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DNA Product Formation in Female Sprague–Dawley Rats Following Polyhalogenated Aromatic Hydrocarbon (PHAH) Exposure

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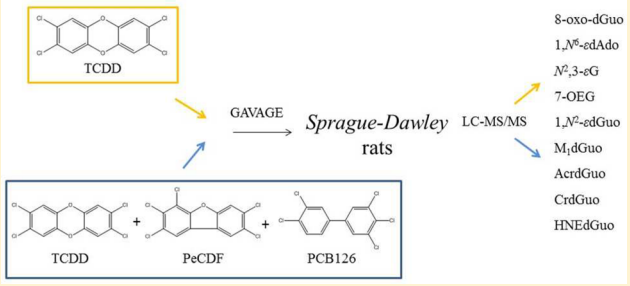
ABSTRACT: DNA oxidation damage has been regarded as one of the possible mechanisms for the hepatic carcinogenesis of dioxin-like compounds (DLCs). In this study, we evaluated the toxic equivalency factor (TEF) from the standpoint of induced DNA oxidation products and their relationship to toxicity and carcinogenicity. Nine DNA oxidation products were analyzed in the liver of female Sprague–Dawley rats exposed to 2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD) alone or the tertiary mixture of TCDD, 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) by gavage for 14, 31, and 53 weeks (5 days/week) by LC–MS/MS: 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo); 1,N^6-etheno-2′-deoxyadenosine (1,N^6-εdAdo); N^2,3-ethenoguanine (N^2,3-εG); 7-(2-oxoethly)guanine (7-OEG); 1,N^2-etheno-2′-deoxyguanosine (1,N^2-εdGuo); malondialdehyde (M_1dGuo); acrolein (AcrdGuo); crotonaldehyde (CrdGuo); and 4-hydroxynonenal (HNEdGuo) derived 2′-deoxyguanosine adducts. Exposure to TCDD (100 ng/kg/day) significantly induced 1,N^6-εdAdo at 31 and 53 weeks, while no increase of 8-oxo-dGuo was observed. Significant increases were observed for 8-oxo-dGuo and 1,N^6-εdAdo at all time points following exposure to the tertiary mixture (TEQ 100 ng/kg/day). Exposure to TCDD for 53 weeks only significantly increased 1,N^6-εdAdo, while increases of N^2,3-εG and 7-OEG were only found in the highest dose group (100 ng/kg/day). Exposure to the tertiary mixture for 53 weeks had no effect on N^2,3-εG in any exposure group (TEQ 0, 22, 46, or 100 ng/kg/day), while significant increases were observed for 1,N^6-εdAdo (all dose groups), 8-oxo-dGuo (46 and 100 ng/kg/day), and 7-OEG (100 ng/kg/day). While no significant increase was observed at 53 weeks for 1,N^2-εdGuo, M_1dGuo, AcrdGuo, or CrdGuo following exposure to TCDD (100 ng/kg/day), all of them were significantly induced in animals exposed to the tertiary mixture (TEQ 100 ng/kg/day). This oxidation DNA product data suggest that the simple TEF methodology cannot be applied to evaluate the diverse patterns of toxic effects induced by DLCs.



INTRODUCTION

Oxidative stress, a common state in pathophysiology, occurs when the number of reactive oxygen species (ROS) being formed is exceeded by those being detoxified. Many endogenous processes, as well as exogenous chemicals or their metabolites, are known to produce ROS. Besides ROS, reactive nitrogen species (RNS) are also generated by macrophages and neutrophils involved in chronic inflammation, which has been recognized as a risk factor in many human cancers.[1] These species or their active metabolites can interact with cellular constituents, especially lipids or nucleic acids, and further induce various DNA oxidation products.[2] These DNA lesions have been implicated in aging, neurodegeneration, and a myriad of diseases including cancer.[2] To date, there are several key ROS/RNS-induced DNA oxidation products: 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo); 1,N^6-etheno-2′-deoxyadenosine (1,N^6-εdAdo); 3,N^4-etheno-2′-deoxycytidine (3,N^4-εdC); N^2,3-ethenoguanine (N^2,3-εG); 1,N^2-etheno-2′-deoxyguanosine (1,N^2-εdGuo); and malondialdehyde (MDA), acrolein, crotonaldehyde, and 4-hydroxynonenal (HNE) derived 2′-deoxyguanosine (dGuo) adducts, designated as M_1dGuo, AcrdGuo, CrdGuo, and HNEdGuo, respectively.[3−7] Among them, the most studied adduct, 8-oxo-dGuo, is formed in relatively high amounts in vivo with steady-state levels usually around $1/10^6$ guanine. It is formed directly by the reaction between dGuo and ROS or carbonate anion radical induced by RNS.[1,8] ROS and RNS metabolites can also abstract hydrogen atoms from polyunsaturated fatty acids producing lipid peroxides and many reactive byproducts such as MDA, HNE, crotonaldehyde, and acrolein. These compounds can further damage DNA and generate multiple oxidation DNA products, which include exocyclic products with either a five-member (etheno products) or a six-member (propano

