Original Article

Synergistic Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin and N-nitrosodiethylamine on Cell Malignant Transformation*

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Abstract

Objective The present paper aims to investigate the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and N-nitrosodiethylamine (DEN) on tumorigenesis and its potential mechanism.

Methods The potentials of TCDD and DEN in separation or in combination to induce malignant transformation were tested in Balb/c 3T3 cells by using a cell transformation assay method. The possible mechanism of observed effects was studied further by adding α-naphthoflavone (α-NF), a competitive binding agent of TCDD, to the Aryl hydrocarbon receptor (AhR) pathway. The mRNA expressions of *Cyp1a1* and *Cyp2a5* gene in Balb/c 3T3 cells treated by DEN and TCDD in separation or in combination with or without presence of α-NF were measured with fluorescence quantification RT-PCR technique.

Results The cell transformation frequency (TF) was significantly higher in case of induction with TCDD in combination with DEN, as compared to that with either TCDD or DEN alone. These effects were not inhibited via α-NF. The mRNA expression levels of both *Cyp1a1* and *Cyp2a5* were enhanced by TCDD treatment alone, but this inducible effect was blocked in cells treated by TCDD and DEN in combination.

Conclusion TCDD and DEN had a significant synergistic effect on tumorigenesis when they were used in combination. AhR pathway may not be the key mechanism of this synergistic effect. Thus, it is necessary to further test the potential mechanism involved in cancer development.

Key words: Dioxin; Nitrosodiethylamine; Cell transformation; Tumorigenesis

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INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a member of the large family of organochlorinated chemicals. In 1997, the International Agency for Research on Cancer (IARC) classified TCDD as one of group 1 carcinogens. This judgment was based on sufficient evidence from experimental animals; however, the evidence from humans was limited and its carcinogenic mechanism remains to be elaborated. As a result, TCDD has been faced with challenges and controversies in terms of its role in human cancer in the past decade[1-4]. Moreover, up to now current research process has not fully considered possible synergistic effects of TCDD and other carcinogens on carcinogenesis. This is partly due to lacking of studies exploring the effect of TCDD in the presence of other carcinogens. Therefore, a combination study will be helpful to further understand the role

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of TCDD in the development of human cancers when it coexists with other carcinogens in a real living environment.

One of such coexisting carcinogens is N-Nitrosodiethylamine (DEN) present in the environment and in tobacco smoke[5]. DEN is one of the nitrosamine compounds that produce tumors in all animal species tested so far[6]. The major target organs are the liver, respiratory and upper digestive tracts and kidney[7]. IARC classified DEN as one of group 2A carcinogens.

Like most of nitrosamine compounds, DEN requires metabolic activation to exhibit its mutagenic and carcinogenic action. This biotransformation process involves hydroxylation of the α-carbon, which is catalyzed by the cytochrome P450 (CYP) oxygenase system. The resultant a-hydroxyalkylnitrosamine breaks down to an alkyldiazonium ion and the corresponding carbonyl compound. The diazonium ion could alkylate a variety of nucleophilic sites such as deoxyribonucleic acid (DNA) and ribonucleic acid(RNA)[8-9].

Meanwhile, TCDD is the well-known potent and persistent aryl hydrocarbon receptor (AhR) agonist, which strongly induces expression of many *Cyp* genes[10-12]. Although nitrosamines are bioactivated by the phase II CYP (such as CYP2E1) while TCDD mainly activates the phase I CYP (such as CYP1A1), studies have shown that TCDD can also up-regulate the expression of mouse phase II CYP2A5 oxygenase, an orthlogue of human CYP2A6[13]. A study on patients with oesophageal cancer found that the expression levels of *Cyp2a6* were promoted in the oesophageal mucosa of these patients and involved in the activation of N-nitroso-diethylamine (DEN)[14]. Therefore, there may be a synergistic effect of TCDD and DEN on regulation of the expression of *Cyp* genes.

The cell transformation assay is an effective method to test synergistic carcinogenic effects of different carcinogens[15]. Cell transformation has been defined as the induction of certain phenotypic alterations in cultured cells that are characteristic of tumorigenic cells. It has been shown to be a multistage process which closely models the various stages of *in vivo* carcinogenesis. A cell transformation assay can provide some crucial evidence specific to the tumorigenic potential of various types of carcinogens, which cannot be supplied by genotoxicity testing. Compared with animal tests, the cell transformation assay can avoid both the time-consuming process and the use of experimental animals. It is widely acknowledged that this method has good concordance between transformation and *in vivo* carcinogenicity, and is highly sensitive and specific for detecting the carcinogenic activity of chemicals. The European Centre for the Validation of Alternative Methods (ECVAM) summarized that the concordance, sensitivity and specificity of Balb/c 3T3 cell transformation assay were 71%, 80%, and 60%[16], respectively, which are consistent with earlier evaluation made by IARC[17] and more recent evaluation by Sakai[18].

