Published in final edited form as:

Mutat Res. 2013 July 4; 755(1): 61–67. doi:10.1016/j.mrgentox.2013.05.009.

DNA synthesis inhibition in response to benzo[*a*]pyrene dihydrodiol epoxide is associated with attenuation of p34cdc2: Role of p53

Jagat J. Mukherjee* and Subodh Kumar

Environmental Toxicology & Chemistry Laboratory, Great Lakes Center, State University of New York College at Buffalo, 1300 Elmwood Avenue, Buffalo, NY 14222, USA

Abstract

Our previous findings demonstrated that DNA damage by polynuclear aromatic hydrocarbons (PAHs) triggers a cellular protective response of growth inhibition (G1-S cell cycle arrest and inhibition of DNA synthesis) in human fibroblasts associated with accumulation of p53 protein, a growth-inhibitory transcription factor. Here, we report that BPDE (the ultimate carcinogenic metabolite of the PAH benzo[*a*]pyrene) treatment triggers a variable extent of inhibition of DNA synthesis/cell growth, which does not correspond to the extent of increased p53 accumulation. BPDE treatment of cells significantly attenuates expression of p34cdc2, a cell cycle activating protein. Although the role of cdc2 down-regulation in inhibition of cell cycle progression is well known, cdc2 down-regulation in response to cellular insult by PAHs has not been reported. Unlike p53 accumulation, there is a correspondence between DNA synthesis/cell growth inhibition and cdc2 down-regulation by BPDE. BPDE-induced cdc2 down-regulation is p53 dependent, although there is no correspondence between p53 accumulation and cdc2 down-regulation. BPDE-induced cdc2 down-regulation corresponded with accumulation of the cell cycle inhibitor protein p21 (transactivation product of p53). DNA synthesis/cell growth inhibition in response to DNA-damaging PAHs may involve down-regulation of cdc2 protein mediated by p53 activation (transactivation ability), and the extent of p53 accumulation is not the sole determining factor in this regard.

Keywords

(±)-anti-Benzo[*a*]pyrene-7,8-diol-9,10-epoxide; p34cdc2; p53; DNA synthesis; p21; cell growth inhibition

Introduction

The tumorigenic potential of polycyclic aromatic hydrocarbons (PAHs) is well documented [1–10, 51–53]. PAHs require metabolic activation to exert their carcinogenic effects. Benzo[*a*]pyrene (BP), a prototypical PAH, is metabolically activated by cytochrome P450-

© 2013 Elsevier B.V. All rights reserved.

*Corresponding author: Phone: 716-878-4614, Fax: 716-878-5400, mukherjj@buffalostate.edu.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

dependent oxidation to *anti*-BP-7,8-dihydrodiol-9,10-epoxide (BPDE), a reactive electrophile which binds to DNA predominantly at the N² position of deoxyguanosine (dG) and which is implicated as the ultimate carcinogenic metabolite of BP [6].

In response to DNA damage, cells elicit protective measures, such as cell cycle arrest and apoptosis [13]. Although BP carcinogenicity involves initial DNA damage, tumorigenicity is greatly enhanced by tumor promoters [11, 12], which interfere with these protective events [42, 43]. Cell cycle arrest gives cells time to repair DNA damage; apoptosis (programmed cell death) eliminates severely damaged cells.

DNA damage-induced cell cycle arrest involves regulation at different cell cycle checkpoints [40]. Defects in these checkpoints can result in gene mutation, chromosomal damage, and aneuploidy, all of which can contribute to tumorigenesis [39]. Induction of p53 protein is an important component associated with these events [14–16]. DNA damage caused by BP or other PAHs can induce p53 protein expression [17–20]. Cell cycle regulation by p53 is an important downstream event [21]. p53-Dependent arrest in response to DNA damage involves both the G1 and G2 phases of the cell cycle [22–25]. Progression through the S phase is controlled by p53 under the condition of nucleotide imbalance, to avoid DNA damage, and inhibits entry into mitosis when DNA synthesis is blocked [26–28]. Inhibition of p53 induction following DNA damage interferes with p53-mediated protective functions and may lead to aberrant cell growth. Several target genes are influenced by p53 protein [41]. p53-Mediated growth arrest can result from p53-dependent transactivation of p21^{WAF1} (p21) [29]. p21 binds and inactivates cyclin-cdk complexes that mediate G₁ phase progression, resulting in pRB hypo-phosphorylation, E2F sequestration, and cell cycle arrest at the G₁/S transition [30–32]. Many other proteins are involved in phases of the cell cycle, and modulation of these proteins may influence the progression of cell growth. The product of the *cdc2* gene, designated p³⁴cdc2, is a serine-threonine protein kinase; it acts at cell cycle control points and is necessary for entry into and passage through mitosis [33, 34]. It also operates in G1 and is involved in the commitment of cells into the proliferative cycle [35]. Thus, *cdc2* is thought to be an essential cell cycle regulator in all eukaryotic cells [38]. Although DNA damage by ionizing radiation or by the DNA intercalating drug doxorubicin can modulate *cdc2* expression [36, 37], there is no information regarding regulation of *cdc2* expression by DNA-damaging PAHs. We investigated the effect of BPDE on *cdc2* protein expression and cell growth in different cell lines, and the role of p53 in this regard.