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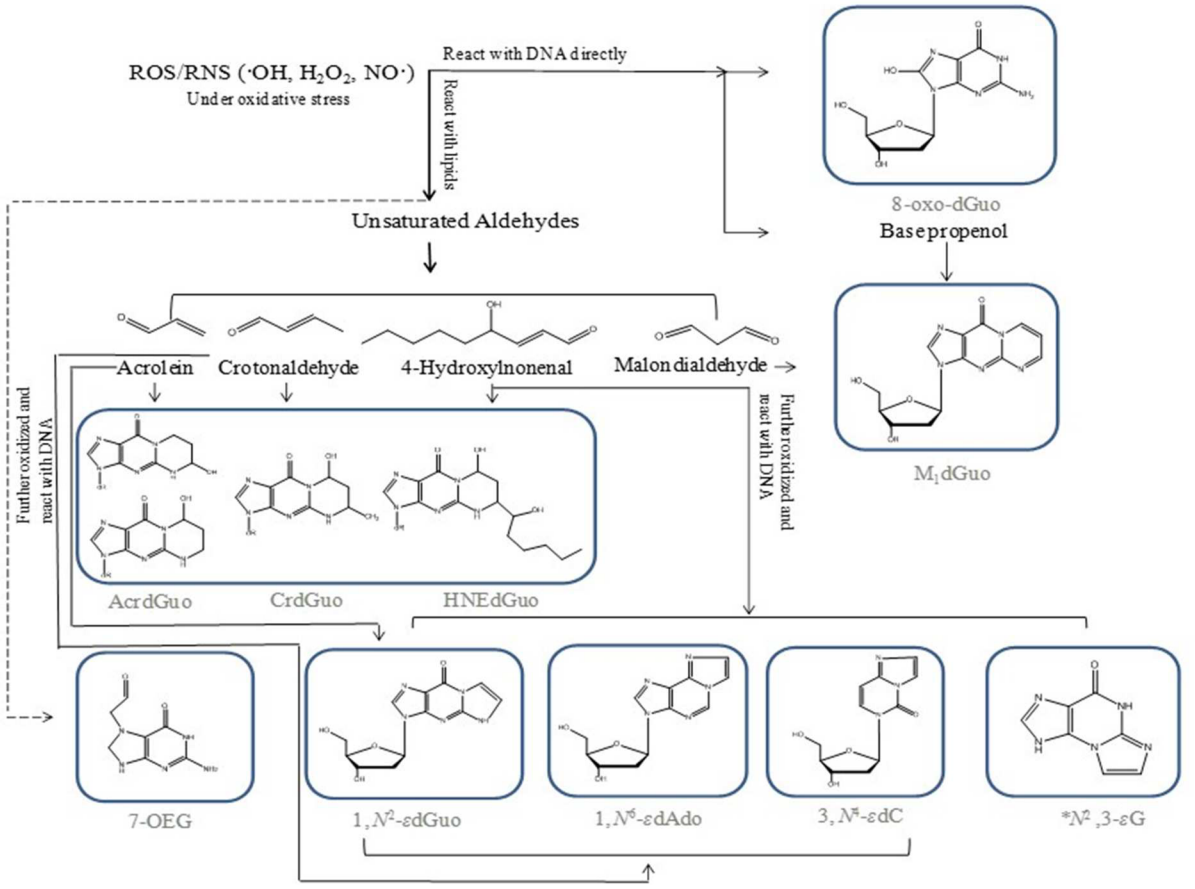

Figure 1. Illustration of the major DNA adducts induced by ROS/RNS.

products) ring attached to DNA bases, as depicted in Figure 1.[1,3−7] Recently, 7-(2-oxoethly)guanine (7-OEG) has been identified as a new DNA product formed by lipid peroxidation (LPO) with steady-state levels around 1−10 adduct/10^6 guanine.[9] In addition to these distinct formation pathways, site-directed mutagenicity studies found that most of these DNA products can induce specific transition or transversion point mutations in bacteria or mammalian cells.[8,10−12] 7-OEG can induce apurinic/apyrimidinic sites (AP sites) in biological systems, although it has no miscoding properties.[9] Considering the diverse metabolic pathways and the mutation spectrum induced by those important ROS/RNS-induced DNA products, evaluating their profile data could compensate for biased results induced by a single product. Growing evidence also indicates that these DNA lesions are significantly induced in patients and animals with various chronic inflammatory diseases including certain cancers.[3,7,8,13,14] Although DNA oxidation product formation is generally regarded as a key event for the carcinogenesis of genotoxic chemicals, it may also be a significant contributor for the tumorigenesis of nongenotoxic chemicals, especially chemicals capable of enhancing the formation of endogenous active metabolites, RNS and ROS, such as polyhalogenated aromatic hydrocarbons (PHAHs).[15−20]

PHAHs comprise a large class of compounds such as polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs).[21−24] While PHAHs are regarded as one of the most prevalent groups of pollutants in the environment due to industrial use, PCDDs and PCDFs are also produced as byproducts during anthropogenic activities such as chlorine bleaching of paper and combustion of wastes and fuels.[21−23] PCBs were commercially produced and widely used for various industrial purposes including heat transfer agents, dielectric insulating fluids for capacitors and transformers, plasticizers, and paint additives.[24] Because of PHAHs' resistance to degradation and persistence in environment, their ability to bioaccumulate in humans and wildlife animals may result in chronic lifetime exposure, possible toxicity, and carcinogenicity.[21−24] Depending on the location and type of halogenations, some PHAHs induce a similar spectrum of biochemical and toxic responses in experimental animals. These responses are considered to be mediated through a common mechanism of action initiated by binding to a cytosolic receptor known as the aryl hydrocarbon receptor (AhR) and triggering the expression of a variety of genes, the so-called AhR gene battery.[21]

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), commonly referred to as "dioxin", is the prototype of these structurally related compounds and exhibits the highest potency of binding to the AhR. Hence, these structurally related compounds are commonly referred to as dioxin-like compounds (DLCs). Because of similarities in toxicity, the concept of the toxic equivalency factor (TEF) has been applied for the risk assessment and regulatory control for DLCs.[25] Therefore, the toxicity of PHAH mixtures is expressed in terms of its total toxic equivalent quotient (TEQ), which is the amount of TCDD that would produce the equal toxic effect of all contributing congeners within the mixture. This allows for the estimation of the potential dioxin-like activity of PHAH mixtures in the environment.

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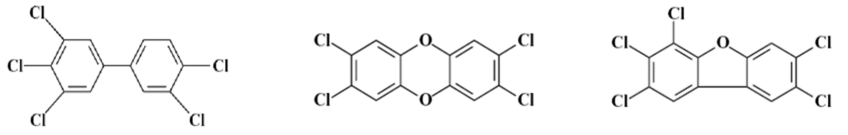

3,3′,4,4′,5-Pentachlorobiphenyl PCB 126 | 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin TCDD | 2,3,4,7,8-Pentachlorodibenzofuran PeCDF

Figure 2. Chemical structures of PCB 126, TCDD, and PeCDF.

The association of oxidative stress and PHAHs, especially TCDD, has been studied for several decades.[17−19,26−39] Substantial evidence has accumulated to support that TCDD can induce oxidative stress in mammalian cells in vitro and in rodents in vivo. Different biomarkers have been applied to assess the oxidative stress induced by TCDD in the liver of animals including hepatic LPO, DNA single strand breaks, hepatic membrane fluidity, glutathione, nonprotein sulfhydryl, and NADPH.[30,31] Significantly increased LPO with large strain differences has also been detected in other organs of rats and mice exposed to TCDD including kidney, thymus, heart, testes, and brain. Similarly, PCB-induced oxidative stress was also observed in numerous studies.[17,20,26−29,32−36,38,39] These studies indicate that oxidative stress is a ubiquitous side effect produced by these compounds. Although many PHAHs have been shown to have very weak initiating activity without direct genotoxic effects, it has been postulated that they may be indirectly genotoxic through the formation of DNA lesions induced by ROS and RNS.[15,17−20,26−39] Because the TEF has been widely applied for the evaluation of the toxic effects of PHAHs and oxidative stress is universally induced in the animals exposed to these compounds, it is meaningful to evaluate the application of the TEF approach in the toxicity of PHAHs using DNA oxidation products, especially in chronic animal carcinogenesis studies.

