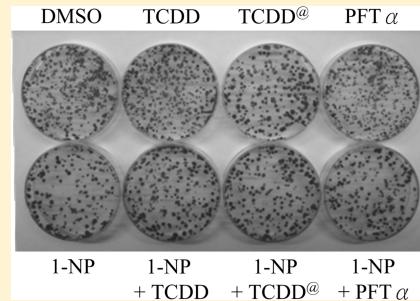


2,3,7,8-Tetrachlorodibenzo-*p*-dioxin's Suppression of 1-Nitropyrene-Induced p53 Expression Is Mediated by Cytochrome P450 1A1

Jyan-Gwo Joseph Su,* Min-Cong Huang,[†] and Fei-Yun Chen[†]

Department of Biochemical Science and Technology, National Chiayi University, Chiayi 600, Taiwan, ROC

ABSTRACT: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), 1-nitropyrene (1-NP), and benzo[*a*]pyrene (BaP) are toxic environmental pollutants. TCDD was shown to suppress p53 expression in response to genotoxic stress and hypoxic conditions. However, the mechanism of TCDD's actions is not clearly understood. Our data showed that pretreatment with TCDD abolished 1-NP- but not BaP-induced p53 and mouse double minute 2 (MDM2; HDM2 in humans) expressions. TCDD suppressed 1-NP- but not BaP-induced p53 activity, and in contrast, pifithrin-alpha (PFT- α), a p53 inhibitor, suppressed both 1-NP- and BaP-induced p53 activity. In the presence of nutlin-3, an HDM2 inhibitor, TCDD was still able to suppress 1-NP-induced p53 expression. However, TCDD-activated HDM2 did not distinctly cause the degradation of BaP- or nutlin-3-induced p53 expression. Accordingly, TCDD's suppression of 1-NP-induced p53 expression was compound-specific, and the contribution of HDM2 to the abolition of 1-NP-induced p53 was limited. β -Naphthoflavon (β -NF), an aryl hydrocarbon receptor (AHR) agonist, mimicked TCDD's action and abolished 1-NP-induced p53 expression. In the presence of CH-223191, an AHR antagonist, TCDD was unable to abolish 1-NP-induced p53 expression. Results indicate that activation of the AHR is required for TCDD's suppression of 1-NP's induction of p53. Cytochrome P450 (CYP) 1A1 is an AHR-targeting gene and a xenobiotic-metabolizing enzyme. TCDD was unable to abolish 1-NP's induction of p53 in CYP1A1-deficient cells, the CYP1A1 transcript of which was degraded by small hairpin RNA-CYP1A1. Both TCDD and PFT- α are potent CYP1A1 inducers and decreased 1-NP-induced cell death and mutagenesis. In summary, TCDD induced detoxification of 1-NP's toxicity, which was mediated by the CYP1A1 enzyme.



INTRODUCTION

Dioxins, polycyclic aromatic hydrocarbons (PAHs), and nitro-PAHs are environmental pollutants, produced in great quantities by combustion and pyrolysis of organic compounds.¹ Many deregulated physiologic functions, including skin rashes, immune deficiency, reproductive and developmental abnormalities, liver damage, and endocrine disruption, are associated with dioxin.² 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic dioxin.³ Although dioxins are considered risk factors for cancer,² the carcinogenicity of TCDD in humans is controversial.⁴ The aryl hydrocarbon receptor (AHR), the only known receptor for TCDD, is a cytosolic receptor, and becomes a transcriptional factor upon activation by its ligands.^{5,6}

Cytochrome P-450s (CYPs) are major phase-I xenobiotic metabolizing enzymes.^{7,8} CYP1A1, the best-known CYP member, is one of the major AHR-sensitive targets and is highly induced by TCDD and benzo[*a*]pyrene (BaP).⁹ The monooxygenase activity of CYP1A1 initiates xenobiotic metabolism by oxidizing PAHs, such as BaP, into epoxide-derivatives.⁹ BaP is a well-known representative PAH, and the BaP-induced CYP1A1 enzyme mediates BaP-induced toxicity.¹⁰ Therefore, CYP1A1, as a representative CYP, is widely used in studies of xenobiotic metabolism.

Diesel exhaust particles (DEPs) contain hundreds of times more 1-nitropyrene (1-NP) than benzo[*a*]pyrene (BaP), a potent

mutagen.^{11–13} 1-NP contributes 30% of the directly acting mutagenicity produced by extracts of DEPs.¹⁴ The extents to which both 1-NP and BaP induce DNA damage in human endothelial cells are comparable.¹⁵ 1-NP induces p53 expression and apoptosis, and S-phase retention in human hepatoma HepG2 cells.¹⁶

The p53 transcription factor is a tumor suppressor and is functionally impaired in nearly 50% of human cancers.¹⁷ When cells encounter DNA damage, nongenotoxic stress, and activation of oncogenes, p53 is induced and activated.¹⁸ Activated p53 is transported from the cytosol into nuclei and binds to the p53-response element (p53RE) of target genes.¹⁹ p53 regulates multiple biological functions, including cell growth arrest, apoptosis, DNA repair, angiogenesis, stress responses, and feedback control.¹⁸ The most distinct feature of p53 is its ability to elicit both apoptotic death and cell-cycle arrest.²⁰ p21 is the most prominent target of p53 for cell-cycle control and is a cyclin-dependent kinase (CDK) inhibitor, resulting in G1-phase arrest, which provides an opportunity for DNA repair.²⁰ The oncoprotein, mouse double minute 2 (MDM2; HDM2 in humans), is transcribed by p53 and acts as a negative feedback control for p53 by promoting ubiquitination and proteasomal degradation of p53.^{21,22} Pifithrin-alpha (PFT- α) is a synthetic p53 inhibitor

Received: July 27, 2011

Published: November 01, 2011

which was shown to protect mice from lethal genotoxic stress associated with antitumor treatment without promoting the formation of tumors.²³ In addition, PFT- α is a potent agonist of the AHR and induces CYP1A1 expression.²⁴

TCDD was shown to suppress p53 expression in response to the DNA-damaging agent, diethylnitrosamine, and hypoxic conditions.^{25,26} However, the mechanism of TCDD's actions is not clearly understood. This is the first report that elucidates a mechanism for TCDD's abolition of p53 expression. In the present study, results showed that TCDD greatly reduced 1-NP- but not BaP- or nutlin-3-induced p53 expression and that the contribution of HDM2 to the abolition of 1-NP-induced p53 was limited. We proved that the AHR signal and CYP1A1 enzyme are required for TCDD's suppression of 1-NP-induced p53 expression. In addition, we also analyzed TCDD's and PFT- α 's decrease in 1-NP-induced cell death and mutagenesis. Evidence implies that TCDD induced the detoxification of 1-NP's toxicity, which was mediated by CYP1A1.