In this paper, we used an improved cell transformation assay protocol and fluorescence quantification RT-PCR technique to study the synergetic effect of TCDD and DEN and its possible tumorigenic mechanism.

MATERIALS AND METHODS

Cells and Media

Balb/c 3T3 clone A31-1-1 cells (ATCC, U.S.) were routinely grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, U.S.), supplemented with 10% fetal bovine serum (FBS, YHSM, China), 1.1 g/L $NaHCO_3$, 100 μg/mL streptomycin and 100 U/mL penicillin (D10F-medium) in a humidified incubator (5% CO_2 plus 95% air). Another culture medium DMEM/F-12 (1:1) (Hyclone, U.S.) supplemented with 5% FBS, 0.5 g/L $NaHCO_3$, 100 μg/mL streptomycin and 100 U/mL penicillin (DF5F-medium) was used for the cell transformation assay.

Chemicals

TCDD (50 μg/mL, Cambridge isotope Laboratories, U.S.) was stocked in dimethyl sulfoxide (DMSO). DEN (liquid, sigma, U.S.) was dissolved and stocked as 10 mmol/L solution in sterile deionized water, and α-naphthoflavone (α-NF, 1.0 g, sigma, U.S.) was dissolved and stocked as 3.5 mmol/L in 20% ethanol. These chemicals were serially diluted by culture medium to prepare final test concentrations as follows: DEN 100 μmol/L, TCDD 0.01 mg/L, α-NF 7.0 μmol/L. (According to our preliminary experiment, these doses were the ones with the highest cell survival rate of 80%-100% in the cell toxic test and the highest cell transformation frequency in the cell transformation assay for DEN.) The concentration of DMSO or ethanol was less than 0.1% of that of the medium, which would not affect cell growth and induction of transformed foci.

Cytotoxicity and Cell Transformation Assay

In the cytotoxicity assay, Balb/c 3T3 cells in the state of exponential growing were seeded at 400 cells/flask in two culture flasks (T25 with filtered cap) for each of the treatment groups. After incubation in D10F-medium for 24 h, the cells were exposed to the corresponding chemicals for 7 days, with the medium with chemicals changed once in this duration. At the end of the treatment, cells were fixed with methanol and stained with Giemsa solution. The endpoints of the assay were formation of cell colonies (larger than 50 cells) and determination of the clonal efficiency (CE) in each treatment group.

In the cell transformation assay, Balb/c 3T3 cells in the state of exponential growing were seeded at 2×10^4 cells per flask, incubated in D10F-medium for 24 h, and exposed to the chemicals for 7 days, with the medium with the chemicals changed once during this period. After exposure, the medium was removed, and the exposed and control cells were reseeded in six T25 flasks at 2×10^4/flask for each treatment group, and cultured in DF5F-medium for 28 days, with the medium changed every three days. The cells were then fixed with methanol and stained with Giemsa solution. Transformed foci were scored according to morphological criteria: deep basophilic staining and dense multi-layering of cells; random orientation of cells at the edge of foci and large than 2 mm[19].

Cell transformation frequency (TF) was calculated by dividing the number of foci/flask by the number of cells at risk estimated from the CE obtained in the cytotoxicity assays. The formula was shown as follows:

TF ($\times10^{-4}$)=Number of foci per flask /(CE×2).

RNA Isolation and Reverse Transcription

Exponentially growing cells were seeded at 5×10^4/flask and incubated in D10F-medium for 24 h, and then exposed to the chemicals for 48 h. The control and exposed cells were collected and all RNA was isolated by using the commercial kit (QIAGEN, GER). The total isolated RNA (0.8 μg) was then reversely transcribed into cDNA in a 20 μL reaction mixture containing MMLV reverse transcriptase, *oligo dT* primer and random 6 primers via incubation at 37 °C for 15 min, followed by 85 °C for 5 s. The cDNA product was stored at 4 °C and ready for amplification.

cDNA Amplification and Purification

Five μL cDNA for *Cyp1a1, Cyp2a5, and β-actin* each was amplified by PCR in a 50 μL reaction mixture containing 2U Taq DNA polymerase (TaKaRa, Dalian, China), 0.3 μmol/L of each primer pair for the amplification of *Cyp1a1*, *Cyp2a5* and *β-actin*, and 0.25 mmol/L each of *dATP, dGTP, dCTP,* and *dTTP*. The reaction was first incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 57 °C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 7 min. The sequences of PCR primer specific for *Cyp1a1, Cyp2a5* and *β-actin* were designed according to previous studies[20-21], as shown in Table 1.

