ROS-INDUCED DNA ADDUCTS IN THE RODENTS AFTER EXPOSURE TO SUPERFUND HAZARDOUS CHEMICALS

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ABSTRACT

LINA GAO: ROS-Induced DNA Damage in Rodents after Exposure to Superfund
Hazardous Chemicals
(Under the direction of James A. Swenberg)

The accumulation of oxidative DNA damage has been hypothesized as a key event in chemical carcinogenesis. In this study, oxidative DNA damage was evaluated in the livers of rats exposed to vinyl chloride (VC), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB153). Eight oxidative DNA adducts were measured, 8-hydroxyl-2′-deoxyguanosine (8-OHdG), 1, N^6-etheno-2'-deoxyadenosine (εdA), N^2, 3-εG, 1, N^2-etheno-2'-deoxyguanosine (1, N^2-εdG), 3-(2′-deoxy-β-d-erythro-pentofuranosyl) pyrimido[1,2-α]purin-10(3H) (M_1dG), acrolein, crotonaldehyde and 4-HNE -derived dG adducts (assigned as AcrdG, CrdG, and 4-HNEdG respectively).

εdA is one of the promutagenic DNA adducts formed by VC, which can also be formed by lipid peroxidation. In this study, both adult and weanling *Sprague-Dawley* rats were exposed to 1100 ppm ($^{13}C_2$)-VC for 1 week (6 h/day, 5 days/week). The results indicated that NA-εdA concentration did not show significant difference in the liver of adult and weanling rats after VC exposure. The distribution pattern of ($^{13}C_2$)-εdA in liver, lung and kidney indicated that liver was the dominant target organ for VC toxicity in both

adult and weanling rats. ROS-induced DNA adducts were detected in the liver of female intact, ovariectomized (OVX) and male *Sprague-Dawley* rats, including 8-OHdG, 1, N^6-εdA, AcrdG, and CrdG. These animals were exposed to TCDD for 30 weeks after diethylnitrosamine (DEN) initiation. Induction of these adducts was consistently found in liver DNA of TCDD-treated intact female rats and 17β-estradiol (E$_2$) supplemented OVX female rats, but not detected in OVX rats without E$_2$ supplement or male rats. These results further confirmed that the induction of these adducts occurs via a sex-specific and estrogen-dependent mechanism reported previously. Oxidative DNA damage was measured in liver DNA of female *Sprague-Dawley* rats following 53-week exposure of PHAHs, including PCB153, PCB126, TCDD, and the ternary mixture of TCDD, PCB126 and PeCDF. Increases of 8-OHdG, N^2, 3-εG and 1, N^6-εdA were observed in PCB153 or PCB126 exposed animals. Significant increases of 1, N^6-εdA were observed in all animals exposed to TCDD and the ternary mixture. Increases of 1, N^2-εdG, CrdG, AcrdG, 4-HNEdG and M$_1$dG were detected in animals exposed to the ternary mixture, but not the TCDD treated rats compared to the control.

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LIST OF ABBREVIATIONS

AcrdG	Acrolein-derived dG adducts
AP	Alkaline Phosphatase
ARP	Aldehyde Reactive Probe
BER	Base Excision Repair
CE	Collision energy
CrdG	Crotonaldehyde-derived dG adducts
DBP	Dibenzo[*a, l*]pyrene
DLCs	Dioxin-like compounds
dA	2´-Deoxyadenosine
dC	2´-Deoxycytidine
εdC	3, N^4-Etheno-2'-deoxycytidine
DEN	Diethylnitrosamine
dG	2´-Deoxyguanosine
dT	Thymidine
E_2	17β-Estradiol
ESCODD	The European Standards Committee on Oxidative DNA Damage
ESI	Electrospray ionization
GC-MS	Gas chromatography-mass spectrometry
4-HNE	*trans*-4-Hydroxy-2-Nonenal
4-HNEdG	*trans*-4-hydroxy-2-nonenal-derived dG adducts
IARC	International Agency for Research on Cancer
LC	Liquid chromatography

