediated regulation of AhR signaling in MSK-Leuk1 cells

A–C, cells were transfected with 2 μ g of control siRNA or siRNA to p53 for 48 hours. A, cell lysates were subjected to Western blotting and the blots probed as indicated. B, cell lysate protein (500 μ g) was subjected to immunoprecipitation with an antibody to Hsp90. Immunoprecipitates were then subjected to Western blotting and the blots probed for Aha1 and Hsp90 as indicated. C, cell lysates were used to measure Hsp90 ATPase activity. D and E, cells were transfected with 2 μ g of Aha1 siRNA or control siRNA. D, Hsp90 ATPase activity was measured in cell lysates. Inset, cell lysates were subjected to Western blotting for Aha1 and β -actin. E, cell lysate protein (2 mg) was subjected to immunoprecipitation separately with antibodies to CYP1A1, CYP1B1 and β -actin, respectively. The immunoprecipitates were then subjected to Western blotting and the blots probed as indicated. F and G, cells were stably transfected with control vector or Aha1 expression vector. F, cell lysates were used to measure Hsp90 ATPase activity. Inset, cell lysates were subjected to Western blotting and the blots probed for Aha1 and β -actin as indicated. G, cells were treated with vehicle or 1 μ mol/L B[a]P. Twelve hours later, cells were harvested for Western blot analysis and the blots probed as indicated. H, cells were treated with 1 μ mol/L 17-AAG or 0.5 μ mol/L PUH-H71 for 24 hours. Cell lysate protein (2 mg) was subjected to immunoprecipitation separately with antibodies to CYP1A1, CYP1B1 and β -actin, respectively. The immunoprecipitates were then subjected to Western blotting and the blots probed as indicated. In C, D and F, mean \pm SD are shown, $n = 6$. *, $P < 0.01$.