Table 1. Primers Used for Real-time PCR and the Amplification of PCR Products

Genes	Primers (5' to 3')	Fragment Length (bp)
Cyp1a1	Forward GTGTCTGGTTACTTTGACAAGTGG Reverse AACATGGACATGCAAGGACA	206
Cyp2a5	Forward GGCAGCTCTATGAGATGTTC Reverse TTATGAAGTCCTCCAGGCCC	96
β-actin	Forward CGTTGACATCCGTAAAGACC Reverse AACAGTCCGCCTAGAAGCAC	281

The amplification products were then purified by using StarPrep Gel extraction kit (GenStar Biosolutions Co., Ltd) according to the manufacturer's instructions. The purified gene fragments were identified by gel electrophoresis. As shown in Figure 1, the sizes of gene fragments were consistent with those of expected gene fragments. These purified gene fragments were diluted at 1:10 and used for the real-time PCR as standard series.

Real-time Quantitative PCR Analyses

Real time quantitative PCR analyses were performed with SYBR® Primescript ™RT-PCR Kit II (TaKaRa, Dalian, China) in a real-time fluorescence quantitative PCR system (ABI 7500 Fast) for *Cyp1a1*, *Cyp2a5,* and *β-actin.* The cumulative fluorescence of the *Cyp1a1* and *Cyp2a5* products was normalized to that of *β-actin* as the housekeeping gene. Two μL of cDNA was amplified by PCR in 20 μL of a mixture

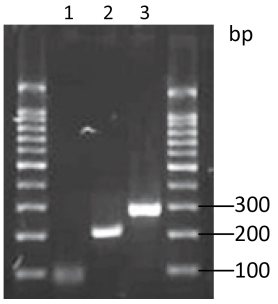

Figure 1. Identification of the purified gene fragments by gel electrophoresis. Line 1: *Cyp2a5*, Line 2: *Cyp1a1*, Line 3: *β-actin.*

containing 10 μL SYBR premix Ex Taq II, 6 μmol/L each of the forward and reverse primers, and 0.4 μL ROX Reference Dye II. The reaction was first incubated at 95 °C for 10 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 31 s, and ended with a melting curve analysis.

Standards were measured at the same time and the standard curves for each target gene were automatically generated (Figure 2). As the slope difference between the measured gene and the housekeeping gene was larger than 0.1, indicating a difference in the amplification efficiency, the hyperbolic relative quantitative method was used to evaluate the results.

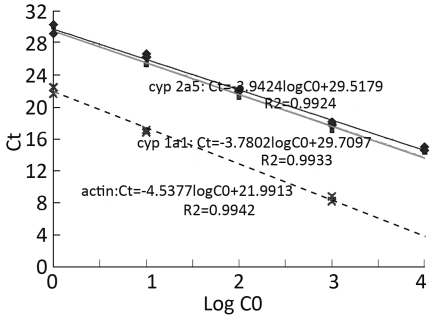

Figure 2. Real-time PCR standard curves for *Cyp1a1*, *Cyp2a5*, and *β-actin*.

Statistical Analysis

For most of the experiment settings, statistical evaluations were performed with the one way analysis of variance (ANOVA). The comparison of *Cyp1a1* or *Cyp2a5* mRNA expression between each chemical tested with or without the addition of α-NF was performed by using the student *t*-test. All statistics were performed at the 95% confidence level and with SPSS (vision 13.0) for Windows (SPSS, Inc. USA).

RESULTS

Synergistic Effect on Inducing Cell Malignant Transformation between DEN and TCDD

The ability of DEN and TCDD alone or in their combination to induce malignant transformation in Balb/c 3T3 cells were tested by using the cell transformation assay (Figure 3). Compared with the control group, TF in the DEN treatment group increased significantly (P<0.01), which indicated that DEN alone had carcinogenic potential. Moreover, no significant difference was observed in TF between the TCDD treatment group and the control group, suggesting a weak carcinogenic potential of TCDD alone. However, when cells were treated by TCDD and DEN together, TF was three times higher than that in the DEN treatment group (P<0.01), and 15 times higher than that in the TCDD treatment group (P<0.01).