LC-APCIMS/MS	Liquid chromatography-atmosphere pressure chemical ionization-tandem mass spectrometry
LC-ECD	Liquid chromatography-electrochemical detection
LC-ESIMS/MS	Liquid chromatography-electrospray tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
LPO	Lipid peroxidation
MDA	Malondialdehyde
M_1dG	3-(2´-deoxy-β-D-erythropentofuranosyl) pyrimido[1,2-α]purin-10(3H)-one
MRM	Multiple reaction monitoring
NDLCs	Non-dioxin-like compounds
NER	Nucleotide excision repair pathway
NICI	Negative ion chemical ionization
NLM	Neutral loss monitor
NP1	Nuclease P1
NTP	National Toxicology Program
$N^2, 3\varepsilon G$	N^2, 3-Etheno-guanine
1, N^6-εdA	1, N^6-Etheno-2'-deoxyadenosine
1, N^2-εdG	1, N^2-Etheno-2'-deoxyguanosine
OVX	Ovariectomized
8-OHdG	7, 8-Dihydro-8-oxo-2′-deoxyguanosine
PDE	Phosphodiesterase I
PFB	Pentafluorobenzylation
PCBs	Polychlorinated biphenyls

PCB126	3,3´,4,4´,5-Pentachlorobiphenyl
PCB153	2,2´,4,4´,5,5´-Hexachlorobiphenyl
PCDDs	Polychlorinated dibenzodioxins
PCDFs	Polychlorinated dibenzofurans
PeCDF	2,3,4,7,8-Pentachlorodibenzofuran
PHAHs	Polyhalogenated aromatic hydrocarbons
ROS	Reactive oxygen species
SIM	Selected ion monitoring
SPE	Solid phase extraction
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TEF	Toxic Equivalency Factor
TEMPO	2,2',6,6'-Tetramethylpiperidinyl-1-oxy
TEQ	Toxic Equivalent
VC	Vinyl chloride

CHAPTER I

INTRODUCTION: OVERVIEW OF ROS-INDUCED DNA DAMAGE, VINYL
CHLORIDE (VC) AND POLYHALOGENATED AROMATIC HYDROCARBONS
(PHAHS)

A. ROS, DNA DAMAGE AND MUTATION

Oxidative stress was defined in 1991 as "an imbalance of oxidants and antioxidants in favor of the former" (1). It can induce considerable amounts of reactive oxygen species (ROS) *in vivo*, which have the ability to permanently alter the structure and even the function of biomolecules. Under conditions of oxidative stress, DNA has received the most study in terms of oxidative damage among all potential targets for ROS (2-10). ROS can react with DNA directly and form various DNA lesions. One of the most studied types of DNA damage is 8-hydroxyl-2′-deoxyguanosine (8-OHdG), as shown in figure 1.1. It is formed in relatively high amounts *in vivo* with background concentrations around $1/10^6$ guanine (11). ROS can also abstract a hydrogen atom from polyunsaturated fatty acids to initiate lipid peroxidation (LPO) and produce multiple relevant adducts. The most well-studied DNA adducts induced by LPO are exocyclic adducts with a five-member ring (etheno adducts) or a six-member ring (propano adducts) attached to DNA bases, as depicted in figure 1.1, including 1, N^6-etheno-2'-deoxyadenosine (εdA), 3, N^4-etheno-2'-deoxycytidine (εdC), 1, N^2-etheno-2'-deoxyguanosine (1, N^2-εdG), and N^2, 3-

ethenoguanine (N^2, 3-εG), 3-(2-deoxy-β-D-erythropentofuranosyl) pyrimido[1,2-α]purin-10(3H)-one (M_1dG) and 1, N^2-propano-2'-deoxyguanosine generated from acrolein, crotonaldehyde and 4-hydroxy-2-nonenal (4-HNE). The specific chiral isomers for propano-derived DNA adducts are shown in figure 1.2.