Several PHAHs were chosen by the National Toxicology Program (NTP) as model compounds including TCDD, PCB 126, and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF).[21−24] PCB 126 is a non-*ortho*-substituted PCB with a TEF value of 0.1. As the most potent DLC in the environment, PCB 126 accounts for 40−90% of the toxic potency of dioxin-like PCBs.[22] PeCDF, with a TEF value of 0.5, represents the most potent PCDF present in human tissues.[23] The structures of these compounds are shown in Figure 2. An important assumption for the TEF methodology is that the toxicity of a mixture of DLCs is dose additive based on the TEF value of the individual components. This study has evaluated this assumption from the standpoint of the number of induced DNA oxidation products and their relationship to toxicity and carcinogenicity of PHAHs.

In this study, we collaborated with the NTP to understand the DNA oxidation product profile in hepatic DNA of female Sprague−Dawley rats that were exposed to TCDD and the tertiary mixture of TCDD, PCB 126, and PeCDF (Figure 2) for 14, 31, and 53 weeks. Nine DNA oxidation lesions (7-OEG; 8-oxo-dGuo; 1,N^6-εdAdo; 1,N^2-εdGuo; N^2,3-εG; M_1dGuo; AcrdGuo; CrdGuo; HNEdGuo) were measured in hepatic DNA isolated from female Sprague−Dawley rats. Since each product has distinct metabolic pathways in vivo, our assessment of a battery of DNA oxidation lesions provides extensive information on DNA oxidation damage. This knowledge enables us to better estimate the toxicity of PHAHs and improve the scientific basis of human risk assessment of PHAHs in the environment.

EXPERIMENTAL PROCEDURES

Chemicals. Nucleic acid purification grade lysis buffer, protein precipitation solution, and proteinase K were purchased from Gentra Systems (Minneapolis, MN). HPLC-grade water and methanol were from Thermo Fisher Scientific Company (Raleigh, NC). $^{15}N_5$-8-Oxo-dGuo, $^{15}N_5$-dGuo, and $^{13}C_{10}$-dGuo were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Other chemical reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO). $^{15}N_5$-1,N^6-εdAdo standard was synthesized as described by Ham et al.[40] 1,N^2-εdGuo and $^{13}C_{10}$-1,N^2-εdGuo were synthesized as reported by Kusmierek et al.[41] MDA modified $^{15}N_5$ and $^{14}N_5$ DNA were made using the method in Jeong's study.[42] AcrdGuo, CrdGuo, and HNEdGuo standards and their $^{15}N_5$ labeled internal standards were synthesized according to previous studies.[43−45] 7-OEG, $^{15}N_5$-7-OEG, and N^2,3-εG were synthesized as described previously by Mutlu et al.[9,46]

Animal Exposure and DNA Isolation. Rat liver tissues were provided by Battelle Laboratories (Columbus, OH) and State University of New York at Buffalo, which conducted the studies under NIEHS contract (N01-ES-75411).[21−24] Female Sprague−Dawley rats were exposed to either TCDD alone or the tertiary mixture by gavage 5 days per week for 14, 31, and 53 weeks. The doses used for TCDD were 0 and 100 ng/kg/day for 14, 31, 53 weeks; 0, 22, 46, and 100 ng/kg/day for 53 weeks. The TEQ doses used for the tertiary mixture were 0 and 100 ng/kg/day for 14, 31, 53 weeks; 0, 22, 46, and 100 ng/kg/day for 53 weeks. Further explanation of the TEQ doses can be found in the NTP technical report on the toxicology and carcinogenesis studies of a mixture of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Cas No. 1746−01−6), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (Cas No. 57117−31−4), and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) (Cas No. 57465−28−8) in female Harlan Sprague−Dawley rats (gavage studies).[24] Liver tissues were collected from 4−8 female rats per group/day after the final exposure and stored frozen at −80 °C. DNA was isolated as described previously.[46]

8-Oxo-dGuo and 1,N^6-εdAdo Assay. The assay was performed as previously described by Pang et al.[7] with minor modifications. A 100 μg sample of DNA in NaOAc buffer I (sodium acetate 30 mM, 0.2 mM $ZnCl_2$, pH 5.6) was incubated with nuclease P1 (5 μg) at 37 °C for 1 h. Immediately after incubation, DNA solutions were spiked with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, 5 μL, 1.5 M), $^{15}N_5$-8-oxo-dGuo (500 fmol), and $^{15}N_5$-1,N^6-εdAdo (20 fmol) followed by addition of NaOAc buffer II (sodium acetate 30 mM, pH 8.1), alkaline phosphatase (20 units), and phosphodiesterase (0.012 units) then incubated at 37 °C for an additional hour. Enzymes and undigested DNA were removed by Microcon-10 filtration (11500 rpm, 4 °C, 50 min), and the filtrate was concentrated using a SpeedVac.

Samples were enriched for 8-oxo-dGuo and 1,N^6-εdAdo using an Agilent 1200 HPLC system equipped with a Atlantis T3 column (5 μm, 4.6 mm × 150 mm).[47] The nucleosides were monitored at 264 nm. The column was eluted at a flow rate of 1 mL/min at 30 °C with a 5−80% MeOH gradient in 10 mM ammonium acetate buffer as follows: hold at 5% MeOH for 5 min, 5−10% MeOH over 5 min, 10−20% MeOH over 10 min, 20−80% over 10 min; re-equilibrate at 5%

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Table 1. Number of 8-Oxo-dGuo Adducts/10^6 dGuo and 1,N^6-εdAdo Adducts/10^8 dAdo Measured in Female Sprague−Dawley Rat Hepatic DNA Following Exposure to TCDD (100 ng/kg/day) or the Tertiary Mixture of TCDD, PCB 126, and PeCDF (TEQ 100 ng/kg/day) for 14, 31, and 53 Weeks

		TCDD			tertiary		
		14 weeks	31 weeks	53 weeks	14 weeks	31 weeks	53 weeks
8-oxo-dGuo add/10^6 dGuo	control	2.41 ± 1.28	2.44 ± 0.80	3.20 ± 0.67	2.09 ± 0.75	2.54 ± 0.76	2.81 ± 0.49
	exposed[a]	2.55 ± 0.91	2.72 ± 0.88	3.87 ± 0.47	3.93 ± 1.27[c]	4.06 ± 1.56[c]	4.87 ± 0.81[b]
1,N^6-εdAdo add/10^8 dAdo	control	1.47 ± 0.65	1.32 ± 0.44	0.91 ± 0.48	1.36 ± 0.32	1.31 ± 0.63	0.93 ± 0.56
	exposed[a]	1.56 ± 0.23	2.76 ± 1.07[b]	2.13 ± 0.52[c]	2.75 ± 1.37[c]	6.55 ± 3.34[c]	4.13 ± 0.87[c]

[a]Indicates the exposures of TCDD, 100 ng/kg/day, and tertiary mixture (TCDD + PCB 126 + PeCDF), TEQ 100 ng/kg/day. [b]Indicates $p \leq 0.05$ compared to corresponding control groups. [c]Indicates $p \leq 0.01$ compared to corresponding control groups.