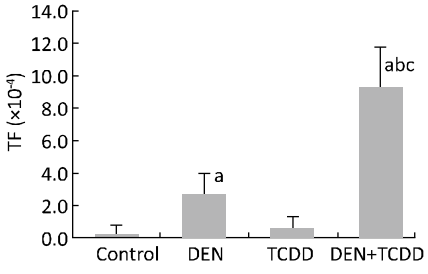

Figure 3. The transformation frequency (TF) of malignant transformation in Balb/c 3T3 cells induced by 100 μmol/L DEN alone, 0.01 mg/L TCDD alone, or 100 μmol/L DEN plus 0.01 mg/L TCDD. a: significant difference compared with the control group (P<0.01); b: significant difference compared with the DEN treatment group (P<0.01); c: significant difference compared with the TCDD treatment group (P<0.01).

Effect of α-NF on the Synergism between DEN and TCDD

α-NF of different concentrations was tested with DEN plus TCDD to investigate whether α-NF, known as an AhR antagonist, could inhibit the synergistic effect of DEN and TCDD on cell malignant transformation. TF in groups with α-NF of different concentrations added was all higher than that in the control group (DEN plus TCDD without the addition of α-NF), but no statistically significant differences were observed. Moreover, no dose-response relationship was observed by adding α-NF to the

DEN and TCDD combination (Figure 4).

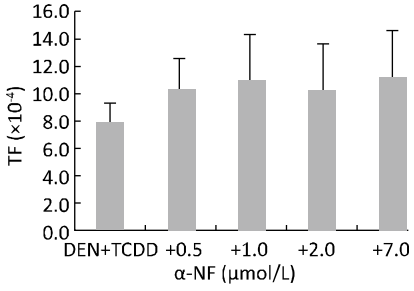

Figure 4. The effect of α-NF on the synergism between DEN and TCDD was tested by exposing Balb/c 3T3 cells to 100 μmol/L DEN plus 0.01 mg/L TCDD plus α-NF with a concentration sequence of 0 μmol/L, 0.5 μmol/L, 1.0 μmol/L, 2.0 μmol/L, and 7.0 μmol/L respectively. There are no significant differences among different treatment groups (P>0.05).

Induction of Cyp2a5 mRNA Expression by DEN, TCDD, and α-NF

Induction of *Cyp2a5* mRNA expression in Balb/c 3T3 cells by DEN or TCDD alone or their combination was determined and compared with that in the control group. Meanwhile, the effects of α-NF on the *Cyp2a5* mRNA expression in each of the groups were also determined (Figure 5). The *Cyp2a5* mRNA level of DEN treated cells was not significantly different from that in the control group, while the *Cyp2a5* expression in TCDD treated cells was significantly higher than that in the control and DEN groups (P<0.05). Interestingly, when cells were co-treated by DEN and TCDD, the *Cyp2a5* mRNA level was reduced dramatically to about 50% of that in the TCDD treatment group and was significantly lower than that in either the control or the DEN treatment group. When α-NF was present, the *Cyp2a5* mRNA levels in the control, DEN and TCDD treated groups were all down-regulated, but no further reduction was observed in the DEN plus TCDD treatment group in comparison to the level without α-NF.

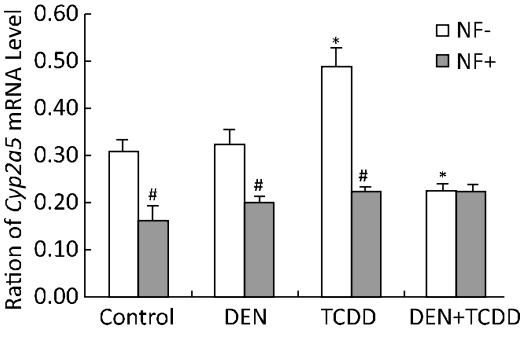

Figure 5. Induction of *Cyp2a5* mRNA expression in Balb/c 3T3 cells after treatment with 100 μmol/L DEN alone, 0.01 mg/L TCDD alone, or 100 μmol/L DEN plus 0.01 mg/L TCDD, and with 7.0 μmol/L α-NF (NF+) or without α-NF (NF-) treatment for each group. *Significant difference compared with all the other treatment groups without the addition of α-NF (P<0.05). #Significant difference compared with the same chemical treatment without the addition of α-NF (P<0.05).

Induction of Cyp1a1 mRNA Expression by DEN, TCDD, and α-NF

Induction of *Cyp1a1* mRNA expression by DEN, TCDD and α-NF was also investigated (Figure 6). The results were similar with those in the *Cyp2a5* expression study above. The up-regulation of *Cyp1a1* mRNA expression as a result of application of TCDD alone was significantly lowered when the cells were treated by both DEN and TCDD. The *Cyp1a1* mRNA levels in the control and TCDD treated groups were significantly down-regulated when the cells were treated additionally with α-NF (P<0.05), but no significant changes were observed in the DEN plus TCDD treatment groups.