EXPERIMENTAL PROCEDURES

Reagents. 1-NP and β -naphthoflavone (β -NF) were obtained from Aldrich (St. Louis, MO). BaP was obtained from ChemService (West Chester, PA). TCDD was purchased from Supelco (St. Louis, MO). PFT- α , nutlin-3, and Dulbecco's modified Eagle's medium/nutrient mixture F12 phenol-free (DMEM/F12) were obtained from Sigma (St. Louis, MO). Minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). The specific primary antibody against β -actin was obtained from Sigma. Antiphospho-p53 (ser15), anti-p53, and antiphospho-HDM2 (ser166) were obtained from Cell Signaling (Danvers, MA). Anti-HDM2 and CH-223191 were obtained from Calbiochem (San Diego, CA). Donkey antigoat immunoglobulin G (IgG)-horseradish peroxidase (HRP), goat antimouse IgG-HRP, and goat antirabbit IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. The human hepatoma cell line, HepG2, was obtained from American Type Tissue Collection (Rockville, MD). Cells were cultured in MEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin. When treated with BaP, 1-NP, TCDD, and PFT- α , cells were cultured in phenol red-free DMEM/F12 medium. All cultured cells were kept at 37 °C in a 95% air/5% CO₂ environment. Agents were dissolved in dimethyl sulfoxide (DMSO).

Cell Lysis and Western Blotting. HepG2 cells were seeded in 6-well plates at 10⁶ cells/well with 2 mL of medium. At the end of the desired treatments, cell lysates were prepared, and Western blotting was performed as described previously.¹⁶

Plasmid Construction and Luciferase Assay. p53-TA-Luc contains a p53 response element (p53RE), located upstream of the minimal TA promoter (Clontech, Mountain View, CA). The other reporter (RSV-lacZ) expresses a lacZ gene-encoded β -galactosidase, driven by a Rous sarcoma virus long terminal repeat (LTR). Luciferase activity indicates transcription activity of the promoter, and β -galactosidase activity of RSV-lacZ was used to normalize the luciferase activity. The procedure for DNA transfection into HepG2 cells and the activity assay of luciferase and β -galactosidase were performed as described previously.¹⁶

RNA Interference (RNAi). To perform RNAi and knock down CYP1A1, a 21-nucleotide duplex corresponding to the human CYP1A1 messenger (m)RNA (GenBank: NM_000499) was carried by a lentivirus (National RNAi Core Facility, Taipei, Taiwan). The two nucleotide duplexes for human CYP1A1 were CYP1A1-a (GCCTAGTCAC CCTGAATAATA, clone ID: TRCN0000064619) and CYP1A1-b

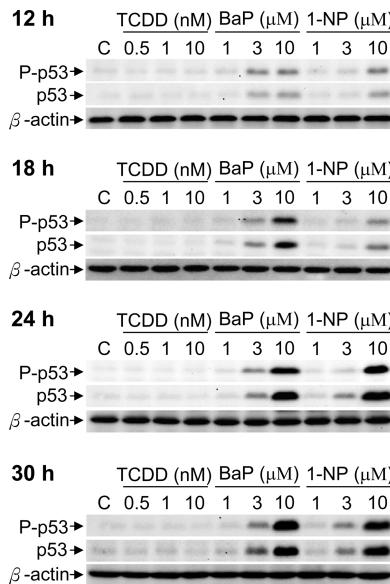


Figure 1. Dose and time course for the induction of p53 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo[a]pyrene (BaP), and 1-nitropyrene (1-NP). HepG2 cells were individually treated with TCDD (0.5, 1, and 10 nM), BaP (1, 3, and 10 μ M), and 1-NP (1, 3, and 10 μ M) for 12, 18, 24, and 30 h. Afterward, cells were harvested, and cell lysates were analyzed by Western blots using antibodies against p53, phosphor-p53 (Ser15), and β -actin.

(CGACAAGGTGTTAAGTGAGAA, clone ID: TRCN0000064620). A 21-nucleotide duplex for firefly luciferase (Luc; CAAATCACAGAACGTCGTAT, clone ID: TRCN0000072246) was used as a control for viral infection. In addition, the pLKO.1 vector, without short hairpin (sh)RNA, carried by a lentivirus was also used as a control for viral infection. HepG2 (1.6 \times 10⁵ cells/well) cells were seeded in 6-well plates overnight and then infected by a lentivirus (4 \times 10⁵ cells/well) for 24 h. Forty-eight hours after infection, cells with shRNA were selected by puromycin (0.5 μ g/ml).

Cytotoxicity Assay. HepG2 cells were seeded at 10⁶ cells/100-mm culture dish overnight and then treated with test compounds for 24 h. Afterward, cells were trypsinized, collected, and seeded at 300 cells/60-mm culture dish, in triplicate, followed by a 14-day culture. Cell colonies were stained with a 1% crystal violet solution (in 30% ethanol). The colony-forming ability was calculated as the cloning efficiency (number of colonies scored per number of cells seeded).²⁷ Percent survival was determined by the colony-forming ability of drug-treated cells relative to the control.

Mutagenesis Assay. A mutagenesis assay was performed by measuring mutations in the *hprt* gene.²⁷ The toxic purine analogue, 6-thioguanine (6-TG; Sigma), was used to select *hprt* gene mutants. Cells were seeded at 10⁶ cells/100-mm culture dish overnight and then treated with test compounds for 24 h, followed by a 7-day culture for the expression period. Afterward, these cells were reseeded at 10⁵ cells/100-mm culture dish (10 dishes/treatment) in a medium with 40 μ M 6-TG and cultured for 14 days, allowing 6-TG-resistant mutant cells to form colonies. In addition, for the purpose of determining the reseeding plating efficiency, 500 cells/treatment were seeded in 60-mm dishes, in triplicate, with nonselective medium for 14 days. The mutant frequency (MF) was determined as the number of mutants counted on each mutation plate divided by the number of cells seeded corrected by the reseeding plating efficiency.

Statistical Analysis. Data are representative of at least three independent experiments under identical conditions and are expressed as the mean \pm SEM. Differences among the data of the control and

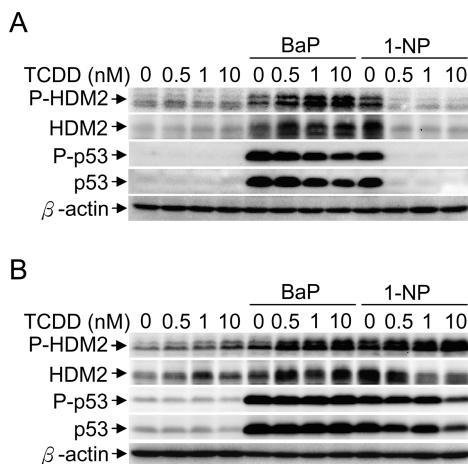


Figure 2. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) abolished the 1-nitropyrene (1-NP)-induced expressions of p53 and HDM2. (A) HepG2 cells were pretreated for 24 h with 0.5, 1, and 10 nM TCDD, followed by treatment with or without 10 μ M 1-NP and 10 μ M benzo[*a*]pyrene (BaP) for 24 h. (B) HepG2 cells were treated at the same time with TCDD (0.5, 1, and 10 nM) alone, a binary mixture of TCDD and 10 μ M 1-NP, or a binary mixture of TCDD and 10 μ M BaP for 24 h. Afterward, cells were harvested, and cell lysates were analyzed by Western blotting using antibodies against p53, HDM2, phosphor-p53 (Ser15), phosphor-HDM2 (Ser166), and β -actin.

further treatments with various compounds were analyzed by Student's *t*-test. Statistical probability (*p*) was expressed as *** *p* < 0.001; ** *p* < 0.01; and * *p* < 0.05; or #** *p* < 0.001; #* *p* < 0.01; and # *p* < 0.05. Means were considered to significantly differ at *p* < 0.05.