Materials and Methods

Cells and reagents

Promotion-sensitive mouse epidermal JB6 cl 41 cells, HCT 116 colorectal carcinoma cells, and normal human lung fibroblast CCD-8Lu were obtained from American Type Culture Collection (ATCC, VA, USA); human dermal fibroblast GM03349 from Coriell Cell Repository, Camden, N.J.; and p53 knockout HCT 116 (HCT 116 p⁵³−/−) cells as described [43]. (+/−)-anti-BPDE was purchased from the NCI Chemical Carcinogen Reference Standard Repository. Modified Eagle's Medium (MEM) and fetal calf serum (FCS) were obtained from Invitrogen Life Technologies (CA, USA). All other chemicals were of analytical grade.

Cell culture

JB6 cl 41 cells were cultured as monolayers at 37°C in an atmosphere of 5% CO₂, in modified Eagle's medium (MEM) containing 5% fetal calf serum, 2 mM L-glutamine, 10 mM sodium pyruvate, and penicillin/streptomycin (50 µg/ml each). CCD-8Lu cells were cultured in MEM containing 15% fetal calf serum. Human fibroblast GM03349 was grown

as a monolayer in MEM supplemented with 15% fetal calf serum, non-essential amino acids, and pyruvate, in an atmosphere of 5% CO₂ in air at 37°C and 85% humidity. The cell cultures were checked for *Mycoplasma* contamination (Gibco Mycotect).

[³H]Thymidine incorporation into DNA

[³H]Thymidine incorporation into DNA was determined as described [44]. Cells were grown in 12-well tissue culture dishes to 50–60% confluency, treated with 0.2 μM BPDE for 1 h (or untreated), followed by treatment with 2 μCi [³H]thymidine in fresh medium, and then cells were allowed to grow for 4, 8, or 20 h. Cells were washed twice with phosphate-buffered saline, four times with 5% trichloroacetic acid, and finally twice with absolute ethanol. The acid-insoluble material was redissolved in 0.3 M NaOH, and an aliquot was taken to measure DNA-associated radioactivity in a liquid scintillation counter.

Cell proliferation assay

The cell proliferation assay was performed using CellTiter 96 AQueous One Solution assay kit (Promega), following the supplier's manual. Cells (~4,000 cells/well) were seeded in 96-microwell plates. At ~50% confluency, cells were treated with 0.2 μM BPDE for 1 h (or untreated), followed by changing the medium, and then allowed to grow. After BPDE treatment, 20 h, cells were incubated with AQueous One Solution reagent as described in the supplier's manual and the absorbance of the formazan product was measured at 490 nm, using a plate reader. As suggested in the manual the very slight background absorbance due to interaction of an identical volume of medium with AQueous One Solution reagent was subtracted from the absorbance values.

Transient transfection with p53-targeted siRNA

JB6 cl41 cells were transiently transfected with p53-targeted siRNA [41]. Cells were seeded in 6-well tissue culture plates (3 × 10⁵ per well) in 2 ml antibiotic-free medium supplemented with 5% fetal bovine serum. At 50–60% confluency, cells were transfected with p53 siRNA (mouse) duplex (Santa Cruz Biotechnology Inc., CA), following the supplier's manual. Briefly, cells were transfected with 100 pmol siRNA in presence of 5 μl siRNA transfection reagent for 5 h at 37°C, followed by addition of fresh growth medium (1 ml) containing 10% FBS, without removing the transfection mixture, and further incubation for 24 h. Control cells were transfected with negative control siRNA-A (Santa Cruz Biotechnology Inc., Sc-37007) consisting of scrambled sequence. Medium was replaced and cells were harvested 24 h after transfection for p53 Western immunoblot assay to confirm p53 down-regulation. For chemical exposure, cells were treated 24 h after transfection, and the respective response was monitored.

Western immunoblotting

After treatments, cells were lysed in lysis buffer consisting of 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM sodium β-glycerophosphate, 1 mM Na₃VO₄, 10 μg/ml pepstatin, 1 mM phenylmethanesulfonyl fluoride, 5 μg/ml leupeptin, and 100 μg/ml aprotinin (Sigma). An equal amount of the cell extract was separated by 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P (Millipore) membrane. The membrane was blocked in 5% skim milk powder. For p53 detection, the membrane was incubated with rabbit polyclonal antibody p53 (Pab 240) (Santa Cruz Biotechnology Inc., CA), 0.7 μg antibody/ml solution. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) was used as secondary antibody. The protein bands were detected and analyzed in a Storm Phosphorimager using Amersham's ECL enhanced chemiluminescence detection reagents (Amersham Biosciences, NJ, USA). For p³⁴cdc2

detection, cell extracts were immunoblotted with anti- p³⁴cdc2 primary antibody (SantaCruz Biotechnology Inc. CA).