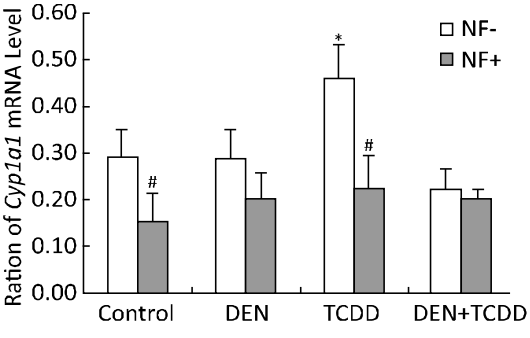

Figure 6. Induction of *Cyp1a1* mRNA expression in Balb/c 3T3 cells after treatment with 100 μmol/L DEN alone, 0.01 mg/L TCDD alone, or 100 μmol/L DEN plus 0.01 mg/L TCDD, and with 7.0 μmol/L α-NF (NF+) or without α-NF (NF-) treatment for each group. *Significant difference compared with all the other treatment groups without the addition of α-NF (P<0.05). #Significant difference compared with the same chemical treatment without the addition of α-NF (P<0.05).

DISCUSSION

Synergistic Effect of DEN and TCDD on Inducing Malignant Transformation of Balb/c 3T3 Cells

The cell transformation assay showed that DEN alone significantly induced malignant transformation of the Balb/c 3T3 cells. This result is consistent with findings of other studies, in which some nitrosamine compounds, such as N-Methyl-N'-nitro-N-nitrosoguanidine, N-methyl-N-nitrosourea, and N-ethyl-N-nitrosourea, had been tested and all had exhibited positive outcomes in Balb/c 3T3 cell transformation tests[22-25]. TCDD was shown to exert effects as a tumor promoter in a study using an improved two-stage Balb/c 3T3 cell transformation model[26]. However, such a tumorigenic effect was not observed in our assay. One possible reason for the phenomenon could be that the cell transformation protocol used in this study was designed to find out combined tumorigenic effects of the two chemicals instead of distinguishing the tumor promoter from the initiator according to the two-stage model. However, our results showed that there was a strong synergistic tumorigenic effect of TCDD and DEN when cells were treated by TCDD and DEN together, and such effect might be explained in part by the role of TCDD as a tumor promoter in the carcinogenic process, as observed by other researchers adopting the DEN-initiated two-stage experimental animal model[27-28].

Other than the two-stage model, the combined effect of DEN and TCDD had not been well studied. One study showed that the combination of TCDD and 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a kind of nitrosamine usually found in tobacco, significantly increased the lung tumor incidence in female A/J mice when compared to groups treated with TCDD or NNK alone[29]. Findings of the present study using the *in vitro* cell assay further demonstrated the synergistic effect of the combination of nitrosamine compounds and TCDD quantitatively.

Role of Cyp1a1 and Cyp2a5 in the Synergistic Tumorigenic Effect of DEN and TCDD

CYP1A1, known as aryl hydrocarbon hydroxylase (AHH), is a major extra hepatic CYP enzyme involved in metabolic activation of many carcinogens, especially poly aryl hydrocarbons (PAHs). Moreover, PAHs, such as benzo[a]pyrene, can induce expression of *Cyp1a1* gene by binding with AhR[30]. Thus, it is commonly known that *Cyp1a1* plays an important role in cancers induced by xenobiotics. However, a recent study showed that *Cyp1a1* might play an essential protective role in benzo[a]pyrene-induced carcinogenesis in *Cyp1a1* knockout mice[31]. Our results revealed that TCDD as a powerful AhR agonist could effectively induce the expression of *Cyp1a1* gene, which was consistent with the common understanding on the toxic and tumorigenic mechanism of TCDD. Nevertheless, when TCDD was used together with DEN, the expression level of *Cyp1a1* was decreased significantly compared with that in the TCDD treatment group, but the decrease was not statistically significant as compared with the control and DEN treatment group, which suggested blocking of the up-regulation effect of TCDD in the presence of DEN. These results indicate that *Cyp1a1* may exert an inhibitive effect on the process of carcinogenesis. Our findings support the conclusions of the study on *Cyp1a1* gene knockout mice.