RESULTS

TCDD Abolishes 1-NP-Induced p53 and HDM2 Expressions. In response to genotoxic stress, p53 plays a central role in cellular decisions of DNA repair and apoptosis; therefore, we detected the expression patterns of p53. TCDD (0.5, 1, and 10 nM) itself did not stimulate p53 protein expression in HepG2 cells (Figure 1). BaP (10 μ M) and 1-NP (10 μ M) greatly and dose-dependently enhanced p53 protein levels, which were detectable 12 h after induction and were greatly enhanced at 24 and 30 h after induction. The expression patterns of phosphorylated p53 (ser15) paralleled those of p53 protein expression. However, when 1-NP and BaP were added to cells pretreated with TCDD (0.5, 1, and 10 nM) for 24 h, 1-NP- but not BaP-induced p53 and HDM2 were totally abolished by TCDD, even when the concentration of TCDD was as low as 0.5 nM (Figure 2A). In contrast, when cells were simultaneously cotreated with TCDD plus either 1-NP or BaP, TCDD did not interrupt 1-NP's or BaP's induction of p53, except that a high dose (10 nM) of TCDD decreased 1-NP-induced p53 and HDM2 expressions (Figure 2B). However, TCDD increased phosphorylation of HDM2 in cells treated with TCDD alone and with TCDD plus BaP or 1-NP.

To confirm the time course of the suppressive effect of TCDD on 1-NP's induction, cells were pretreated for 0, 6, 12, and 24 h with TCDD (1 nM), followed by 1-NP treatment (Figure 3A). Results indicated that the increase in TCDD's suppressive effect on 1-NP-induced p53 paralleled the increase in the TCDD pretreatment period. To further confirm the persistence of the suppressive effect of TCDD on 1-NP's induction, cells were

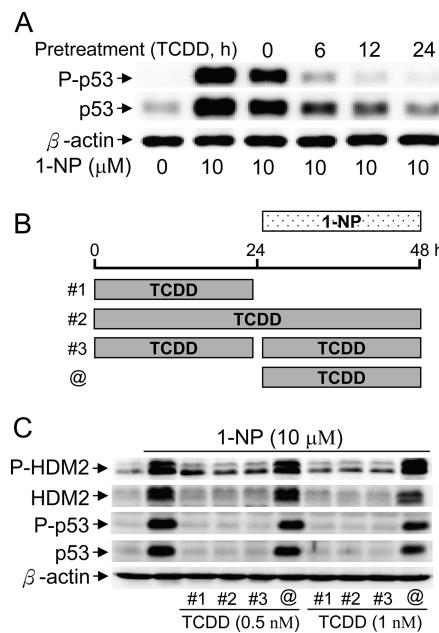


Figure 3. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)'s counteraction of 1-NP's induction requires a lag period. (A) HepG2 cells were pretreated with TCDD (1 nM) for 0, 6, 12, and 24 h, followed by 1-NP (10 μ M) treatment. (B,C) A schematic description of treatment with TCDD and 1-NP is shown. Cells were pretreated for 24 h with TCDD (0.5 and 1 nM), and then TCDD was either removed (#1), was not removed (#2), or was removed but cells were replenished with fresh TCDD (#3), followed by 1-NP (10 μ M) treatment for 24 h. @ Indicates that cells were cotreated with TCDD and 1-NP for 24 h. The expressions and phosphorylations of p53 and HDM2 of cell lysates were analyzed by Western blotting.

pretreated for 24 h with TCDD (0.5 and 1 nM), and then TCDD was removed, not removed, or removed, but cells were replenished with fresh TCDD, followed by 1-NP (10 μ M) treatment for 24 h. The other condition was that cells were cotreated with TCDD and 1-NP for 24 h. A schematic description of TCDD and 1-NP treatment is given in Figure 3B. 1-NP-induced p53 and HDM2 expression and phosphorylation were abolished by TCDD treatment in all three experiments in which cells were pretreated with TCDD (Figure 3C). Adding TCDD at the time of 1-NP treatment caused a slight decrease in 1-NP-induced p53 expression.

TCDD Suppresses 1-NP- but Not BaP-Induced p53 Activity. The reporter plasmid, p53-TA-Luc, was applied to quantify p53 activity. PFT- α is a p53 inhibitor. Expression of p21 is driven by activated p53. BaP (10 μ M) and 1-NP (10 μ M) together with TCDD (10 nM) or PFT- α (10 μ M) were added to cells. Both BaP and 1-NP stimulated the transcriptional activity of p53RE (Figure 4A). As expected, PFT- α abolished BaP- and 1-NP-stimulated transcriptional activity of p53RE in the p53-TA-Luc reporter. In contrast, TCDD decreased 1-NP- but not BaP-induced transcriptional activity of p53RE. Both TCDD and PFT- α themselves induced minor increases in p21 expression (Figure 4B). Both TCDD and PFT- α abolished 1-NP- but not BaP-induced p21 protein expression.

HDM2 Is Not the Only Factor in TCDD's Suppression of 1-NP-Induced p53 Expression. Nutlin-3 is an HDM2 inhibitor. Nutlin-3 itself induced minor p53 expression and high HDM2 expression (Figure 5). Cotreatment with 1-NP and nutlin-3 caused further increases in 1-NP-induced p53 and HDM2 expressions.

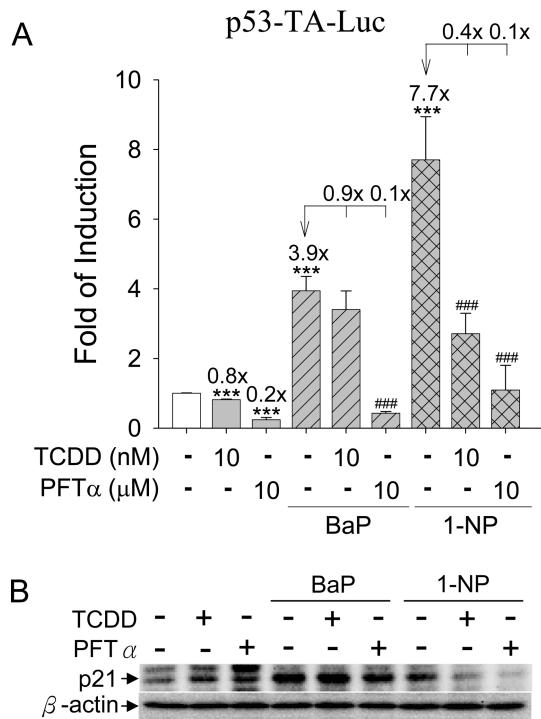


Figure 4. Differential regulation of the 1-nitropyrene (1-NP)- and benzo[a]pyrene (BaP)-induced transcriptional activity of p53RE and p21 expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and pifithrin-alpha (PFT- α). HepG2 cells were transfected with plasmids of (A) p53RE-TA-Luc plus RSV-lacZ, as described in Experimental Procedures, then were incubated with 10 nM TCDD, 10 μ M PFT- α , 10 μ M 1-NP, and 10 μ M BaP for 24 h, followed by the activity assay of luciferase and β -galactosidase. TCDD was added together with 1-NP and BaP, and PFT- α was added 1 h before treatment with 1-NP and BaP. Data are the mean \pm SEM of three replicate experiments. *** $p < 0.001$, ** $p < 0.01$, *** $p < 0.001$, and ## $p < 0.01$. * Indicates a comparison with the negative control (DMSO-treated cells). # Indicates a comparison with the group treated with either 1-NP or BaP alone, as indicated by an arrow. (B) Cells were treated with 10 nM TCDD, 10 μ M PFT- α , 10 μ M BaP, and 10 μ M 1-NP for 24 h. TCDD was added at the same time with 1-NP and BaP treatment, and PFT- α was added 1 h before treatment with 1-NP and BaP. p21 protein expression of cell lysates was analyzed by Western blotting.