Table 2. Number of N^2,3-εG Adducts/10^8 G, 7-OEG Adducts/10^7 G, 8-Oxo-dGuo Adducts/10^6 dGuo, and 1,N^6-εdAdo Adducts/10^8 dAdo Measured in Female Sprague−Dawley Rat Hepatic DNA Following Exposure to Multiple Concentrations of TCDD or the Tertiary Mixture of TCDD, PCB 126, and PeCDF for 53 Weeks

		N^2,3-εG add/10^8 G	7-OEG add/10^7 G	8-oxo-dGuo add/10^6 dGuo	1,N^6-εdAdo add/10^8 dAdo
TCDD 53 weeks	control	2.18 ± 0.44	4.92 ± 1.07	3.20 ± 0.67	1.19 ± 0.39
	22 ng/kg	2.55 ± 0.37	6.96 ± 0.81	3.34 ± 0.62	2.37 ± 0.84[b]
	46 ng/kg	2.11 ± 0.56	7.47 ± 5.96	4.32 ± 0.14	2.51 ± 0.83[b]
	100 ng/kg	4.44 ± 2.04[a]	29.8 ± 16.47[b]	3.87 ± 0.47	2.13 ± 0.52[a]
tertiary (TCDD+PCB 126+ PeCDF) 53 weeks	control	2.07 ± 1.15	7.88 ± 3.77	2.81 ± 0.49	0.93 ± 0.56
	22 ng/kg[c]	1.78 ± 0.19	7.81 ± 2.96	3.07 ± 0.11	3.93 ± 1.15[b]
	46 ng/kg[c]	2.41 ± 1.04	9.92 ± 7.22	4.11 ± 0.60[a]	4.94 ± 1.63[b]
	100 ng/kg[c]	2.55 ± 0.37	22.4 ± 14.84[a]	4.87 ± 0.80[a]	4.13 ± 0.87[b]

[a]Indicates $p \leq 0.05$ compared to corresponding control groups. [b]Indicates $p \leq 0.01$ compared to corresponding control groups. [c]Indicates TEQ dose.

for 5 min. 8-oxo-dGuo and 1,N^6-εdAdo fractions were collected at 24−26 min and 33−34 min, respectively.

AcrdGuo, 1,N^2-εdGuo, M_1dGuo, CrdGuo, and HNEdGuo Assay. An assay similar to 8-oxo-dGuo and 1,N^6-εdAdo was applied to measure AcrdGuo, 1,N^2-εdGuo, M_1dGuo, CrdGuo, and HNEdGuo with minor modifications. Considering their similar chromatography behaviors, these oxidation products were analyzed simultaneously. A 100 μg sample of DNA in NaOAc buffer I was incubated with nuclease P1 (5 μg) at 37 °C for 1 h. Immediately after incubation, DNA solutions were spiked with TEMPO (5 μL, 1.5 M), $^{15}N_5$−AcrdGuo (50 fmol), $^{13}C_{10}$-1,N^2-εdGuo (100 fmol), MDA modified internal standard DNA corresponding to 400 fmol $^{15}N_5$-M_1dGuo, $^{15}N_5$−CrdGuo (50 fmol), and $^{15}N_5$−HNEdGuo (50 fmol), followed by addition of NaOAc buffer II, alkaline phosphate (20 units), and phosphodiesterase (0.012 units) then incubated at 37 °C for an additional hour.[42] Enzymes and undigested DNA were removed by Microcon-10 filtration (11 500 rpm, 4 °C, 50 min), and the filtrate was concentrated using a SpeedVac.

Samples were enriched for AcrdGuo, 1,N^2-εdGuo, and M_1dGuo by the same HPLC method as described for 8-oxo-dGuo and 1,N^6-εdAdo with a 100% methanol and 5 mM ammonium formate−0.1% formic acid gradient.[47] CrdGuo and HNEdGuo were eluted at a flow rate of 0.5 mL/min, with a 35−70% MeOH gradient in 10 mM ammonium acetate buffer over 25 min. Fraction collection times for AcrdGuo, 1,N^2-εdGuo, CrdG, and HNEdGuo were 28−30 min, 32−34 min, 15−18 min, and 34−36 min, respectively. All fractions of the enzyme-digestion, MDA-treated DNA were collected every minute from the HPLC. Following concentration via SpeedVac the fraction containing M_1dGuo was determined by using nanoLC−MS/MS.[42]

N^2,3-εG and 7-OEG Assay. N^2,3-εG and 7-OEG were analyzed as described previously by Mutlu et al.[9,46]

LC−MS/MS Analysis. 8-Oxo-dGuo was analyzed by a Waters Acquity UPLC coupled to a Thermofinnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer in a positive selected reaction mode (SRM) monitoring the signals m/z 284.1 → 168.0 for 8-oxo-dGuo and m/z 289.1 → 173.0 for $^{15}N_5$-8-oxo-dGuo. Separation was performed on a T3 HSS column (1.7 μm, 2.1 mm × 100 mm) with a flow rate of 200 μL/min using gradient (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in methanol. MS settings were as follows: electrospray voltage (3000 V), ion transfer capillary temperature (285 °C), the vaporizer temperature (250 °C), sheath and auxiliary gas pressures (35 and 30 arbitrary units), and collision energy (12 eV).