Similar results were demonstrated in the *Cyp2a5* expression study. The expression of *Cyp2a5* was down-regulated significantly compared with that in the TCDD, DEN and control groups. CYP2A5 enzyme is involved in metabolism of many carcinogens, such as N-nitrosamine compounds and aflatoxin. High expression of CYP2A5 can be found in hepatic cells as it is a hepatic enzyme[32]. The regulation of *Cyp2a5* is complex and also different from that of other Cytochrome P450 genes. The expression of *Cyp2a5* gene is inducible by a number of chemicals and pathophydiological conditions that usually inhibit expression of other *Cyp* genes. *Cyp2a5* can be induced by some liver toxic substances, including TCDD, phenobarbital and heavy metals. The increase of the *Cyp2a5* mRNA level was observed in chemical-induced hepatoma cells[20,32-33]. Studies also showed that CYP2A5 was a metabolic activating enzyme of DEN and could enhance the carcinogenic activity of DEN[34-35]. However, the down-regulation of *Cyp2a5* found in the TCDD and DEN co-treated group indicated that the synergetic tumorigenic effect of DEN and TCDD was not realized by TCDD enhancing the metabolic activation process of DEN.

Possible Mechanisms of the Synergetic Tumorigenic Effect of DEN and TCDD

TCDD is a well-known toxin which damages the liver, skin and immune system, causes reproductive and developmental abnormality and thus induces cancers. Although a host of studies have demonstrated that diverse toxicities of TCDD are

related to AhR[36], they have failed to explain the tumor promoting effects of TCDD and whether there are mechanistic relations between *Cyp* genes induction and TCDD toxicity or not[2,37-38]. AhR is a cytosolic transcription factor with basic helix-loop-helix structure. Upon binding to TCDD, AhR will get its structure changed, get translocated into the nucleus and dimerized with the AhR nuclear translocator (ARNT). After that, the dimer further binds to the xenobiotic responsive element (XRE) sequences in the promoter regions of the target genes to regulate their expression[39-40].

Alpha-naphthoflavone is a kind of flavone derivative with a molecular structure similar to that of TCDD. It inhibits the biological effect of TCDD by binding competitively to AhR[41]. The possible inhibitory effect of α-NF on synergism between TCDD and DEN was investigated in this study. The results showed that α-NF could inhibit TCDD from inducing the expression of *Cyp1a1* and *Cyp2a5* genes, but it failed to inhibit the synergetic effect of TCDD and DEN, which indicated that the synergetic tumorigenic effect was not achieved through the AhR pathway. The roles of AhR and subsequent induction of the *Cypla* family in tumorigenesis were studied by using mouse strains differing in AhR phenotype and hepatocarcinogen sensitivity[28]. It can be concluded that whereas AhR is required for liver tumor promotion, "the degree of activation as measured by induction of *Cypla* is not directly related to the degree of tumor-promoting capability. Other genetic factors must play a role in mediating the final tumor outcome." Meanwhile, another study has found that the down-regulation of nuclear P16 partially contributes to synergistic effects of TCDD and NNK in lung tumorigenesis[29].

Therefore, the synergetic tumorigenic effect of TCDD in combination with DEN is not achieved simply by TCDD up-regulating the expression of CYP enzymes to enhance the carcinogenic potential of DEN. It is well known that the cell transformation consists of at least four stages: (1) a block in cellular differentiation; (2) acquisition of immortality; (3) acquisition of tumorigenicity; and (4) full malignancy including metastasis. The malignant transformation of Balb/c 3T3 is a conversion from the immortal and non-tumorigenic stage to the tumorigenic phenotype stage. This conversion occurs following either the activation of oncogenes or the inactivation of tumor suppressing genes[42], which involves a series of complex regulation processes. It is possible that TCDD interacts with DEN through another pathway during this process. Studies have shown that TCDD can enhance the activity of NF-kB, an inhibitory factor for expression of CYP mRNA. In addition, the NF-kB signal pathway is related to the health effects of xenobiotics and the interaction between NF-kB and AhR signal pathways is found to be a potential mechanism of dioxin toxicity[43-45]. Therefore, the synergetic effect of DEN and TCDD may involve interactions among some molecular signal pathways and gene regulation mechanisms. The down-regulation of the expression of *Cyp1a1* and *Cyp2a5* genes is only an indirect representation of the interaction between DEN and TCDD at the molecular level. To clarify the synergetic effect of TCDD in combination with DEN is helpful to understand the role of TCDD in human cancers and the process of cancer occurrence and development.