1-NP induced more HDM2 than did TCDD. TCDD highly suppressed 1-NP- but not nutlin-3-induced p53 and HDM2 expressions. In addition, TCDD increased the phosphorylation of nutlin-3- but not 1-NP-induced HDM2. p53 expression in cells treated with TCDD, nutlin-3, and 1-NP was less than that in cells treated with nutlin-3 and 1-NP, indicating the suppressive effect of TCDD. In the presence of nutlin-3, 1-NP-induced p53 expression was still suppressed in cells cotreated with 1-NP and TCDD.

Activation of the AHR Is Essential for TCDD's Suppression of 1-NP-Induced p53 Expression. β -NF is an AHR agonist, and CH-223191 is an AHR antagonist. Induction of p53 expression by 1-NP was abolished in cells pretreated with β -NF (10 μ M) for 8 h but not in cells pretreated with β -NF (10 μ M) for 1 h (Figure 6A). Pretreatment of TCDD for 24 h abolished the induction of p53 expression by 1-NP (Figure 6B). However, when TCDD was cotreated with CH-223191, p53 expression induced by 1-NP was not abolished by TCDD.

TCDD Cannot Abolish 1-NP-Induced p53 Expression in CYP1A1-Deficient Cells. To identify the necessity of CYP1A1 in

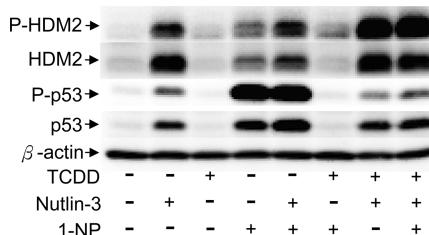


Figure 5. Even when HDM2 activity was blocked, p53 expression induced by 1-nitropyrene (1-NP) was still suppressed by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). HepG2 cells were pretreated with TCDD (10 nM) for 24 h and then pretreated with nutlin-3 (10 μ M) for 1 h, followed by treatment with 1-NP (10 μ M) for 24 h. Afterward, cells were harvested, and cell lysates were analyzed by Western blotting using antibodies against p53, HDM2, phosphor-p53 (Ser15), phosphor-HDM2 (Ser166), and β -actin.

TCDD's suppression of 1-NP-induced p53, shRNAs of CYP1A1 were introduced into HepG2 cells by a lentivirus to knock down CYP1A1 levels. Lentiviruses were used to individually produce an shRNA of firefly luciferase (Luc) and two different shRNAs of CYP1A1, designated CYP1A1-a and -b, respectively, in virus-infected cells. Viruses with the vector and shRNA-Luc were used as a control of viral infection. CYP1A1 induced by TCDD was seen in cells without and with the vector and shRNA-Luc but not in cells with shRNA-CYP1A1-a or -b, which indicated that CYP1A1 expression was knocked down by shRNA-CYP1A1 (Figure 7A). Relative TCDD-induced CYP1A1 protein levels in cells, without and with shRNAs, were revealed by Western blotting and quantified (Figure 7B). There was not much difference in induced CYP1A1 protein levels between cells without and with shRNA-Luc. However, only 13% and 17% of CYP1A1 were left in cells treated with shRNA-CYP1A1-a and -b, respectively.

Cells without and with shRNA-CYP1A1s were monitored for 1-NP-induced p53 in the presence of TCDD. Results indicated that p53 induced by 1-NP was abolished in cells pretreated with TCDD for 24 h, without and with the vector and shRNA-Luc (Figure 7C). However, p53 induced by 1-NP was not abolished in cells pretreated with TCDD for 24 h with shRNA-CYP1A1-a and -b.

TCDD and PFT- α Reduce 1-NP-Induced Cytotoxicity and Mutagenesis. How TCDD and PFT- α influence 1-NP-induced cytotoxicity and mutagenesis was examined here by a colony-forming ability assay and mutagenesis assays. When cells were cultured for an additional 14 days after treatment with the test compounds, the cell survival rate was determined by the colony-forming ability. Cell survival did not change under treatment with TCDD (24 and 48 h) or PFT- α (24 h) but was reduced under treatment with 1-NP (24 h) (Figure 8A,B). Pretreatment (24 h) with TCDD and individual cotreatment with TCDD or PFT- α neutralized the 1-NP-decreased cell survival rate. Treatment with TCDD (for 24 and 48 h), PFT- α , and 1-NP, respectively, led to 16-, 11-, 8.5-, and 22-fold increases in the *hprt* mutation frequency in HepG2 cells (Figure 8C). A longer exposure to TCDD (48 h) caused a lower *hprt* mutation frequency than did a shorter exposure to TCDD (24 h). Compared to treatment with 1-NP alone, treatment with 1-NP plus TCDD did not further increase the *hprt* mutation frequency, and treatment with 1-NP plus pretreatment (24 h) with TCDD led to a lower *hprt* mutation frequency. In addition, cotreatment with 1-NP plus PFT- α led to a lower *hprt* mutation frequency than did treatment with 1-NP alone.

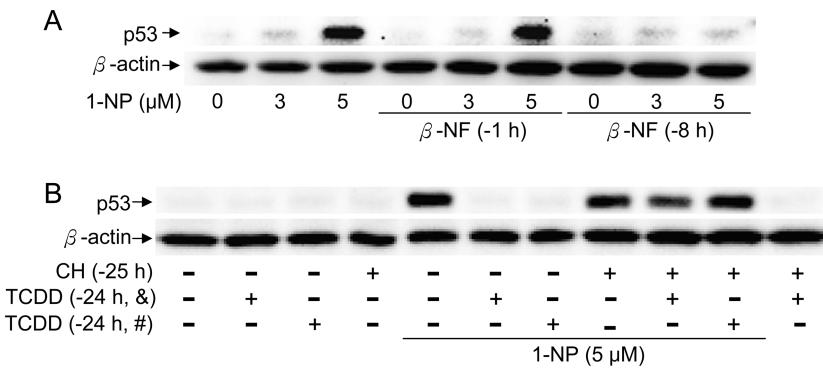


Figure 6. Effects of an aryl hydrocarbon receptor (AHR) agonist and antagonist on TCDD's suppression of 1-NP-induced p53 expression. (A) HepG2 cells were pretreated with β -naphthoflavon (β -NF) (10 μ M) for 1 and 8 h, followed by treatment with 1-NP (3 and 5 μ M) for 24 h. (B) HepG2 cells were pretreated with CH-223191 (CH) (10 μ M) for 25 h and pretreated with TCDD for 24 h, and then they were either removed (#) or were not removed (&), followed by treatment with 1-NP (5 μ M) for 24 h. Afterward, cells were harvested, and cell lysates were analyzed by Western blotting using antibodies against p53 and β -actin.