Apoptosis assay

The cell death enzyme-linked immunosorbent assay kit obtained from Roche Applied Science (Indianapolis, IN) was used for apoptosis assay [43]. Cells were cultured in a 96-well plate, and, after treatment, incubated in serum-containing medium for 20 h. Cells were washed with phosphate-buffered saline (PBS) and the extent of apoptosis was followed by measuring the levels of cytosolic histone-bound DNA fragments, as described in the manufacturer's manual. Briefly, the cells were lysed with 200 μ l lysis buffer; 20 μ l lysate was mixed with 80 μ l antibody solution in a well of the enzyme-linked immunosorbent assay plate and incubated at room temperature for 2 h. After washing the substrate was added and incubated at 37°C for 10–20 min, and the optical density was measured using a microplate reader at a wavelength of 405 nm with a reference wavelength of 492 nm. The readings were used to represent the degree of apoptosis. Cells treated with 20 μ M hydrogen peroxide represent the positive control for the apoptosis experiment using the Roche ELISA kit.

Results

Inhibition of DNA synthesis and cell proliferation by BPDE does not correspond to the extent of p53 accumulation

Our previous study showed that DNA damage by BPDE can induce G1-S cell cycle arrest and p53 accumulation [44]. Here, we investigated cell growth inhibition after BPDE treatment in different cell lines and examined the relationship between extent of cell growth inhibition/DNA synthesis inhibition and extent of p53 accumulation. BPDE treatment inhibited DNA synthesis to different extents in the three cell lines, as measured by [³H]thymidine incorporation into DNA (Fig. 1A). BPDE also inhibited cell proliferation to different extents ($p < 0.05$; two-tailed paired t-test) for JB6 and GM03349 cells and $0.2 > p > 0.05$ for CCD8-Lu cells (Fig. 1B). Each data point of Fig. 1A and Fig. 1B represents the mean $\pm$ S.D. of three parallel experiments. The extent of inhibition of either DNA synthesis or cell proliferation was in the order GM03349>JB6>CCD-8Lu at each time point after BPDE treatment. BPDE treatment caused variable extent of p53 accumulation in the cell lines, as evidenced from p53 immunoblot data (Fig. 1C, representative of three independent immunoblot experiments). Quantification of p53 band intensities (after β -actin normalization) in three cell lines is shown in Fig. 1C, inset. The extent of p53 accumulation does not show the same cell-specific order as was obtained with DNA synthesis/cell growth inhibition (Fig. 1A and 1B). GM03349 cells, which showed highest DNA synthesis/cell growth inhibition 20 h after BPDE treatment had the lowest p53 accumulation. p53 accumulation was in the order JB6>CCD-8Lu>GM03349 (Fig. 1C inset), whereas the extent of DNA synthesis/cell growth inhibitions was in the order GM03349>JB6>CCD-8Lu, as described above.

Since the observed DNA synthesis ([³H]thymidine incorporation) inhibition may be due to apoptosis induction without inhibition of cell proliferation, we tested whether there is any variation in BPDE apoptosis induction among the cell lines. Apoptosis induction by 0.2 μ M BPDE was negligible in all three cell lines, and differences were insignificant (Figure 1D). These findings rule out a possible role of apoptosis in this regard, and also establish that the data in Fig. 1B reflect differences in cell proliferation rather than differences in apoptosis.

BPDE attenuation of p³⁴cdc2 levels reflects cell growth inhibition

The lack of correspondence between p53 accumulation and DNA synthesis/cell growth inhibition prompted us to examine the effect of BPDE exposure on other cell cycle regulatory proteins. BPDE treatment attenuates p³⁴cdc2 expression to variable extents in the different cell lines (Fig. 2). Quantification of β -actin normalized p³⁴cdc2 band intensities in the three cell lines is shown in Fig. 2, inset. Attenuation of p³⁴cdc2 protein level by BPDE was time-dependent; maximum attenuation was observed 20 h after BPDE treatment. p³⁴cdc2 is a cell growth activating protein and inhibition of p³⁴cdc2 is associated with cell growth inhibition [33–35]. The extent of p³⁴cdc2 attenuation in the cell lines was in the same order (GM03349>JB6>CCD-8Lu) (Fig. 2, inset) as was seen with DNA synthesis and cell growth inhibition.