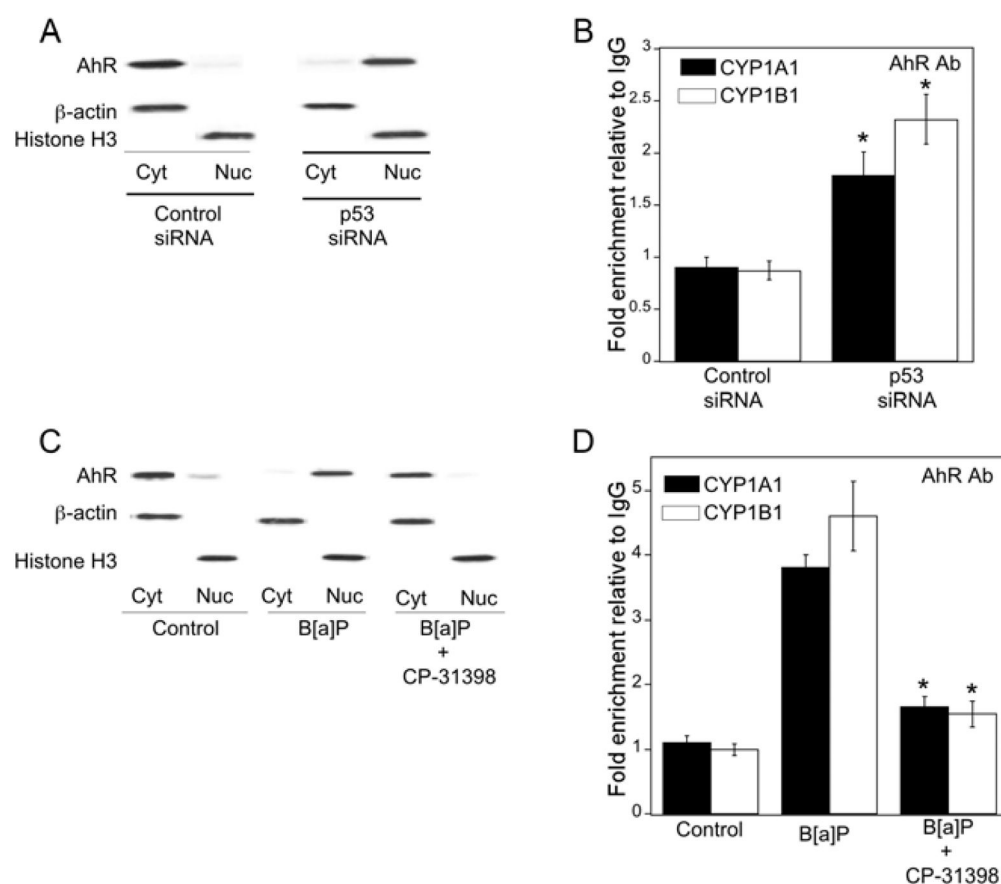


Figure 4. p53 regulates the localization of AhR in MSK-Leuk1 cells

In A and B, cells were transfected with 2 μ g of control siRNA or siRNA to p53 for 48 hours. A, cells were lysed, cytosolic (Cyt) and nuclear (Nuc) fractions were isolated and subjected to Western blotting and the blots probed as indicated. B, ChIP assays were performed. Chromatin fragments were immunoprecipitated with antibodies against AhR, and the *CYP1A1* and *CYP1B1* promoters were amplified by PCR. C, cells were treated vehicle or 20 μ mol/L CP-31398 for 2 hours. Subsequently, the cells were treated with vehicle (control) or 1 μ mol/L B[a]P for 2 hours. Cytosolic (Cyt) and nuclear (Nuc) fractions were isolated and subjected to Western blotting and the blots were probed as indicated. D, cells were treated with vehicle or 20 μ mol/L CP-31398 for 2 hours. Subsequently, cells received vehicle (control) or 1 μ mol/L B[a]P for an additional 4 hours. ChIP assays were then performed. Chromatin fragments were immunoprecipitated with antibodies against AhR, and the *CYP1A1* and *CYP1B1* promoters were amplified by PCR. In B and D, DNA sequencing was carried out, and the PCR products were confirmed to be the correct promoters. In B and D, mean \pm SD are shown, n = 6. *, P < 0.01 compared with control siRNA (B) or B[a]P-treated cells (D).

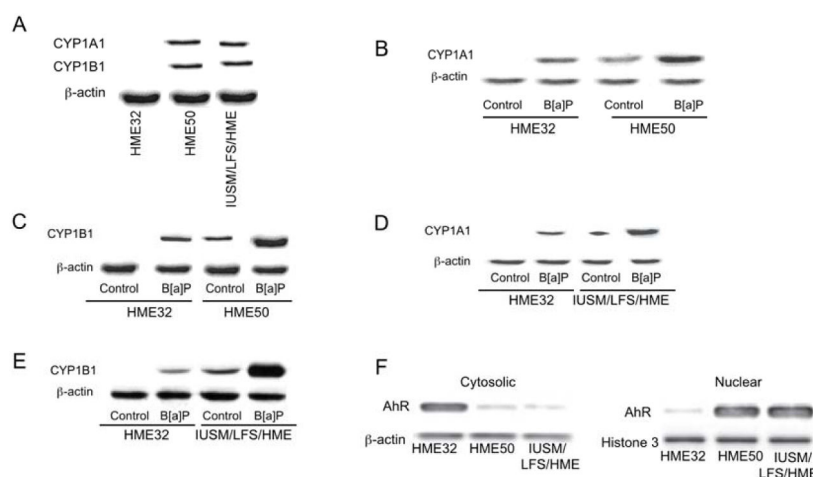


Figure 5. AhR signaling is activated in cells derived from Li-Fraumeni Syndrome patients LFS epithelial cells (HME50, IUSM/LFS/HME) and epithelial cells wild type for p53 (HME32) were compared. A, Cell lysate protein (1 mg) was subjected to immunoprecipitation separately with antibodies to CYP1A1, CYP1B1 and β-actin, respectively. The immunoprecipitates were then subjected to Western blotting and the blots probed as indicated. In B–E, cells were treated with DMSO (control) or 1 μmol/L B[a]P for 12 hours. Cell lysates were then subjected to immunoblotting and the blots probed as indicated. F, cytosolic and nuclear fractions were isolated and subjected to Western blotting. The blots were probed as indicated.

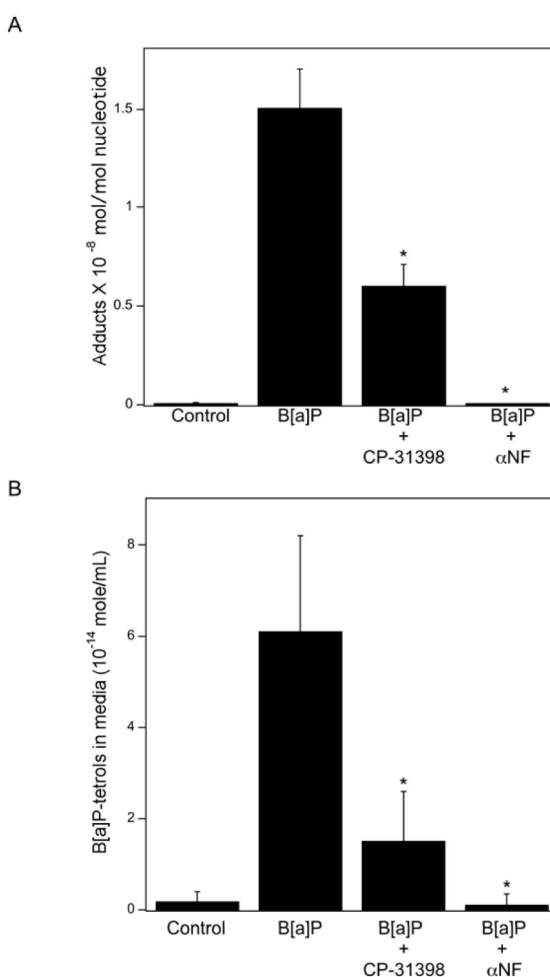


Figure 6. CP-31398 inhibits B[a]P-induced DNA adduct formation

MSK-Leuk1 cells were treated with vehicle, 20 $\mu\text{mol/L}$ CP-31398 or 2 $\mu\text{mol/L}$ αNF for 2 hours. Subsequently, the cells received vehicle or 1 $\mu\text{mol/L}$ B[a]P for 10 hours. DNA was isolated for quantification of DNA adducts (A); B[a]P-tetrol formation was measured in the media (B). In A and B, mean \pm SD are shown, $n = 4$. *, $P < 0.01$ compared with B[a]P treated cells.