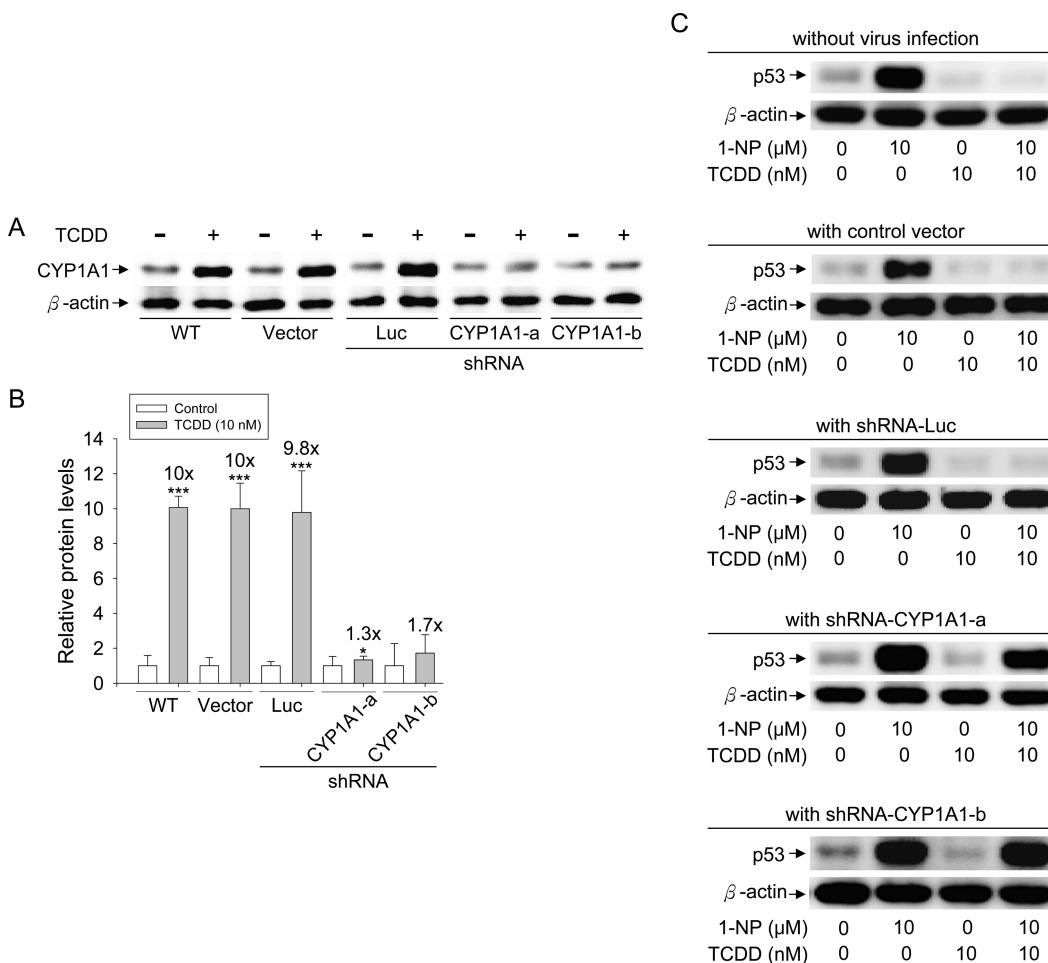


Figure 7. Effect of cytochrome P450 1a1 (CYP1A1) RNAi (interference) on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)'s abolition of p53 expression induced by 1-nitropyrene (1-NP). Lentiviruses individually carrying a short hairpin (sh)RNA of firefly luciferase (Luc) and two different shRNAs of CYP1A1, respectively, designated CYP1A1-a and -b, were used to infect cells and create respective shRNAs, as described in Experimental Procedures. A virus with the vector or shRNA-Luc was used as the control for viral infection. (A) HepG2 cells, without and with the vectors, shRNA-Luc and shRNA-CYP1A1s, were treated with TCDD (10 nM) for 9 h. Expressions of CYP1A1 and β -actin proteins were analyzed by Western blotting. (B) CYP1A1 protein levels revealed by the Western blots were quantified and standardized against the amount of β -actin protein. Results are expressed as the mean \pm SEM, $n = 3$. *** $p < 0.001$; * $p < 0.05$. (C) Cells with or without shRNA were pretreated with TCDD (10 nM) for 24 h and then treated with 1-NP (10 μ M) for 24 h. p53 protein expression of their cell lysates was analyzed by Western blotting.

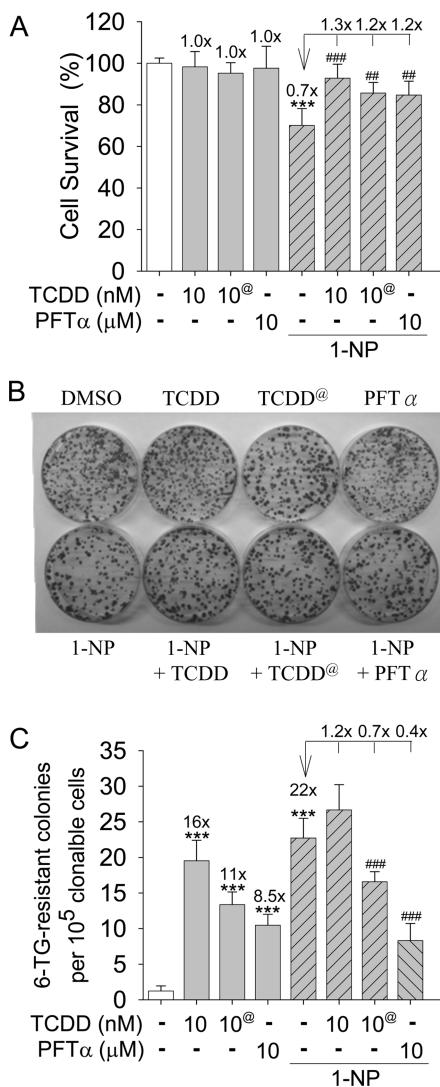


Figure 8. Cytotoxicity and mutagenesis induced by 1-nitropyrene (1-NP), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and pifithrin-alpha (PFT- α). Cells were treated with 10 μ M 1-NP, 10 nM TCDD, 10 μ M PFT- α , or binary mixtures of these compounds as indicated in the figure for 24 h. [@] Indicates cells pretreated with TCDD for 24 h before the addition of 1-NP; therefore, the treatment period of TCDD was 48 h. (A) Percent survival was determined by the colony-forming ability of drug-treated cells relative to the control. A colony-forming ability assay was performed as described in the cytotoxicity assay in Experimental Procedures. Data are the mean \pm SEM of three replicate experiments. (B) Representative photograph of culture dishes showing the colony-forming ability assay for cell survival. (C) Mutagenesis induced by 1-NP, TCDD, and PFT- α was determined by the induced 6-TG-resistant colonies per 10^5 clonable cells. The mutagenesis assay was performed as described in Experimental Procedures. Data are expressed as the mean \pm SEM where $n = 10$. * Indicates a comparison with the negative control (DMSO-treated cells). # Indicates a comparison with the group treated with 1-NP alone, as indicated by an arrow. *** $p < 0.001$; # $p < 0.01$; and ## $p < 0.05$.

DISCUSSION

Both 1-NP and BaP, but not TCDD, highly induced p53 expression and phosphorylation. Advance treatment (24 h) with TCDD completely abolished 1-NP- but not BaP-induced p53. When pretreatment (24 h) of TCDD was stopped before treatment with 1-NP, signals responsive to TCDD for suppressing

1-NP's action still persisted and were highly effective. The results indicated that pretreatment is required to turn on TCDD-induced signals to suppress 1-NP's but not BaP's action. TCDD was also shown to attenuate p53's response to the DNA-damaging agent, diethylnitrosamine,²⁵ and hypoxic conditions.²⁸ The reported TCDD-induced suppression of p53 expression is suspected to occur due to HDM2-mediated degradation of p53, which was not yet proven by direct evidence. In contrast, TCDD did not attenuate BaP- or nutlin-3-induced p53 expression, an indication that TCDD's action on p53 induction is selective.