The accumulation of ROS-induced DNA adducts was shown to potentially represent a hazard *in vivo* because of its association with mutagenesis (2-10). Site-directed mutagenicity studies found that many can induce specific transition and/or transversion point mutations in bacteria and/or mammalian cells, as shown in table 1.1. The promutagenicity of etheno DNA adducts was further demonstrated by studying their behavior in vinyl chloride (VC) exposed humans and animals (13, 14). Genetic changes similar to those induced by VC induced DNA adducts were demonstrated in p53 and H-ras genes in animal and human angiosarcomas. The presence of 8-OHdG during DNA replication can cause G to T transversions (8). Replication of MDA-modified single-stranded M13 genomes in *E coli* caused G→T, A→G, and C→T mutations (9, 15). M_1dG is the predominant DNA adduct formed by MDA in *vivo*. The mutagenic properties of γ-AcrdG have been investigated in *E. coli* in three independent studies and the mutations (G→T transversion) were consistently observed at very low frequencies (<0.1%) or not detected at all (16-18). α-AcrdG was found to be moderately mutagenic in COS-7 and XPA cells (19, 20). The mutagenicities of (6R, 8R) - and (6S, 8S)-CrdG were similar in COS-7 system, with G→T transversions as the dominant mutations (21). Although 4-HNE was inactive in bacterial mutagenesis assays, it caused mutations in Chinese

hamster lung cells and human lymphoblastoid cells with G→T transversions as the most prevalent base changes (22, 23).

B. THE DETECTION OF ROS-INDUCED DNA ADDUCTS AND THEIR CONNECTION WITH DISEASE

Many different methods have been developed to detect ROS-induced DNA adducts. For etheno adducts, the dominant detection technique is the ^{32}P-postlabeling method, which has been successfully applied to measure εdA and εdC in rodent and human tissues (24). Persistent increases of εdA and εdC were found in premalignant target organs affected by various diseases, such as genetic metal storage disorders (Wilson's disease and hemochromatosis), chronic pancreatitis, and chronic hepatitis (24). 8-OHdG was ever detected by LC-ECD (electrochemical detector), GC-MS, immunoassay and LC-MS/MS (25). Using different detection techniques, elevated 8-OHdG has been detected in patients with various malignancies, including acute leukemia, colorectal cancer, hepatic cancer, oral squamous cell carcinoma, and breast cancer (25-29). M_1dG was measured in human and animal tissues by ^{32}P-postlabling, immunoslot blot, GC-MS and LC-MS/MS techniques (30-32). Higher M_1dG was detected in smoking patients with lung cancer compared with control smokers (30). In two studies, the level of MDA–DNA adducts from patients with breast cancer and patients with larynx cancer have been found to be higher than in controls (31, 32). HPLC-^{32}P-postlabeling has been the dominant technique used for the detection of propano-derived DNA adducts, but recently more studies have used LC-MS/MS to detect them (33-35). To date, there is still a lack of evidence for the

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importance of propano adducts in chemical carcinogenesis or chronic inflammatory diseases (24). Using different techniques, the endogenous levels of these DNA adducts in human and rodent tissues were obtained, as depicted in table 1.2.

C. DNA REPAIR PATHWAYS FOR ROS-INDUCED DNA ADDUCTS

Base excision repair (BER) is regarded as the dominant repair pathway for ROS-induced small DNA adducts, such as 1, N^6-εdA, 3, N^4-εdC and 8-OHdG (36-39). It is a critical process for genomic maintenance, as highlighted by the possible association of animals deficient in BER function with biological effects, including cancer, premature aging and metabolic effects (40).The initial step in BER uses DNA glycosylases, which cleave the N-glycosyl bond between the sugar and the base, thus releasing the damaged base to form an abasic site, also termed apurinic/apyrimidinic (AP) site. An individual glycosylase may recognize more than one type of damage, and each specific modification may be recognized by more than one type of glycosylase, giving a degree of redundancy in the process. For example, OGG1 has strong specificity for 8-oxodG, but hMYH and hMPG can also repair this adduct (41, 42), as shown in table 1.3. Although different types of DNA damage require different repair mechanisms, functional overlap between pathways and between proteins in a pathway has been observed. Furthermore, the overall DNA repair system is intimately integrated with cell cycle regulation, transcription and replication (41). Recently it was found that besides BER, global genome repair (a type of nucleotide excision repair, NER) of 8-OHdG was demonstrated in hamster cells and several lines of evidence indicated that mismatch repair was also involved in repair of 8-

OHdG:dA mismatches (41). Similar results were found for 1, N^6-εdA (43), which could be repair by both BER and AlkB, a direct reversal repair pathway, as shown in table 1.3. At present, NER is regarded as the primary repair pathway for propano-adducts, but it is possible that other repair pathways are involved in their repair (10, 44).