1,N^6-εdAdo, AcrdGuo, 1,N^2-εdGuo, M_1dGuo, CrdGuo, and HNEdGuo were analyzed by nanoAcquity UPLC coupled to a Thermofinnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer in positive SRM monitoring the signals m/z 276.0 → 160.0 for 1,N^6-εdAdo, m/z 281.0 → 165.0 for $^{15}N_5$-1,N^6-εdAdo, m/z 304.0 → 188.0 for M_1dGuo, m/z 309.0 → 193.0 for $^{15}N_5$-M_1dGuo, m/z 292.0 → 176.0 for 1,N^2-εdGuo, m/z 302.0 → 181.0 for $^{13}C_{10}$-1,N^2-εdGuo, m/z 424.0 → 308.0 for HNEdGuo, m/z 429.0 → 313.0 for $^{15}N_5$-HNEdGuo, m/z 338.0 → 222.0 for CrdGuo, m/z 343.0 → 227.0 for $^{15}N_5$-CrdGuo, m/z 324.0 → 208.0 for AcrdGuo, and m/z 329.0 → 213.0 for $^{15}N_5$-AcrdGuo. Separation was performed on a UPLC BEH C18 column (1.7 μm, 100 μm × 100 mm) with a flow rate of 1 μL/min using gradient (A) 5 mM ammonium formate in water and (B) 1% formic acid in acetonitrile for 1,N^6-εdAdo, or (A) 0.1% formic acid in water and (B) acetonitrile for AcrdGuo, 1,N^2-εdGuo, M_1dGuo, CrdGuo, and HNEdGuo. MS settings were as follows: emitter tip voltage (1500 V), ion transfer capillary temperature (285 °C), and collision energy (12 eV).

Statistical Analysis. Statistical analyses were performed using R (2.11). Considering the limited sample size in certain groups, the nonparametric test was used to assess the differences between control and PHAH-treated rats or various control groups for the number of DNA oxidation products by Wilcox Rank Sum test. Two-sided and one-sided p-values were considered significant if they were less than 0.05.

RESULTS AND DISCUSSION

In this study, we examined the relationship between exposure to either TCDD or the tertiary mixture of TCDD, PCB 126, and PeCDF and the formation of DNA oxidation products. Female Sprague−Dawley rats were exposed to 0 and 100 ng/

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Table 3. Number of 1,N^2-εdGuo Adducts/10^8 dGuo, M_1dGuo adducts/10^8 dGuo, CrdGuo Adducts/10^8 dGuo, HNEdGuo Adducts/10^8 dGuo, and AcrdGuo Adducts/10^8 dGuo Measured in Female Sprague−Dawley Rat Hepatic DNA Following Exposure to TCDD (100 ng/kg/day) or the Tertiary Mixture of TCDD, PCB 126, and PeCDF (TEQ 100 ng/kg/day) for 53 Weeks

	TCDD add/10^8 dGuo	tertiary add/10^8 dGuo
1,N^2-εdGuo control	1.61 ± 0.37	2.07 ± 0.43
1,N^2-εdGuo exposed[a]	2.14 ± 0.51	3.80 ± 1.12[c]
M_1dGuo control	4.16 ± 1.02	4.71 ± 2.23
M_1dGuo exposed[a]	6.43 ± 1.68	12.4 ± 6.73[b]
CrdGuo control	0.24 ± 0.10	0.35 ± 0.07
CrdGuo exposed[a]	0.24 ± 0.06	0.50 ± 0.12[b]
HNEdGuo control	1.12 ± 0.05	0.90 ± 0.22
HNEdGuo exposed[a]	1.18 ± 0.38	1.40 ± 0.38
AcrdGuo control	6.02 ± 1.30	8.26 ± 0.98
AcrdGuo exposed[a]	7.16 ± 0.84	42.9 ± 24.50[b]

[a]Indicates the exposures of TCDD, 100 ng/kg/day, and tertiary mixture (TCDD + PCB 126 + PeCDF), TEQ 100 ng/kg/day, 53 weeks. [b]Indicates $p \leq 0.05$ compared to corresponding control groups. [c]Indicates $p \leq 0.01$ compared to corresponding control groups.

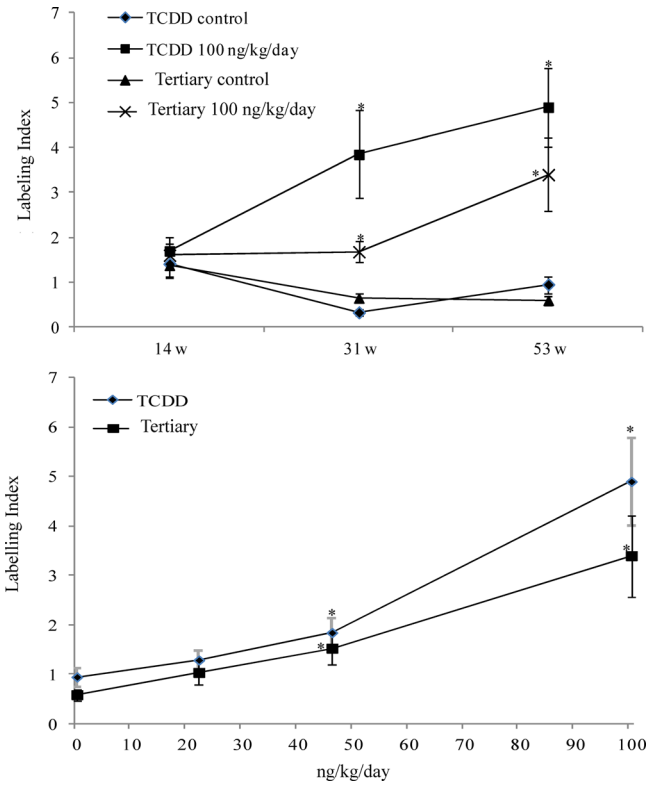

Figure 3. (Top) Cell proliferation in the liver of female Sprague−Dawley rats exposed to TCDD (100 ng/kg/day) or the tertiary mixture of TCDD, PCB 126, and PeCDF (TEQ 100 ng/kg/day) at 14, 31, and 53 weeks.[21,24] (Bottom) Cell proliferation in the liver of female Sprague−Dawley rats exposed to TCDD (0, 22, 46, and 100 ng/kg/day) or the tertiary mixture of TCDD, PCB 126, and PeCDF (TEQ 0, 22, 46, and 100 ng/kg/day) at 53 weeks.[21,24] *, Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's test.

kg/day of TCDD or TEQ doses of 0 and 100 ng/kg/day of the tertiary mixture for 14, 31, and 53 weeks and to 0, 22, 46, and 100 ng/kg/day TCDD or TEQ doses of 0, 22, 46, and 100 ng/kg/day of the tertiary mixture for 53 weeks.