HDM2 is a p53-driven gene and is produced for the negative feedback of p53 expression.²⁹ Phosphorylation of HDM2 at Ser166 is associated with active HDM2.³⁰ In the presence of TCDD, the expression patterns of HDM2 induced by 1-NP and BaP paralleled those of p53. Although p53 is supposedly degraded by the proteasome, mediated by E3 ubiquitin ligase, HDM2,²⁹ our data showed that BaP-induced p53 still existed in the presence of active HDM2. Results indicated that HDM2 is not the only factor involved in TCDD's suppression of 1-NP-induced p53 expression, and it is likely due to TCDD's suppression of 1-NP-induced p53 production.

Further analysis was performed of the contribution of HDM2 to the abolition of 1-NP-induced p53. An HDM2 inhibitor, nutlin-3, was applied to block HDM2's activity. In the presence of nutlin-3, TCDD still suppressed 1-NP's induction of p53 expression, which indicates that TCDD's suppression of 1-NP-induced p53 is not mainly due to the presence of HDM2. Nutlin-3 itself also induced p53 and HDM2 expressions, but TCDD did not abolish nutlin-3-induced p53 or HDM2 expression. These results further imply that TCDD's suppression of p53 expression is specific to 1-NP's action.

Both β -NF, an AHR agonist, and TCDD are able to activate the AHR. Pretreatment with TCDD is required to abolish p53 expression induced by 1-NP. β -NF mimicked the effect of TCDD in abolishing the induction of p53 by 1-NP. Induction of p53 expression by 1-NP was abolished in cells pretreated with β -NF (10 μ M) for 8 h, but not 1 h, which indicates that pretreatment with β -NF for more than 1 h is required to induce the AHR-targeting gene, the protein product of which is involved in abolishing p53 expression induced by 1-NP. CH-223191, a potent AHR antagonist, was applied to block TCDD from binding to the AHR. When the AHR was blocked by CH-223191 and could not be activated by TCDD, 1-NP-induced p53 expression was not suppressed by TCDD. These results further indicate that AHR-targeted gene expression induced by TCDD is required for the abolition of 1-NP's induction of p53.

CYP1A1, CYP1A2, and CYP1B1 are phase-I xenobiotic-metabolizing enzymes and are transcribed by the activated AHR.⁸ TCDD is the most potent known CYP1A1 inducer, the action of which is mediated by the transcriptional activity of the activated AHR.³¹ In addition, TCDD induces much-greater CYP1A1 expression than CYP1A2 or CYP1B1 expressions in HepG2 cells.³² It was reported that genotoxicity, indicated by *umu* gene expression, of 1-NP in *Salmonella typhimurium* TA1535/pSK1002 decreases in the presence of CYP1A1.³³ Therefore, we were interested in analyzing whether CYP1A1 mediates TCDD's suppression of 1-NP-induced p53 expression. By applying shRNA-CYP1A1, which knocked down the CYP1A1 expression level, data showed that TCDD was unable to abolish 1-NP's induction of p53 expression in CYP1A1-deficient cells. These results are consistent with data of experiments applying an AHR agonist, β -NF, and an AHR antagonist, CH-223191, to,

respectively, mimic and block TCDD's action. Accordingly, CYP1A1 is essential for TCDD to abolish 1-NP's action in inducing p53, which implies that CYP1A1 may have a detoxification function against 1-NP. Although 1-NP itself induces CYP1A1,³⁴ before CYP1A1 induced by 1-NP appears, 1-NP is likely to damage cells and induce p53 expression. Therefore, p53 induced by 1-NP was abolished only when CYP1A1 largely preexisted in cells before the presence of 1-NP.

In response to p53, the best-characterized cellular responses are apoptosis and cell cycle arrest. p53 is a transcription factor and harbors a DNA response element, resulting in the control of its target gene expression. p21 is regulated by p53 and mediates cellular responses to p53 for antiapoptotic and repair processes.³⁵ Our data indicated that PFT- α , a p53 inhibitor, decreased 1-NP- and BaP-induced p53 activity and p21 expression. In contrast, TCDD was able to inhibit 1-NP- but not BaP-induced p53 activity and p21 expression. Results imply that TCDD differentially regulates 1-NP- and BaP-induced p53 expression/activity.

Both 1-NP and BaP are mutagens.^{11,14,15} It was reported that TCDD increases BaP-DNA adduct formation in fetal dolphin kidney cells,³⁶ but coexposure to both TCDD and indolo[3,2-b]carbazole, a natural AHR ligand, significantly reduced the amount of BaP-DNA adducts in a human colorectal adenocarcinoma cell line.³⁷ It was reported that TCDD (2 μ g/kg, twice each week) was not mutagenic to rats over a 6-week exposure period³⁸ and that treatment with TCDD completely prevented aflatoxin-induced mutations in female rats.³⁹ Continuous ingestion (2 years) of a high dose of 0.1 μ g TCDD/kg/day decreased the incidence of tumors of the pituitary, uterus, mammary gland, pancreas, and adrenal medulla in rats.⁴⁰ Accordingly, TCDD potentially provides signals preventing mutations. PFT- α was shown to protect mice from lethal genotoxic stress.²³ Our results demonstrated that although PFT- α inhibited 1-NP-induced p53 activity and TCDD abolished 1-NP-induced p53 expression, both PFT- α and TCDD decreased 1-NP-induced cell death and the mutation frequency. Both PFT- α and TCDD are potent agonists of the AHR and induce CYP1A1 expression. Therefore, neutralization of 1-NP-induced cell death and mutagenesis by PFT- α and TCDD are likely due to the induction of CYP1A1, which detoxifies 1-NP.

It was reported that 1-NP is principally metabolized by nitroreduction to 1-aminopyrene and by CYP-mediated C-oxidation to K-region *trans*-dihydrodiols, 1-nitropyrene-3-ol, 1-nitropyrene-6-ol, and 1-nitropyrene-8-ol in uninduced HepG2 cells.⁴¹ In contrast, a complete change in the metabolism of 1-NP with 1-nitropyrene-6-ol and 1-nitropyrene-8-ol formation being 80-fold greater than 1-aminopyrene formation was observed in HepG2 cells pretreated with TCDD for 24 h, which indicated that TCDD-induced CYP-mediated C oxidation dominates the metabolism of 1-NP. A major DNA adduct of 1-NP, *N*-(2'-deoxyguanosin-8-yl)-1-aminopyrene (dG-C8-AP) derived from the nitroreduction pathway of 1-NP, was demonstrated in HepG2 cell culture.⁴¹ TCDD pretreatment of HepG2 cells resulted in a 99% reduction of the dG-C8-AP adduct in HepG2 cells, which was correlated with an increase in CYP-mediated C-oxidation and loss of the nitroreduction of 1-nitropyrene to 1-aminopyrene. Accordingly, Silvers et al. suggested that CYP-mediated C-oxidative pathways are detoxification pathways in HepG2 cells.⁴¹ CYP1A1 is the major CYP1 enzyme induced by the activated AHR in HepG2 cells.⁴² On the basis of the data in this report, we postulated that TCDD and PFT- α induced the detoxification of 1-NP as mediated by CYP1A1.