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REFERENCES

1. Steenland K, Bertazzi P, Baccarelli A, et al. Dioxin revisited: developments since the 1997 IARC classification of dioxin as a human carcinogen. Environ Health Perspect, 2004; 112(13), 1265-8.
2. Mandal PK. Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. J Comp Physiol, 2005; 175(4), 221-30.
3. Kashuba EV, Gradin K, Isaguliants M, et al. Regulation of transactivation function of the aryl hydrocarbon receptor by the Epstein-Barr virus-encoded EBNA-3 protein. J Biol Chem, 2006; 281(2), 1215-23.
4. IARC. IARC working group on the evaluation of carcinogenic risks to humans. Polychlorinated dibenzo-para-dioxins and polychlorinated dibenzofurans. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 69. International Agency for Research on Cancer, Lyon. 1997.
5. Tricker AR and Preussmann R. Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. Mutat Res, 1991; 259, 277-89.
6. Bogovski P and Bogovski S. Animal species in which N-nitroso compounds induce cancer. Int.J. Cancer, 1981; 27, 471-4.
7. WHO/IARC. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol.17. Some N-nitroso compounds. International Agency for Research on Cancer, Lyon. 1998.
8. Wu YN. Present Knowledge in Food Safety. Beijing: Chemical Industry Press, 2003; 204-31. (In Chinese)
9. Nollet LML and Toldra F. Safety analysis of foods of animal origin. New York: CRC Press, 2011; 421-6.
10. Perocco P, Paolini M, Mazzullo M, et al. β-Carotene as enhancer of cell transforming activity of powerful carcinogens and cigarette-smoke condensate on BALB/c 3T3 cells *in vitro*. Mutat Res, 1999; 440(1), 83-90.
11. Perocco P, Iori R, Barillari J, et al. In vitro induction of