D. VINYL CHLORIDE AND ETHENO DNA ADDUCTS

VC is a colorless organic gas used almost exclusively by the plastic industry to produce polyvinyl chloride (PVC) and copolymers. Regarded as carcinogenic to humans by IARC (45), VC can induce angiosarcoma of liver in animals and humans (14). After metabolic activation by CYP2E1 mainly in liver, VC is transformed into an active metabolite, chloroetheylene oxide, which can react with DNA directly and form several DNA adducts, 7-(2-oxoethyl)guanine (7OEG), and the minor adducts, 1, N^6-εdA, N^2,3-εG and 3,N^4-εdC (46-49), as shown in figure 1.3. Because all these etheno-adducts are promutagenic, it is possible for them to cause base-pair substitution mutations in key cancer genes *in vivo,* if not repaired before cell replication. Several changes of oncogenes and tumor suppressor genes were reported in humans and animals exposed to VC, which would be consistent with the promutagenic properties of those DNA adducts (50-57). The accumulation of such mutations could further contribute to the formation of cancer.

Besides chemical exposures as exogenous sources, these etheno adducts can also be formed by lipid peroxidation induced by oxidative stress, which is the source of identical endogenous adducts in cells (14). Several lipid peroxidation products play an important

role during this process, such as 4-HNE. Because of the existence of these background etheno adducts, it is impossible to differentiate adducts arising from VC and those formed endogenously. **In this study, exposure of rats to ^{13}C labeled VC will be utilized to distinguish endogenous NA- from exogenous ($^{13}C_2$)-1, N^6-εdA. The distribution of both NA- and ($^{13}C_2$)-1, N^6-εdA and the persistence of ($^{13}C_2$)-1, N^6-εdA will be evaluated in the primary target (liver) and non-target organs of rats. By this study, the reliability of nanoUPLC-MS/MS will be evaluated, which will applied to detect other LPO-induced adducts in animal tissues exposed to other superfund chemicals.**

E. TCDD, OXIDATIVE STRESS AND ROS-INDUCED DNA DAMAGE

TCDD is a persistent organic pollutant (POP), which is widespread in the environment and formed as an unwanted by-product during many anthropogenic activities (58). It has been shown to act as a multi-site carcinogen and a very potent tumor promoter in rodents, but has very weak initiating activity (58). The central event that triggers the biological effects of TCDD is thought to be its binding to a receptor protein, aryl hydrocarbon receptor (AhR), and subsequently, the ligand-receptor complex binds to the AhR nuclear translocator (ARNT), and is transported to the nucleus, where it binds to DNA, triggering the expression of a variety of genes, especially CYP450, as shown in figure 1.4 (59). The up-regulation of these enzymes may greatly speed up the oxidative transformation of endogenous and exogenous compounds in the body and induce ROS, and oxidative DNA damage. The accumulation and persistence of ROS damage may

contribute to the toxic effects of TCDD, since TCDD is not readily metabolized and can result in a prolonged and amplified response.

The relationship between oxidative stress and the toxicity of TCDD and its congeners has been studied since the 1980's (60). Scientific studies indicated that TCDD-mediated activation of the AhR changed the cellular redox balance to produce an oxidative stress response in liver (60-63). Hepatic lipid peroxidation induced by TCDD occurred at low doses (500ng/kg) in C57BL/6 mice, which carry the high-affinity *Ahr*b1 allele, and only at higher doses (5 µg/kg) in DBA/2 mouse which has a low-affinity allele (64). In addition, inactivation of aconitase activity, a reliable measure of oxidative stress (65), was documented in C57BL/6 but not in DBA/2 mice following TCDD treatment (62). For this reason it has become widely hypothesized that the toxicity induced by TCDD involves an oxidative stress component; an observation that has been reported by several laboratories (60, 66-70). In the review written by Stohs, evidence indicating production of oxidative stress by TCDD in rodents was summarized, which included enhanced *in vitro* and *in vivo* hepatic and extrahepatic lipid peroxidation; increased hepatic and macrophage DNA damage; increased urinary excretion of malondialdehyde; decreased hepatic membrane fluidity; decreased glutathione, nonprotein sulfhydryls, and NADPH content in the liver (60). Although most of these studies were acute or subchronic studies, several relevant chronic studies were also reported. All of them focused on the oxidative stress in the liver of female *Sprague-Dawley* rats, which is a sensitive strain and gender with high hepatic tumor incidence after TCDD exposure. Tritscher et al. (68) first reported increased 8-OHdG in livers of TCDD treated intact and