In contrast to CYP1A1's detoxification of 1-NP, the expression of Cyp1a1 corresponds well with BaP's induction of apoptosis.¹⁰ In addition, suppression of BaP-induced Cyp1a1 results in a concomitant decrease in BaP-induced DNA adducts.⁴³ Pretreatment of animals with TCDD prior to *in vitro* incubation with BaP increases the levels of DNA adduct formation.⁴⁴ BaP is metabolized by CYP-mediated C-oxidation to bay-region diol-epoxide and catechol. Catechol is further oxidized by oxygen radicals to benzo-(*a*)pyrene-7,8-dione.⁴⁵ Both bay-region diol-epoxide and benzo-(*a*)pyrene-7,8-dione potentially interact with nucleotides and form DNA adducts.^{45,46} Therefore, in the presence of TCDD, differential genotoxic response levels will appear between cells treated with 1-NP and BaP.

It was reported that PFT- α decreases NO production, which may be associated with the increased survival of hemopoietic clonogenic cells *in vivo*.⁴⁷ PFT- α reduces BaP metabolism, the covalent binding of BaP, and BaP metabolite-induced apoptosis.^{48,49} In addition, PFT- α induces CYP1B1 expression but highly inhibits CYP1B1 activity in human mammary tumor MCF-7 cells.⁴⁹ Whether NO reduction and the potent inhibitory effect toward CYP1B1 of PFT- α are associated with PFT- α 's action of reducing 1-NP-induced cell death and mutagenesis remains to be determined in future studies.

In conclusion, in addition to HDM2, other factors may be involved in TCDD-induced abolition of p53 expression. We applied an AHR antagonist (CH-223191), AHR agonists (TCDD, β -NF, and PFT- α) which induced CYP1A1, and RNAi-CYP1A1s which knocked down the CYP1A1 protein level, to analyze the cause of TCDD-induced suppression of 1-NP-induced p53, cell death, and mutagenesis. Results indicated that CYP1A1 is required for TCDD-induced detoxification of 1-NP. This report provides an avenue to analyze the novel mechanisms of adaptations which will provide pharmaceutical targets in future investigations.

AUTHOR INFORMATION

Corresponding Author

*Tel: +886-5-271-7785. Fax: +886-5-271-7999. E-mail: jgsu@mail.ncyu.edu.tw.

Author Contributions

[†]These authors contributed equally to this work.

ABBREVIATIONS

AHR, aryl hydrocarbon receptor; BaP, benzo[*a*]pyrene; CYP, cytochrome P450; MDM2 (HDM2 in humans), mouse double minute 2; 1-NP, 1-nitropyrene; p53RE, p53 response element; PAHs, polycyclic aromatic hydrocarbons; PFT- α , pifithrin-alpha; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

REFERENCES

- (1) Singh, S., and Prakash, V. (2007) Toxic environmental releases from medical waste incineration: a review. *Environ. Monit. Assess.* 132, 67–81.
- (2) Schecter, A., Birnbaum, L., Ryan, J. J., and Constable, J. D. (2006) Dioxins: an overview. *Environ. Res.* 101, 419–428.
- (3) Van den Berg, M., Birnbaum, L., Bosveld, A. T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X., Liem, A. K., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T. (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect.* 106, 775–792.