Attenuation of p³⁴cdc2 protein by BPDE is p53-dependent

Using two p53-deficient cell lines, we tested whether p53 has a role in the cdc2 response to BPDE. JB6 cells were transiently transfected with p53 siRNA to impair p53 activity; scrambled siRNA served as the control. Secondly, we used the human colorectal cancer cell line HCT116, deficient in p53 (p53^{-/-}) [43]. In p53 siRNA transfected JB6 cells, p53 accumulation in response to BPDE was significantly inhibited, compared to control cells. In HCT116 (p53^{-/-}) cells, BPDE did not induce p53 accumulation, as opposed to p53-wild-type HCT116 cells (Fig. 3). Impairment of p53 in these cell lines was confirmed by the observation that expression of p21 protein, a transactivation product of active p53, is practically absent both in p53 siRNA transfected JB6 cells and HCT116 (p53^{-/-}) cells, whereas the respective control cells showed p21 accumulation in response to BPDE (Fig. 3). In p53 siRNA transfected JB6 cells, BPDE is practically unable to attenuate cdc2 protein levels, whereas in control JB6 cells, BPDE significantly attenuated cdc2 accumulation (Fig. 3, upper panel). BPDE was also unable to attenuate cdc2 protein levels in HCT116 (p53^{-/-}) cells, whereas normal HCT116 cells showed significant attenuation (Fig. 3, lower panel). These findings suggest that cdc2 attenuation in response to BPDE is p53-dependent and that p21 induction by BPDE depends on the p53 response to BPDE.

BPDE attenuates p³⁴cdc2 protein level in a corresponding manner with p21 induction in different cell lines

Although cdc2 attenuation depends on the p53 response, we observed, among the cell lines, different orders of cdc2 attenuation vs p53 accumulation by BPDE. We examined the effects of BPDE on expression levels of p21 protein. The P21 gene is a transactivation target of p53 protein we also saw p53 dependency of the p21 response to BPDE (Fig. 3). The expression of p21 protein was determined by Western immunoblotting in extracts of BPDE-treated or control cells. The extent of p21 protein induction by BPDE was highest in GM03349 fibroblasts followed by JB6 cells, and lowest in CCD-8Lu cells (Fig. 4A). The p21 band intensities obtained from three independent Western blots is shown in Fig. 4A, inset. Although p21 is a transactivation product of active p53, the observed order of p21 induction (Fig. 4A, inset) was similar to that of cdc2 attenuation (Fig. 2, inset) but differs from that of p53 accumulation (Fig. 1C, inset). These findings suggest that p53 transactivation ability (p53 activation) rather than p53 accumulation, has a role in cdc2 attenuation by BPDE.

Activation of p53 in response to DNA damage involves phosphorylation of p53 at different sites, depending on the DNA-damaging agent (56). Besides p21 expression as indicative of p53 activation, we also examined p53 phosphorylation at ser15 and ser20 by BPDE as indicative of p53 activation. BPDE (0.2 μ M) treatment of cells results in phosphorylation of p53 at ser15 (Fig. 4B) but not at ser20 (data not shown). Phosphorylation of p53 at ser15 in GM03349 cells is greater than in JB6 cells, whereas it is lowest in CCD8-Lu cells (Fig. 4B).

These findings support the role of p53 activation, not p53 accumulation, in cdc2 attenuation by BPDE.

Discussion

The tumor suppressor protein p53 plays a pivotal role in the DNA damage-induced cellular protective response of cell cycle arrest and apoptosis [13]. Induction of p53 in response to DNA damage modulates several target genes. Previously, we showed that treatment of human fibroblast with BPDE causes accumulation of p53 and p21 (transactivation product of p53) proteins and inhibits cell cycle progression at G1-S [44]. Considering the role of p53 in DNA damage-induced cell growth inhibition, in this investigation, we first examined the correspondence between the extent of cell growth inhibition and p53 accumulation in response to BPDE. The extents of inhibition of DNA synthesis (indicative of S phase inhibition) and cell proliferation were not associated with a similar trend of p53 induction among the cell lines. The order of DNA synthesis/cell growth inhibition was GM03349>JB6>CCD-8LU compared to the order of p53 accumulation, JB6>CCD-8LU>GM03349. Two explanations are possible; (a) although p53 accumulation in response to BPDE is different among the cell lines, the activation status of p53 protein dictates its functional dimension; (b) BPDE-induced cell growth inhibition is significantly mediated by a p53-independent mechanism. The p53 response to DNA damage includes two discrete events: accumulation of p53 protein (dependent on transcription of gene and stabilization of proteins) and activation of p53 protein [45]. The lack of correspondence between p53 accumulation and cell growth inhibition (p53 functional attribute) may imply different extents of p53 activation among the cell lines tested. Whether BPDE-induced cell growth inhibition is significantly mediated by a p53-independent signaling pathway remains to be elucidated.