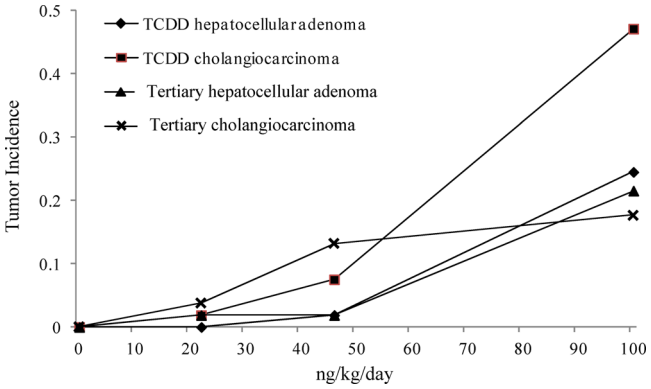

Figure 4. Tumor incidence from 2-year cancer bioassay of female Sprague−Dawley rat livers exposed to TCDD (0, 22, 46, and 100 ng/kg/day) or the tertiary mixture of TCDD, PCB 126, and PeCDF (TEQ 0, 22, 46, and 100 ng/kg/day).[21,24]

Measurements of 8-oxo-dGuo and 1,N^6-εdAdo formation in the liver of the female rats exposed to TCDD (0 and 100 ng/kg/day) or the tertiary mixture (TEQ 0, and 100 ng/kg/day) at 14, 31, and 53 weeks are shown in Table 1. While no significant increase of 8-oxo-dGuo was detected after TCDD exposure for 14, 31, or 53 weeks, 1,N^6-εdAdo concentrations for 31 (p = 0.03) and 53 (p = 0.003) weeks were higher in comparison to their respective control groups. Exposure to the tertiary mixture for 14 (p = 0.004 for 8-oxo-dGuo, and p = 0.004 for 1,N^6-εdAdo), 31 (p = 0.0001 for 8-oxo-dGuo, and p = 0.0002 for 1,N^6-εdA), and 53 weeks (p = 0.02 for 8-oxo-dGuo, and p = 0.0002 for 1,N^6-εdAdo) all showed statistically significant increases in 8-oxo-dGuo and 1,N^6-εdAdo. These increases correspond to a 1.5−1.8-fold increase in 8-oxo-dGuo and a 1.5−5.0-fold increase in 1,N^6-εdAdo in the hepatic DNA of female rats.

The accumulations of N^2,3-εG, 7-OEG, 8-oxo-dGuo, and 1,N^6-εdAdo were evaluated in the hepatic DNA of the female Sprague−Dawley Rats exposed to TCDD at 0, 22, 46, 100 ng/kg/day or the tertiary mixture at TEQ doses of 0, 22, 46, 100 ng/kg/day for 53 weeks (Table 2). No accumulation of 8-oxo-dGuo was observed after TCDD exposure, but a significant increase of 8-oxo-dGuo was observed following exposure to the tertiary mixture at TEQ doses of 46 (p = 0.03) and 100 ng/kg/day (p = 0.02). 1,N^6-εdAdo concentrations increased significantly at all doses (22, 46, 100 mg/kg/day) following either TCDD (p = 0.008, p = 0.008, and p = 0.02, respectively) or tertiary (p = 0.003, p = 0.003, and p = 0.002, respectively) exposures. Table 2 shows the significant increase in the number of 7-OEG in the liver at the highest dose exposures of both TCDD (100 ng/kg, p = 0.0098) and the tertiary mixture (TEQ 100 ng/kg, p = 0.014). Endogenous 7-OEG was measured to be 4.92 ± 1.07 adducts/10^7G in control samples, while exposure to 100 ng/kg/day TCDD induced 29.79 ± 16.47 adducts/10^7G of 7-OEG. Additionally, a three-fold increase was observed in 7-OEG formation from exposure to the tertiary mixture. No significant increase was observed for N^2,3-εG concentration after exposure to the tertiary mixture, including the highest TEQ dose group, while a two-fold increase in N^2,3-εG was observed after 100 ng/kg/day TCDD exposure (p = 0.015).

Several other LPO-induced DNA products (1,N^2-εdGuo; M_1dGuo; CrdGuo; HNEdGuo; AcrdGuo) were analyzed in the hepatic DNA of female rats exposed to TCDD (100 ng/kg/day) and the tertiary mixture (TEQ 100 ng/kg/day) for 53

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weeks (Table 3). Concentrations of 1,N^2-εdGuo (p = 0.008), M_1dGuo (p = 0.03), CrdGuo (p = 0.05), and AcrdGuo (p = 0.03) were significantly higher in animals exposed to the tertiary mixture, while no significant increase was observed in any of the LPO-induced product concentrations in animals that were exposed to TCDD (100 ng/kg/day) for 53 weeks.

As one of the predominant nongenotoxic pollutants in the environment, the mechanism of PHAH-related carcinogenesis has been studied for decades. Among them, TCDD is the most researched. DNA oxidation products and oxidative stress induced by TCDD have been measured extensively both in vitro and in vivo.[18,19,26−31] Hassoun et al. reported the induction of LPO in the liver of female Sprague−Dawley rats exposed to TCDD (100 ng/kg/day) for 13 and 30 weeks.[27,29] LPO became more pronounced with increasing exposure time, which is similar to our results for 1,N^6-εdAdo (Table 1). In our study, no significant increase of 1,N^6-εdAdo was observed in TCDD treated animals at 14-week exposure, but a two-fold significant increase of this product was detected at 31 weeks exposure. In the study by Hassoun et al., higher increases of LPO were detected in animals exposed to TCDD for 30 weeks (6-fold) than 13 weeks (1.5-fold).[26] Similarly, DNA oxidation product results found in our study showed statistically significant increases (7-OEG, p = 0.00008; N^2,3-εG, p = 0.0025; 1,N^6-εdAdo, p = 0.0016) following 53-week exposure to TCDD, which are shown in Table 2. Although an increase of hepatic 8-oxo-dGuo was previously reported to be gender and estrogen dependent in a two-stage carcinogenesis model with diethylnitrosamine as initiator and TCDD as promotor,[18] we observed no significant induction of 8-oxo-dGuo in the female rat livers following exposure to TCDD for 14, 31, or 53 weeks (Table 1). These results may be due to differences between the animal study designs or the analytical assays (LC−MS/MS vs LC-ECD).[18]

Induction of superoxide anions, LPO and DNA single-strand breaks following exposure to the tertiary mixture (TEQ 100 ng/kg/day) for 13 and 30 weeks in female rats was also previously reported by Hassoun et al.[26,29] In their study, exposure for 30 weeks to the tertiary mixture induced more superoxide anions and LPO (8.2-fold, 2.3-fold) than 13 week exposure (6-fold, 1.9-fold), which indicated that production of these biomarkers is time-dependent.[26,29] Consistently, more DNA oxidation products were induced in liver DNA of our studied animals after exposure to the tertiary mixture for 31 weeks than 14 weeks, as indicated by 1,N^6-εdAdo and 8-oxo-dGuo results in Table 1. Significant dose-dependent increases in the production of superoxide anions, LPO and DNA single-strand breaks in the liver of female rats were also reported in the liver DNA of female rats exposed to the tertiary mixture for 13 weeks and 30 weeks in Hassoun et al.'s studies.[26,29] Consistently, we found a significant positive association between exposure dose and concentrations of 7-OEG (p < 0.05), 8-oxo-dGuo (p = 0.00041), and 1,N^6-εdAdo (p < 1×10^{-5}) following exposure to the tertiary mixture for 53 weeks (Table 2).