- (4) Cole, P., Trichopoulos, D., Pastides, H., Starr, T., and Mandel, J. S. (2003) Dioxin and cancer: a critical review. *Regul. Toxicol. Pharmacol.* 38, 378–388.
- (5) Bock, K. W., and Kohle, C. (2006) Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. *Biochem. Pharmacol.* 72, 393–404.
- (6) Connor, K. T., and Aylward, L. L. (2006) Human response to dioxin: aryl hydrocarbon receptor (AhR) molecular structure, function, and dose-response data for enzyme induction indicate an impaired human AhR. *J. Toxicol. Environ. Health, Part B* 9, 147–171.
- (7) Guengerich, F. P. (2008) Cytochrome p450 and chemical toxicology. *Chem. Res. Toxicol.* 21, 70–83.
- (8) Nakata, K., Tanaka, Y., Nakano, T., Adachi, T., Tanaka, H., Kaminuma, T., and Ishikawa, T. (2006) Nuclear receptor-mediated transcriptional regulation in Phase I, II, and III xenobiotic metabolizing systems. *Drug Metab. Pharmacokinet.* 21, 437–457.
- (9) Shimada, T. (2006) Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. *Drug Metab. Pharmacokinet.* 21, 257–276.
- (10) Solhaug, A., Refsnes, M., Lag, M., Schwarze, P. E., Husoy, T., and Holme, J. A. (2004) Polycyclic aromatic hydrocarbons induce both apoptotic and anti-apoptotic signals in Hepa1c1c7 cells. *Carcinogenesis* 25, 809–819.
- (11) Aoki, Y., Hashimoto, A. H., Amanuma, K., Matsumoto, M., Hiyoshi, K., Takano, H., Masumura, K., Itoh, K., Nohmi, T., and Yamamoto, M. (2007) Enhanced spontaneous and benzo(a)pyrene-induced mutations in the lung of Nrf2-deficient gpt delta mice. *Cancer Res.* 67, 5643–5648.
- (12) NIST (2000) *Certificate of Analysis, Standard Reference Material (SRM) 2975, Diesel Particulate Matter (Industrial Forklift)*, pp 1–8, National Institute of Standards and Technology (NIST), Gaithersburg, MD.
- (13) NIST (2008) *Certificate of Analysis, Standard Reference Material (SRM) 1975, Diesel Particulate Extract*, pp 1–9, National Institute of Standards and Technology (NIST), Gaithersburg, MD.
- (14) Schuetzle, D., Lee, F. S., and Prater, T. J. (1981) The identification of polynuclear aromatic hydrocarbon (PAH) derivatives in mutagenic fractions of diesel particulate extracts. *Int. J. Environ. Anal. Chem.* 9, 93–144.
- (15) Andersson, H., Piras, E., Demma, J., Hellman, B., and Brittebo, E. (2009) Low levels of the air pollutant 1-nitropyrene induce DNA damage, increased levels of reactive oxygen species and endoplasmic reticulum stress in human endothelial cells. *Toxicology* 262, 57–64.
- (16) Su, J. G., Liao, P. J., Huang, M. C., Chu, W. C., Lin, S. C., and Chang, Y. J. (2008) Aldo-keto reductase 1C2 is essential for 1-nitropyrene's but not for benzo[a]pyrene's induction of p53 phosphorylation and apoptosis. *Toxicology* 244, 257–270.
- (17) Feki, A., and Irminger-Finger, I. (2004) Mutational spectrum of p53 mutations in primary breast and ovarian tumors. *Crit. Rev. Oncol. Hematol.* 52, 103–116.
- (18) Guimaraes, D. P., and Hainaut, P. (2002) TP53: a key gene in human cancer. *Biochimie* 84, 83–93.
- (19) Jegga, A. G., Inga, A., Menendez, D., Aronow, B. J., and Resnick, M. A. (2008) Functional evolution of the p53 regulatory network through its target response elements. *Proc. Natl. Acad. Sci. U.S.A.* 105, 944–949.
- (20) Olivier, M., Petitjean, A., Marcel, V., Petre, A., Mounawar, M., Plymoth, A., de Fromentel, C. C., and Hainaut, P. (2009) Recent advances in p53 research: an interdisciplinary perspective. *Cancer Gene Ther.* 16, 1–12.
- (21) Honda, R., Tanaka, H., and Yasuda, H. (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* 420, 25–27.
- (22) Shangary, S., and Wang, S. (2008) Targeting the MDM2-p53 interaction for cancer therapy. *Clin. Cancer Res.* 14, 5318–5324.
- (23) Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V., and Gudkov, A. V. (1999) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* 285, 1733–1737.
- (24) Hoagland, M. S., Hoagland, E. M., and Swanson, H. I. (2005) The p53 inhibitor pifithrin-alpha is a potent agonist of the aryl hydrocarbon receptor. *J. Pharmacol. Exp. Ther.* 314, 603–610.
- (25) Paajarvi, G., Viluksela, M., Pohjanvirta, R., Stenius, U., and Hogberg, J. (2005) TCDD activates Mdm2 and attenuates the p53 response to DNA damaging agents. *Carcinogenesis* 26, 201–208.
- (26) Seifert, A., Taubert, H., Hombach-Klonisch, S., Fischer, B., and Navarrete Santos, A. (2009) TCDD mediates inhibition of p53 and activation of ERalpha signaling in MCF-7 cells at moderate hypoxic conditions. *Int. J. Oncol.* 35, 417–424.
- (27) Klein, C. B., Broday, L., and Costa, M. (1999) *Mutagenesis Assays in Mammalian Cells*, John Wiley and Sons, Inc., Hoboken, NJ.
- (28) Seifert, A., Taubert, H., Hombach-Klonisch, S., Fischer, B., and Navarrete Santos, A. (2009) TCDD mediates inhibition of p53 and activation of ERalpha signaling in MCF-7 cells at moderate hypoxic conditions. *Int. J. Oncol.* 35, 417–424.
- (29) Mayo, L. D., and Donner, D. B. (2002) The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. *Trends Biochem. Sci.* 27, 462–467.
- (30) Zhou, B. P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M. C. (2001) HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat. Cell Biol.* 3, 973–982.
- (31) Whitlock, J. P., Jr. (1999) Induction of cytochrome P4501A1. *Annu. Rev. Pharmacol. Toxicol.* 39, 103–125.
- (32) Iwanari, M., Nakajima, M., Kizu, R., Hayakawa, K., and Yokoi, T. (2002) Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitro-polycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. *Arch. Toxicol.* 76, 287–298.
- (33) Yamazaki, H., Hatanaka, N., Kizu, R., Hayakawa, K., Shimada, N., Guengerich, F. P., Nakajima, M., and Yokoi, T. (2000) Bioactivation of diesel exhaust particle extracts and their major nitrated polycyclic aromatic hydrocarbon components, 1-nitropyrene and dinitropyrenes, by human cytochromes P450 1A1, 1A2, and 1B1. *Mutat. Res.* 472, 129–138.
- (34) Chu, W. C., Hong, W. F., Huang, M. C., Chen, F. Y., Lin, S. C., Liao, P. J., and Su, J. G. (2009) 1-Nitropyrene stabilizes the mRNA of cytochrome P450 1A1, a carcinogen-metabolizing enzyme, via the Akt pathway. *Chem. Res. Toxicol.* 22, 1938–1947.
- (35) Weiss, R. H. (2003) p21Waf1/Cip1 as a therapeutic target in breast and other cancers. *Cancer Cell* 4, 425–429.
- (36) Carvan, M. J., III, Flood, L. P., Campbell, B. D., and Busbee, D. L. (1995) Effects of benzo(a)pyrene and tetrachlorodibenzo-(p)dioxin on fetal dolphin kidney cells: inhibition of proliferation and initiation of DNA damage. *Chemosphere* 30, 187–198.
- (37) de Waard, P. W., de Kok, T. M., Maas, L. M., Peijnenburg, A. A., Hoogenboom, R. L., Aarts, J. M., and van Schooten, F. J. (2008) Influence of TCDD and natural Ah receptor agonists on benzo[a]pyrene-DNA adduct formation in the Caco-2 human colon cell line. *Mutagenesis* 23, 67–73.
- (38) Thornton, A. S., Oda, Y., Stuart, G. R., Glickman, B. W., and de Boer, J. G. (2001) Mutagenicity of TCDD in Big Blue transgenic rats. *Mutat. Res.* 478, 45–50.
- (39) Thornton, A. S., Oda, Y., Stuart, G. R., Holcroft, J., and de Boer, J. G. (2004) The dioxin TCDD protects against aflatoxin-induced mutation in female rats, but not in male rats. *Mutat. Res.* 561, 147–152.
- (40) Kociba, R. J., Keyes, D. G., Beyer, J. E., Carreon, R. M., and Gehring, P. J. (1979) Long-term toxicologic studies of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in laboratory animals. *Ann. N.Y. Acad. Sci.* 320, 397–404.
- (41) Silvers, K. J., Couch, L. H., Rorke, E. A., and Howard, P. C. (1997) Role of nitroreductases but not cytochromes P450 in the metabolic activation of 1-nitropyrene in the HepG2 human hepatoblastoma cell line. *Biochem. Pharmacol.* 54, 927–936.
- (42) Iwanari, M., Nakajima, M., Kizu, R., Hayakawa, K., and Yokoi, T. (2002) Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitro-polycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. *Arch. Toxicol.* 76, 287–298.

- (43) Kang, Z. C., Tsai, S. J., and Lee, H. (1999) Quercetin inhibits benzo[a]pyrene-induced DNA adducts in human Hep G2 cells by altering cytochrome P-450 1A1 gene expression. *Nutr. Cancer* 35, 175–179.
- (44) Harrigan, J. A., Vezina, C. M., McGarrigle, B. P., Ersing, N., Box, H. C., MacCubbin, A. E., and Olson, J. R. (2004) DNA adduct formation in precision-cut rat liver and lung slices exposed to benzo[a]pyrene. *Toxicol. Sci.* 77, 307–314.
- (45) Jin, Y., and Penning, T. M. (2007) Aldo-keto reductases and bioactivation/detoxication. *Annu. Rev. Pharmacol. Toxicol.* 47, 263–292.
- (46) Baird, W. M., Hooven, L. A., and Mahadevan, B. (2005) Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *Environ. Mol. Mutagen.* 45, 106–114.
- (47) Proskuryakov, S. Y., Konoplyannikov, A. G., Konoplyannikova, O. A., Shevchenko, L. I., Verkhovskii, Y. G., and Tsyb, A. F. (2009) Possible involvement of NO in the stimulating effect of pifithrins on survival of hemopoietic clonogenic cells. *Biochemistry (Moscow)* 74, 130–136.
- (48) Solhaug, A., Ovrebo, S., Mollerup, S., Lag, M., Schwarze, P. E., Nesnow, S., and Holme, J. A. (2005) Role of cell signaling in B[a]P-induced apoptosis: characterization of unspecific effects of cell signaling inhibitors and apoptotic effects of B[a]P metabolites. *Chem.-Biol. Interact.* 151, 101–119.
- (49) Sparfel, L., Van Grevenynghe, J., Le Vee, M., Aninat, C., and Fardel, O. (2006) Potent inhibition of carcinogen-bioactivating cytochrome P450 1B1 by the p53 inhibitor pifithrin alpha. *Carcinogenesis* 27, 656–663.