We investigated whether BPDE has modulating effects on other proteins which are associated with cell cycle check points. BPDE treatment attenuates the basal level of p34cdc2 protein expression to different extents in the different cell lines. P34cdc2 is a cell cycle activating protein [33–35]. The cdc2 gene product p34cdc2 is a serine-threonine protein kinase. P34cdc2 is required for commitment to the cycle both at the ‘start’ control point in G1 and the onset of mitosis (G2-M) [33–35; 48–50]. Attenuation of p34cdc2 by BPDE may inhibit cell growth, and our observation shows that this inhibition is associated with attenuation of cdc2 protein. Although a regulatory role of p34cdc2 in cell cycle inhibition by DNA damaging agents (e.g. ionizing radiation or the intercalating drug doxorubicin) has been reported [36, 37], the role of p34cdc2 in PAH-induced cell growth inhibition has not been documented. We observed that the extent of attenuation of cdc2 protein, unlike p53 accumulation, is associated with a similar trend in the inhibition of DNA synthesis and cell proliferation among the cell lines.

Cdc2 attenuation by BPDE was p53 dependent, although the extent of cdc2 attenuation by BPDE does not follow the same trend as that of p53 accumulation. These findings corroborate the previous assertion of a possible role of p53 activation rather than p53 accumulation, in this regard. In support of this assertion, we observed that BPDE induces p21 protein to a variable extent, and the extent of p21 induction follows the same trend as the extent of inhibition of DNA synthesis and cell growth in these cell lines. Activation of p53 protein is essential for binding to the p21 gene to elicit its transactivation function. p21 expression levels in different cell lines may not indicate p53 activation status, since there are reports of p53-independent p21 activation (54, 55). However, our observations show that p53 impairment severely inhibits the BPDE-induced p21 response in JB6 and HCT 116 cells. Our findings also show that the order of BPDE-induced p53 ser15 phosphorylation (indicative of p53 activation) among the cell lines follows the same order as observed with

cdc2 attenuation and p21 accumulation. Thus, our results indicate that functionally active p53, not the extent of p53 accumulation, may have a role in the attenuation of cdc2 protein by BPDE. This supports the previous report that p53 may repress cdc2 promoter and inhibit its transcription through induction of p21 [46].

Besides p53's possible regulatory effect on cdc2 transcription, association of p53 with p34cdc2 at the protein level has also been indicated [47]. There is speculation of the possibility of p53 inactivation due to complex formation. If p53 is inactivated due to complex formation with p34cdc2 protein, then attenuation of cdc2 by BPDE (as we observed) will relieve p53 inactivation resulting from complex formation (increased free p53) and thereby cell growth inhibition by p53 will be augmented. In this situation, the intracellular level of cdc2 protein will be one of the determining factors of p53's correspondence to cell growth inhibition. Our findings show that the extent of cell growth inhibition corresponds to the extent of attenuation of cdc2 protein by BPDE among the cell lines. Also, the extent of p53 accumulation may not be a predictive marker of the extent of cell growth inhibition in different cell lines. Further studies are needed with regard to mechanistic understanding of p34cdc2's role in cell growth inhibition by PAH-mediated DNA damage and the involvement of p53 in this regard.

Acknowledgments

This work was partially supported by National Cancer Institute (NCI) Grant R15CA125630 (To J.J.M.).

References

1. Orris L, et al. Carcinogenicity for mouse skin and aromatic hydrocarbon content of cigarette-smoke condensate. *J Natl Cancer Inst.* 1958; 21:557–561. [PubMed: 13576107]
2. Hoffmann, D.; Hecht, SS. Advances in tobacco carcinogenesis. In: Cooper, CS.; Grover, PL., editors. *Handbook of Experimental Pharmacology*. Vol. 94/1. Springer-Verlag; Heidelberg: 1990. p. 63-102.
3. Hecht SS. Approaches to chemoprevention of lung cancer based on carcinogens in tobacco smoke. *Environ Health Perspect.* 1997; 105:955–963. [PubMed: 9255587]
4. Pelkonen O, Nebert DW. Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. *Pharmacol Rev.* 1982; 34:189–222. [PubMed: 6287505]
5. Stampfer MR, Bartley JC. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo(a)pyrene. *Proc Natl Acad Sci USA.* 1985; 82:2394–2398. [PubMed: 3857588]
6. Harvey, RG. *Polycyclic Aromatic Hydrocarbons: Chemistry and Carcinogenicity*. Cambridge University Press; Cambridge: 1991. p. 396
7. Van Durren BL. Identification of some polynuclear aromatic hydrocarbons in cigarette-smoke condensate. *J Natl Cancer Inst.* 1958; 21:1–16. [PubMed: 13564193]
8. Gellhorn A. The carcinogenic activity of cigarette tobacco tar. *Cancer Res.* 1958; 18:510–517. [PubMed: 13547042]
9. Pelkonen O, Nebert DW. Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. *Pharmacol Rev.* 1982; 34:189–222. [PubMed: 6287505]
10. Heidelberger C. Chemical carcinogenesis. *Annu Rev Biochem.* 1975; 44:79–121. [PubMed: 1094923]
11. Lasne C, Gentil A, Chouroulinkov I. Two-stage carcinogenesis with rat embryo cells in tissue culture. *Br J Cancer.* 1977; 35:722–729. [PubMed: 405981]
12. Miller ML, et al. The tumor promoter TPA enhances benzo[a]pyrene and benzo[a]pyrene diol epoxide mutagenesis in Big Blue mouse skin. *Environ Mol Mutagen.* 2000; 35:319–327. [PubMed: 10861950]

13. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. *Nature*. 2004; 432:307–315. [PubMed: 15549092]
14. Fritsche M, Haessler C, Brandner G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene*. 1993; 8:307–318. [PubMed: 8426740]
15. Maltzman W, Czyzyk L. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol Cell Biol*. 1984; 4:1689–1694. [PubMed: 6092932]
16. Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci USA*. 1992; 89:7491–7495. [PubMed: 1323840]
17. Bjelogrić NM, Makinen M, Stenback F, Vahakangas K. Benzo[a]pyrene-7,8-diol-9,10-epoxide-DNA adducts and increased p53 protein in mouse skin. *Carcinogenesis*. 1994; 15:771–774. [PubMed: 8149494]
18. Stuart D, Khan QA, Brown R, Dipple A. Hydrocarbon carcinogens induce p53 activity in normal mouse tissue. *Cancer Lett*. 2001; 173:111–114. [PubMed: 11597784]
19. Binkova B, Giguere Y, Rossner P, Dostal M, Sram RJ. The effect of dibenzo[a,h]pyrene and benzo[a]pyrene on human diploid lung fibroblasts: the induction of DNA adducts, expression of p53 and p21(WAF1) proteins and cell cycle distribution. *Mutat Res*. 2000; 471:57–70. [PubMed: 11080661]
20. Venkatachalam S, Denissenko M, Wani AA. Modulation of (+/-)-anti-BPDE mediated p53 accumulation by inhibitors of protein kinase C and poly(ADP-ribose) polymerase. *Oncogene*. 1997; 14:801–809. [PubMed: 9047387]
21. Shackelford RE, Kaufmann WK, Paules RS. Cell cycle control, checkpoint mechanisms, and genotoxic stress. *Environ Health Perspect*. 1999; 107:5–24. [PubMed: 10229703]
22. Kastan MB, et al. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*. 1991; 51:6304–6311. [PubMed: 1933891]
23. Agarwal ML, et al. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci U S A*. 1995; 92:8493–8497. [PubMed: 7667317]
24. Stewart N, Hicks GG, Paraskevas F, Mowat M. Evidence for a second cell cycle block at G2/M by p53 oncogene. 1995; 10:109–115.
25. Bunz F, et al. Requirement for p53 and p21 to sustain G₂ arrest after DNA damage. *Science*. 1998; 282:1497–1501. [PubMed: 9822382]
26. Chernova OB, et al. The role of p53 in regulating genomic stability when DNA and RNA synthesis are inhibited. *Trends Biochem Sci*. 1995; 20:431–434. [PubMed: 8533158]
27. Agarwal ML, et al. A p53-dependent S-phase checkpoint helps to protect cells from DNA damage in response to starvation for pyrimidine nucleotides. *Proc Natl Acad Sci U S A*. 1998; 95:14775–14780. [PubMed: 9843965]
28. Taylor WR, et al. p53 inhibits entry into mitosis when DNA synthesis is blocked. *Oncogene*. 1999; 18:283–295. [PubMed: 9927185]
29. El-Deiry WS, et al. WAF1, a potential mediator of p53 tumor suppression. *Cell*. 1993; 75:817–825. [PubMed: 8242752]
30. Xiong Y, et al. p21 is a universal inhibitor of cyclin kinases. *Nature*. 1993; 366:701–704. [PubMed: 8259214]
31. Harper JW, et al. The p21 Cdk-interacting protein Cipl is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*. 1993; 75:805–816. [PubMed: 8242751]
32. Shackelford RE, Kaufmann WK, Paules RS. Cell cycle control, checkpoint mechanisms, and genotoxic stress. *Environ Health Perspect*. 1999; 107:5–24. [PubMed: 10229703]
33. Furukara Y, et al. CDC2 gene expression at the G1 to S transition in human T lymphocytes. *Science*. 1990; 250:805–808. [PubMed: 2237430]
34. Draetta G, Beach D. Activation of cdc2 protein kinase during mitosis in human cells: Cell cycle-dependent phosphorylation and subunit rearrangement. *Cell*. 1988; 54:17–26. [PubMed: 3289755]
35. Giordano A, et al. A 60 kd cdc2-associated polypeptide complexes with the E1A proteins in adenovirus-infected cells. *Cell*. 1989; 58:981–990. [PubMed: 2570639]