Similar to other toxic effects of TCDD, DNA oxidation damage and oxidative stress have been assumed to be connected with the AhR and activation of cytochrome P450 superfamily of enzymes, especially CYP1A1.[37,38] The upregulation of these enzymes triggers metabolic changes of endogenous compounds in vivo, which may further produce DNA oxidation damage. Since the TEF methodology is based on the AhR affinity of PHAHs and oxidative stress was reported in diverse biological systems exposed to PHAHs, the TEF methodology may also be valuable for the evaluation of oxidative stress and oxidative damage induced by mixtures of PHAHs, especially DLCs. However, the DNA oxidation product findings in this study, and several other studies, indicate that a simple TEF value cannot be applied to evaluate oxidative stress and oxidative damage.[26,48] Hassoun et al. showed that following 13-weeks exposure, higher levels of superoxide anions and LPO were induced in the liver of female rats exposed to the tertiary mixture (TEQ 100 ng/kg/day) than TCDD alone (100 ng/kg/day).[26] With similar background concentrations in control samples, the concentrations of superoxide anion were ∼1.3 and 0.4 nmol cytochrome c reduced/mg in the animals treated with the tertiary mixture or TCDD alone, respectively. The concentrations of LPO were ∼3 and 2 nmol 2-thiobarbituric acid substances (TBARS) formed/mg protein detected in these two groups, respectively.[26,27] Additionally, synergistic effects of TCDD, PeCDF, and PCB126 were indicated in the production of superoxide anion in hepatic tissues, which required only 0.25−0.5% of the doses of the three individual congeners within the tertiary mixture to produce similar effects for each congener alone.[26] The interactions of these three congeners were also seen in the process of superoxide anion production in female rats exposed to the tertiary mixture for 30 weeks.[29] A significant difference was observed when comparing the dose−response curves of superoxide anion production in the hepatic tissues exposed to the tertiary mixture versus TCDD, PeCDF or PCB126 alone, with larger effects produced by an equivalent dose of the tertiary mixture than the three congeners alone.

In our study, we compared oxidation DNA product formation induced by either TCDD (100 ng/kg/day) or the tertiary mixture (TEQ 100 ng/kg/day) for 14, 31, and 53 weeks. A significant production of hepatic 8-oxo-dGuo and 1,N^6-εdAdo was observed following exposure to the tertiary mixture at all time points, while TCDD-exposed animals showed a significant increase in 1,N^6-εdAdo (31 and 53 weeks) but no significant difference in 8-oxo-dGuo. After 53-week exposure, significant induction of 1,N^2-εdGuo, M_1dGuo, AcrdGuo, and CrdGuo was observed in the tertiary mixture-treated rat liver DNA; however, none of the products showed significant changes in TCDD-treated animals. Conversely, a significant induction of 7-OEG and N^2,3-εG was observed in the liver following 53-week exposure to TCDD, while only 7-OEG was significantly induced in tertiary mixture-exposed animals, as indicated in Table 2.

Complex results were also reported in the toxicogenomic analysis by Vezina et al.[48] and Ovando et al.[49] Vezina et al. examined the gene expression in the hepatic DNA of female rats exposed to TCDD, PCB 126, and PeCDF for 14 weeks.[48] With the same TEQ dose used in this study (100 ng/kg/day), a limited subset of genes, which included CAT, cytochrome b5 (CYPB5), and COX oxidative stress response genes, was activated by PeCDF and PCB 126 alone, but not TCDD. PeCDF and PCB 126 also induced growth arrest and a DNA-damage-inducible gene product, Gadd45, indicating oxidative DNA damage in the liver from those animals exposed to these chemicals. Interestingly, Gadd45 was not induced in TCDD-treated animals. Therefore, PeCDF and PCB126 were more effective in activating the expression of oxidative stress response genes than TCDD in the liver of these animals after a 14-week exposure. Similar genomic studies were conducted in the hepatic tissue of female rats following 53-week exposure to

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TCDD and PCB126 by Ovando et al.[49] A dose-dependent increase in the number of differentially expressed genes was observed in animals exposed to PCB 126 for 53 weeks with 30, 300, and 1000 ng/kg/day. While fewer genes were differentially expressed in animals exposed to PCB 126 for 53 weeks (216) than 13 weeks (371), many more genes showed differential expression in animals exposed to TCDD for 53 weeks (299) than 13 weeks (103) with the same TEQ dose. More ROS or their active metabolite-related detoxification genes were upregulated or downregulated in the chronic TCDD exposed animals including glutathione-S-transferase (GST), glutathione peroxidase, aldehyde dehydrogenase (Aldh), and aldo-keto reductase (Akr).

DNA oxidation damage in rodents exposed to DLCs is probably induced through upregulation of cytochrome P450 superfamily of enzymes, mediated by AhR-dependent pathways.[37,38] The dose−response and different time course induction of CYP1A1 is well characterized in the liver of female Sprague−Dawley rats following exposure to TCDD and the tertiary mixture[21,24,50] CYP1A1 induction occurs in virtually every tissue of the body following exposure to either TCDD or the tertiary mixture.[21,24] By using the continuous nonlinear Hill model, the induction of hepatic CYP1A1 was compared in female rats following exposure to TCDD, PCB 126, and PeCDF alone or their tertiary mixture with doses or TEQ doses ranging from 0−100 ng/kg/day for 14, 31, or 53 weeks.[50] The estimated parameters indicated that congener-specific dose−response shapes were significantly different, and the additivity of TEF methodology failed for these individual congeners and their mixture. Six of the 12 equiv time-dose combinations failed to agree between the tertiary mixture and TCDD alone. The same results were found when liver concentrations of TCDD or TEQ dose for the tertiary mixture were used. The maximum activity of 7-ethoxyresorufin-o-deethylase (EROD) is substantially higher (1.2−3.0-fold) for PeCDF than TCDD with the same TEQ dose. Toyoshiba et al. observed significant, nonadditive interaction for EROD activity at 31 and 53 weeks in the tertiary mixture exposed samples but not at 14 weeks.[50] In summary, although the importance of CYP1A1 in the induction of oxidative DNA damage was implied previously, the complicated association between DNA oxidation damage and induction of CYP1A1 by DLCs or their mixtures cannot be completely described in our study and still requires further detailed exploration. DNA oxidation products, oxidative stress biomarkers, CYP1A1 induction, and genomic studies consistently suggest that the simple TEF methodology cannot be applied to evaluate the diverse patterns of toxic effects induced by DLCs.