benzo(a)pyrene cell-transforming activity by the glucosinolate gluconasturtiin found in cruciferous vegetables. Cancer Lett, 2002; 184(1), 65-71.
12. Wang LW, Zhong WJ, Ying XP, et al. Study on the Effects of Genistein on the Transformation of BALB/c-3T3 Cells. Carcinogenesis, Teratogenesis & Mutagenesis, 2005; 17(1), 48-51.
13. Arpiainen S, Raffalli-Mathieu F, Lang MA, et al. Regulation of the Cyp2a5 gene involves an aryl hydrocarbon-dependent pathway. Mol Pharmacol, 2005; 67(4), 1325-33.
14. Godoy W, Albano RM, Moraes EG, et al. CYP2A6/2A7 and CYP2E1 expression in human oesophageal mucosa: regional and inter-individual variation in expression and relevance to nitrosamine metabolism. Carcinogenesis, 2002; 23(4), 611-6.
15. Sakai A , Iwase Y , Nakamura Y, et al. Use of a cell transformation assay with established cell lines, and a metabolic cooperation assay with V79 cells for the detection of tumor promoters: a review. ATLA, 2002; 30, 33-59.
16. Combes R, Balls M, Curren R, et al. Cell transformation assay as predictors of human carcinogenicity. The report and recommendation of ECVAM Workshop 39. ATLA, 1999; 27, 745-67.
17. McGregor DB, Rice JM & Venitt S, eds. The Use of Short- and Medium-term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation (IARC Scientific Publications No. 146), Lyon: IARC press, 1999. (http://monographs.iarc.fr/ENG/Publications/pub146/IARCpub146.pdf).
18. Sakai A. Balb/c 3T3 cell transformation assays for the assessment of chemical carcinogenicity. AATEX, 2007; 14(Special Issue), 367-73.
19. IARC/NCI/EPA Working group. Cellular and molecular mechanisms of cell transformation and standardization carcinogenic chemicals: overview and recommended protocols. Cancer Res, 1985; 45(5), 2395-9.
20. Abu-Bakar A, Satarug S, Marks GC, et al. Acute cadmium chloride administration induces hepatic and renal Cyp2a5 mRNA, protein and activity in the mouse: involvement of transcription factor NRF2. Toxicol Lett, 2004; 148(3), 199-210.
21. Chun YJ, Lee BY, Yang SA, et al. Induction of Cytochrome P450 1A1 Gene Expression by a Vitamin K3 Analog in Mouse Hepatoma Hepa-1c1c7 Cells. Mol Cells, 2001; 12(2), 190-6.
22. Tsuchiya T, Umeda M. Improvement in the efficiency of the in vitro transformation assay method using BALB/3T3 A31-1-1 cells. Carcinogensis, 1995; 16(8), 1887-94.
23. Kajiwara Y, Ajimi S, Hosokawa A, et al. Improvement of carcinogen detection in the BALB/3T3 cell transformation assay by using a rich basal medium supplemented with low concentration of serum and some growth factors. Mutat Res, 1997; 393(1-2), 81-90.
24. Kajiwara Y, Ajimi S. Verification of the BALB/c 3T3 cell transformation assay after improvement by using an ITES-medium. Toxicol in Vitro, 2003; 17(4), 489-96.
25. Asada S, Sasaki K, Tanaka N, et al. Detection of initiating as well as promoting activity of chemicals by a novel cell transformation assay using v-Ha-ras-transfected BALB/c 3T3 cells (Bhas 42 cells). Mutat Res, 2005; 588(1), 7-21.
26. Fang MZ, Kim DY, Lee HW, et al. Improvement of *in vitro* two-stage transformation assay and determination of the promotional effect of cadmium. Toxicol *in vitro*, 2001; 15(3), 225-31.
27. Viluksela M, Bager Y, Tuomisto JT, et al. Liver Tumor-promoting Activity of 2,3,7,8-Tetrachloro- dibenzo-p-dioxin (TCDD) in TCDD-sensitive and TCDD-resistant Rat Strains. Cancer res, 2000; 60, 6911-20.
28. Beebe LE, Fornwald LW, Diwan BA, et al. Promotion of N-Nitrosodiethylamine-initiated Hepatocellular Tumors and Hepatoblastomas by 2,3,7,8-Tetrachlorodibenzo-p-dioxin or Aroclor 1254 in C57BL/6, DBA/2, and B6D2F1 Mice. Cancer Res, 1995; 55(21), 4875-80.
29. Wang YJ, Chang H, Kuo YC, et al. Synergism between 2,3,7,8-tetrachlorodibenzo-p- dioxin and 4-(methylnitrosamino)-1- (3-pyridyl)-1-butanone on lung tumor incidence in mice. J Hazard Mater, 2011; 186(1), 869-75.
30. Whitlock JP Jr. Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol, 1999; 39, 103-25.
31. Ma Q, Lu AY. CYP1A Induction and Human Risk Assessment: An Evolving Tale of in Vitro and in Vivo Studies. Drug Metab Dispos, 2007; 35(7), 1009-16.
32. Su T, Ding X. Regulation of the cytochrome P450 2A genes. Toxicol Appl Pharmacol, 2004; 199(3), 285-94.
33. Seubert JM, Webb CD, and Bend JR. Acute sodium arsenite treatment induces Cyp2a5 but not Cyp1a1 in the C57B1/6 mouse in a tissue (kidney) selective manner. J Biochem Mol Toxicol, 2002; 16(2), 96-106.
34. Hukkanen J. Xenobiotic-Metabolizing Cytochrome P450 Enzymes in Human Lung. Oulu: Oulu university press, 2000; 15-24.
35. Wastl UM, Rossmanith W, Lang MA, et al. Expression of cytochrome P450 2A5 in preneoplastic and neoplastic mouse liver lesions. Mol Carcinog, 1998; 22(4), 229-34.
36. El Gendy MA, Soshilov AA, et al. Transcriptional and posttranslational inhibition of dioxin-mediated induction of Cyp1a1 by harmine and harmol. Toxicol Lett, 2012; 208(1), 51-61.
37. Cole P, Trihopoulos D, pastides H, et al. Dioxin and cancer: a critical review. Regul Toxicol Pharmacol, 2003; 38, 378-88.
38. Nukaya M, Moran S, Bradfield CA. The role of the dioxin-responsive element cluster between the Cyp1a1 and Cyp1a2 loci in aryl hydrocarbon receptor biology. Proc Natl Acad Sci USA, 2009; 106(12), 4923-8.
39. Corton JC, Moreno ES, Hovis SM, et al. Identification of a cell-specific transcription activation domain within the human Ah receptor nuclear translocator. Toxicol Appl Pharmacol, 1996; 139(2), 272-80.
40. Ma Q, Dong L, Whitlock JP Jr. Transcriptional activation by the mouse Ah receptor. Interplay between multiple stimulatory and inhibitory functions. J Biol Chem, 1995; 270(21), 12697-703.
41. Merchant M, Morrison V, Santostefano M, et al. Mechanism of action of aryl hydrocarbon receptor antagonists: inhibition of 2,3,7,8-tetrachlorodibenzo-p-dioxin- induced Cyp1a1 gene expression. Arch Biochem Biophys, 1992; 298(2), 389-94.
42. Isfort RJ, LeBoeuf RA. Application of *in vitro* cell transformation assays to predict the carcinogenic potential of chemicals. Mutat Res, 1996; 365, 161-73.
43. Kajta M, Wójtowicz AK, Maćkowiak M, et al. Aryl hydrocarbon receptor-mediated apoptosis of neuronal cells: a possible interaction with estrogen receptor signaling. Neuroscience, 2009; 158(2), 811-22.
44. Tian Y, Ke S, Denison MS, et al. Ah Receptor and NF-kB Interactions, a Potential Mechanism for Dioxin Toxicity. J Biol Chem, 1999; 274(1), 510-5.
45. Tian Y, Rabson AB, Gallo MA. Ah receptor and NF-kappaB interactions: mechanisms and physiological implications. Chem Biol Interact, 2002; 141(1-2), 97-115.