36. Azzam EI, et al. CDC2 is down-regulated by ionizing radiation in a p53-dependent manner. *Cell Growth Differ.* 1997; 8:1161–1169. [PubMed: 9372239]
37. Le Gac Gerald, et al. DNA Damage-induced down-regulation of human Cdc25C and Cdc2 is mediated by cooperation between p53 and maintenance DNA (cytosine-5) methyltransferase 1. *J Biol Chem.* 2006; 281:24161–24170. [PubMed: 16807237]
38. Nurse P. Ordering S phase and M phase in the cell cycle. *Cell.* 1994; 79:547–550. [PubMed: 7954820]
39. Paulovich AG, Toczyski DP, Hartwell LH. When checkpoints fail. *Cell.* 1997; 88:315–321. [PubMed: 9039258]
40. Kaufmann WK, Paulest RS. DNA damage and cell cycle checkpoints. *FASEB J.* 1996; 10:238–247. [PubMed: 8641557]
41. Wang L, et al. Analyses of p53 target genes in the human genome by bioinformatic and microarray approaches. *J Biol Chem.* 2001; 276:43604–43610. [PubMed: 11571296]
42. Price BD, Calderwood SK. Increased sequence-specific p53-DNA binding activity after DNA damage is attenuated by phorbol esters. *Oncogene.* 1993; 11:3055–3062. [PubMed: 8414506]
43. Mukherjee JJ, Kumar S, Gocinski R, Williams J. Phenolic fraction of tobacco smoke inhibits BPDE-induced apoptosis response and potentiates cell transformation: Role of attenuation of p53 response. *Chem Res Toxicol.* 2011; 24:698–705. [PubMed: 21480602]
44. Mukherjee JJ, Gupta SK, Kumar S, Sikka HC. Effects of cadmium(II) on (+/-)-anti-benzo[*a*]pyrene-7,8-diol-9,10-epoxide-induced DNA damage response in human fibroblasts and DNA repair: a possible mechanism of cadmium's co-genotoxicity. *Chem Res Toxicol.* 2004; 17:287–293. [PubMed: 15025498]
45. Chernov MV, et al. Stabilization and activation of p53 are regulated independently by different phosphorylation events. *Proc Natl Acad Sci USA.* 1998; 95:2284–2289. [PubMed: 9482877]
46. Taylor WR, et al. p130/E2F4 Binds to and represses the cdc2 promoter in response to p53. *J Biol Chem.* 2001; 276:1998–2006. [PubMed: 11032828]
47. Milner J, et al. p53 is associated with p34cdc2 in transformed cells. *EMBO J.* 1990; 9:2885–2889. [PubMed: 2167834]
48. Norbury CJ, Nurse P. Control of the higher eukaryote cell cycle by p34cdc2 homologues. *Biochim Biophys Acta.* 1989; 989:85–95. [PubMed: 2546600]
49. Nurse P. Universal control mechanism regulating onset of M-phase. *Nature.* 1990; 344:503–508. [PubMed: 2138713]
50. Pines J, Hunter T. Cdc2 p34: the S and M kinase? *New Biol.* 1990; 2:389–401. [PubMed: 2149647]
51. Lin CH, et al. Molecular topology of polycyclic aromatic carcinogens determines DNA adduct conformation: a link to tumorigenic activity. *J Mol Biol.* 2001; 306:1059–1080. [PubMed: 11237618]
52. Wang J, et al. Inhalation cancer risk associated with exposure to complex polycyclic aromatic hydrocarbon mixtures in an electronic waste and urban area in south China. *Environ Sci Technol.* 2012; 46:9745–9752. [PubMed: 22913732]
53. Armstrong B, et al. lung cancer risk after exposure to polycyclic aromatic hydrocarbons: a review and meta-analysis. *Environ Health Perspect.* 2004; 112:970–978. [PubMed: 15198916]
54. Aliouat-Denis CM, et al. p53-independent regulation of p21Waf1/Cip1 expression and senescence by Chk2. *Mol Cancer Res.* 2005; 11:627–674. [PubMed: 16317088]
55. Jeong JH, et al. p53-Independent Induction of G1 Arrest and p21WAF1/CIP1 Expression by ascofuranone, an isoprenoid antibiotic, through downregulation of c-myc. *Mol Cancer Ther.* 2010; 9:2102–2113. [PubMed: 20587660]
56. Ashcroft M, et al. Regulation of p53 function and stability by phosphorylation. *Molec Cell Biol.* 1999; 19:1751–1758. [PubMed: 10022862]

Highlights

- BPDE inhibits cell growth and DNA synthesis in different cell lines
- BPDE attenuates p34cdc2 expression level and induce p53 and p21 accumulation
- p34cdc2 attenuation, not p53 accumulation corresponds to cell growth inhibition
- Interestingly p34cdc2 attenuation by BPDE is p53 dependent
- Role of p53 activation not accumulation is implicated in cdc2 attenuation