Mutation studies have suggested that oxidative stress and DNA oxidation damage, especially from chronic inflammation, are associated with carcinogenesis.[2,3] At present, more than one hundred DNA oxidation products are reported in model studies, but less than 20 of them are measured in cellular DNA.[51] Among them, 8-oxo-dGuo is the most extensively studied in vivo adduct with G to T transversions as the dominant mutation pattern.[8] Many assays have been developed to detect this product in animal or human tissues,[8,52] but artificial formation during sample preparation has hampered its application as a good biomarker.[52,53] 1,N^6-εdAdo is another popular biomarker, widely applied to evaluate chronic inflammation and LPO in animal or human tissues.[3] 1,N^6-εdAdo, 1,N^2-εdGuo, N^2,3-εG, M_1dGuo, AcrdGuo, CrdGuo, and HNEdGuo can also induce distinct mutation spectra, similar to 8-oxo-dGuo.[10−12] However, compared with 1,N^6-εdAdo and 8-oxo-dGuo, the other products examined in our study are less widely applied in risk assessment of carcinogens. 7-OEG, the predominant product formed by vinyl chloride in rodents, was also recently established as an LPO-induced product.[9] Although it is not a direct promutagenic adduct, 7-OEG can cause AP sites and further induce mutations if they cannot be repaired before cell replication.[54] In addition to promutagenic properties, 8-oxo-dGuo, 1,N^6-εdAdo, 1,N^2-εdGuo, N^2,3-εG, M_1dGuo, AcrdGuo, CrdGuo, and HNEdGuo also have distinct metabolic pathways, as shown in Figure 1. Furthermore, the primary repair pathways for these products are also different.[11,55−58] For small products (8-oxo-dGuo; 1,N^6-εdAdo; 1,N^2-εdGuo; N^2,3-εG), base excision repair (BER) is the dominant repair pathway, with different glycosylases involved to repair different products.[55−57,59] For the medium and bulky products (AcrdGuo; CrdGuo; HNEdGuo; M_1dGuo), nuclear excision repair (NER) is the primary repair pathway.[11,58] Therefore, the simple concentration of a DNA oxidation product in animals reflects the complex interactions between environmental and biological systems. The potential adverse effect of a product is dynamically controlled by its formation and repair pathways, which could be determined by numerous factors including, but not limited to, exposure dose, exposure time, exposure pathway, chemical metabolism, tissue, age, sex, and species. A corresponding mutation spectrum study is still necessary for further validation of the biological significance of certain DNA oxidation products in hepatic toxicity of TCDD and the tertiary mixture exposed animals examined in our study.

Besides the possible indirect genotoxicity of oxidative stress, cytotoxicity, stimulation of cell proliferation, inhibition of apoptosis, and induction of enzymes are all suggested to be involved in the toxicity of TCDD and its congeners.[60−63] As depicted in Figure 3, hepatic cells from female rats showed a high rate of proliferation following exposure to TCDD alone (100 ng/kg/day) or the tertiary mixture (TEQ 100 ng/kg/day) for 53 weeks, which is consistent with our DNA oxidation product induction results in Tables 2 and 3. No significant enhancement of cell proliferation was observed in 14-week TCDD or tertiary mixture exposed animals, but it was observed in 31- and 53-week TCDD and tertiary mixture exposed animals.[22,24] Low-dose exposure of TCDD or the tertiary mixture for 53 weeks failed to induce significant production of cell proliferation, which agreed with the observation of dose-dependent accumulation of DNA oxidation products following 53-week exposure. Dose- and time-dependent induction of CYP1A1 and CYP1A2 was detected in the livers of female rats exposed to TCDD or the tertiary mixture, which is also in accordance with our results of DNA oxidation products.[21,24] Additionally, increased incidences and severities of hepatocyte inflammation were observed at 31 and 53 weeks in TCDD or the tertiary mixture exposed animals. All these factors are potential players, which can be combined to induce the dose-dependent incidence of hepatocellular adenoma and cholangiocarcinoma in the 2-year cancer bioassay by the NTP.[21,24]

Recent advances in scientific understanding of cancer biology support the view that environmental chemicals can act through multiple toxicity pathways, modes, or mechanisms of action to induce cancer.[64,65] However, evaluating the relative weight of each possible important contributor to cancer induction is far more complicated than simply identifying them. The role DNA oxidation damage plays in the hepatic toxicity effect of PHAHs

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in female rats may be a good case in point. Although the 2-year tumor incidence data (Figure 4) support the application of the TEF approach in the risk assessment of DLCs based on the generation of similar dose−response curves in response to TCDD and the tertiary mixture exposed female rats,[66] the TEF approach cannot be applied to evaluate the DNA oxidation products in this study, CYP1A1 or CYP1A2 induction, or oxidative biomarkers.[26,27,29,50] Such inconsistency further indicates the complexity of the formation of DNA oxidation damage and carcinogenesis of DLCs in the liver of female rats.

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ABBREVIATIONS

1,N^2-εdGuo, 1,N^2-etheno-2′-deoxyguanosine; 1,N^6-εdAdo, 1,N^6-etheno-2′-deoxyadenosine; 7-OEG, 7-(2-oxoethly)-guanine; 8-oxo-dGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; AcrdGuo, acrolein derived dGuo adducts; AhR, aryl hydrocarbon receptor; AlkB, alpha-ketoglutarate-dependent dioxygenase; ANPG, alkyl-N-purine-DNA glycosylase; CrdGuo, crotonaldehyde derived dGuo adducts; DLCs, dioxin-like compounds; HNEdG, 4-hydroxynonenal derived dG adducts; LPO, lipid peroxidation; M_1dGuo, malondialdehyde derived dGuo adducts; MDA, malondialdehyde; N^2,3-εG, N^2,3ethenoguanine; PCB 126, 3,3′,4,4′,5-pentachlorobiphenyl; PeCDF, 2,3,4,7,8-pentachlorodibenzofuran; PHAHs, polyhalogenated aromatic hydrocarbons; RNS, reactive nitrogen species; ROS, reactive oxygen species; SRM, selected reaction mode; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TEF, toxic equivalency factor; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; TEQ, toxic equivalent quotient

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