Figure 1. Effects of BPDE on DNA synthesis, cell proliferation, p53 accumulation, and apoptosis
 (A) Cells were treated with or without 0.2 μ M BPDE for 1 h followed by treatment with 2 μ Ci [3 H]thymidine in fresh medium, and then the cells were harvested at 4, 8 or 20 h after [3 H]thymidine treatment. Radioactivity associated with cellular DNA was measured as described in Methods. Each data point represents mean $\pm$ SD of triplicate experiments. The extent of Inhibition of DNA synthesis by BPDE was expressed in table format (see the inset). (B) Cells were treated with 0.2 μ M BPDE for 1 h followed by analysis of cell proliferation 20 h after BPDE treatment, using CellTitre 96 AQueous One Solution assay system described in Methods. Each bar indicates the mean $\pm$ SD of three parallel experiments. Statistical analysis of significance with two-tailed paired t-test is shown (inset). (C) Representative Western immunoblot of p53 accumulation. Cells were treated as described and an cell extract (50 μ g protein) was subjected to Western immunoblotting for determination of p53 accumulation 20 h after BPDE treatment, using p53 (Pab 240) antibody (Santa Cruz Biotechnology Inc. CA). (Inset) Bar diagram shows p53 accumulation in response to 0.2 μ M BPDE treatment from three different experiments with each cell line. p53 band intensity after β -actin normalization (STORM data) is shown (inset). Each bar represents mean $\pm$ S.D. of three experiments for each cell line. One-way ANOVA indicates $p < 0.05$. (D). Apoptosis response to BPDE. Cells were treated with 0.2 μ M BPDE as described above and apoptosis was followed 20 h after BPDE treatment. Apoptosis was monitored using a cell death enzyme-linked immunosorbent assay kit (Roche Applied

Science, IN) as described in the text. Treatment of JB6 cells with 20 μM H_2O_2 (known apoptosis inducer) represents the positive control. Each bar shows the mean of triplicate experiments and the error bars indicate S.D.

Figure 2. Effect of BPDE on p³⁴cdc2 protein levels

Cells were treated with 0.2 μ M BPDE followed by harvesting at 4, 8 and 20 h after BPDE treatment. Cell extracts (50 μ g total protein in each lane) were subjected to Western immunoblotting using anti-p³⁴cdc2 primary antibody (Santa Cruz Biotechnology Inc. CA). The membranes were washed and re-probed for β -actin levels with anti- β -actin antibody to normalize protein loading. (Inset) Bar diagram shows cdc2 protein inhibition 20 h after BPDE treatment from three separate experiments of each cell line. p³⁴cdc2 band intensity after β -actin normalization (STORM data) from each control and BPDE treated sample was determined followed by determination of percent inhibition of cdc2 protein level. Each bar represents mean $\pm$ S.D. of three different experiments for each cell line. One-way ANOVA indicates $p < 0.05$.

Figure 3. Inhibition of p53 impairs the ability of BPDE to attenuate p34cdc2 protein expression level

Cells were treated with 0.2 μ M BPDE followed by detection of respective proteins, as described in the text. (Upper panel) Western immunoblot of p53, cdc2, and p21 proteins in response to BPDE in JB6 cells transfected with either scrambled siRNA (control) or p53 targeting siRNA duplex. (Lower panel) Western analysis of expression of above proteins in response to BPDE in p53-WT HCT 116 and p53-null HCT 116 (p53^{-/-}) cells.

Figure 4. Effects of BPDE on p21 protein levels and phosphorylation of p53 at ser15
 (A) Representative (out of three) Western immunoblot of p21 accumulation has been shown. Cells were treated with 0.2 μ M BPDE followed by lysis 20 h after BPDE treatment. Cell extracts (50 μ g total protein per lane) were subjected to Western immunoblotting using anti-p21 primary antibody. The membranes are washed and re-probed for β -actin levels with anti- β -actin antibody to normalize protein loading. (Inset) Bar diagram shows p21 accumulation in response to 0.2 μ M BPDE treatment from three different experiments of each cell line. P21 band intensity after β -actin normalization (STORM data) has been shown in the *inset*. Each bar represents average $\pm$ S.D. of three independent experiments for each cell line. One-way ANOVA indicates $p < 0.05$. (B) Cells (CCD8-Lu- lanes 1 and 4; GM03349- lanes 2 and 5; and JB6- lanes 3 and 6) were either untreated (lanes 1, 2 and 3) or treated with 0.2 μ M BPDE (lanes 4, 5 and 6) followed by lysis 20 h after BPDE treatment. Cell extracts (80 μ g total protein in each lane) were subjected to Western immunoblotting using anti-phospho-p53 (ser15) primary antibody (Cell Signaling). The membranes were washed and re-probed for β -actin levels using anti- β -actin antibody.