not ovariectomized female rats after diethylnitrosamine (DEN) initiatiation. Wyde et al. (69) further found the induction of hepatic 8-OHdG by TCDD in rats after DEN initiation was female-specific, estrogen-dependant and was a chronic effect. Recently, Hassoun et al. (67) observed increased production of superoxide anion, lipid peroxidaiton and DNA single-strand breaks in hepatic tissues of rats after 30 weeks of exposure to TCDD. All these studies supported the importance of oxidative DNA damage in carcinogenesis of TCDD.

Meanwhile, it was also found that TCDD dose and tissue concentration did not necessarily correlate with ROS production. An acute oral dose of TCDD (0.001 to 100 µg/kg) administered to C57BL/6 mice induced a significantly sustained increase in liver superoxide anion and thiobarbituric acid reactive substance (TBARS) only at the highest dose (100 µg/kg), and resulted in hepatic TCDD concentrations of 321 ng/g at 13 weeks post exposure (83). In comparison, subchronic TCDD administration (0.15 to 150 ng/kg; 5 days/week for 13 weeks) produced significantly increased superoxide and TBARS only with the highest (150 ng/kg/day) exposure level, corresponding to TCDD concentration of 12 ng/g in liver (83). Following a dosing regimen capable of producing an oxidative stress response, TCDD did not alter the mutation frequency or the mutation spectrum of the *lacI* transgene in male or female Big Blue rats (70). Recently, studies found that oxidative stress response genes were not activated in livers of animals exposed to 100 ng/kg TCDD for 14 weeks, although significant induction of CYP450 and hepatocyte hypertrophy were found in the liver of these animals (71).

In summary, the contribution of ROS to the toxic effects of TCDD is still controversial. Further scientific studies are necessary to explore whether oxidative stress is important or just a hypothesis of scientists to explain the complicated mechanisms for the toxic effects of chemicals. Because of the high propensity for artifact formation during sample processing, 8-OHdG can be a problematic biomarker for ROS-induced DNA damage. Different DNA adducts have different formation and repair pathways, which may result in different toxicities/responses in treated animals. **Therefore, in this study, a battery of ROS-induced DNA adducts will be applied to further evaluate ROS-induced DNA damage in a two-stage initiation-promotion rat liver model with DEN as the initiator and TCDD as the promoter** (69)**. This information will help us better understand the mode of action of hepatocarcinogensis of TCDD in female rats.**

F. PHAHs, TOXIC EQUAVELENCY FACTOR, OXIDATIVE STRESS AND OXIDATIVE DNA DAMAGE

PHAHs consist of a large class of compounds including polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) (58). They are one of the most prevalent groups of pollutants in the environment. PCDDs and PCDFs have no industrial use but are produced as unwanted by-products of many anthropogenic activities, such as chlorine bleaching of paper and pulp (73). PCBs were commercially produced and widely used for various industrial purposes, including heat transfer agents, dielectric insulating fluids for capacitors and paint additives (74). Because these compounds are resistant to degradation and persistent

in the environment, they accumulate in tissues. Humans and wildlife animals are exposed daily to complex mixtures of these chemicals, primarily via trace amounts present in food, resulting in chronic lifetime exposure which may evoke toxicity and carcinogenicity (74).

Depending on the location and type of halogenation, some PHAHs induce a similar spectrum of biochemical and toxic responses in experimental animals characterized by severe weight loss, thymic atrophy, hepatotoxicity, immunotoxicity and enzyme induction (58). These common biological effects are mediated through a similar mechanism of action as described for TCDD binding to AhR, as shown in figure 1.4. Therefore, these structurally related compounds are commonly referred to as dioxin-like compounds (DLCs) (58, 74). Due to similarity in toxicity and mechanisms of DLCs, the concept of the Toxic Equivalency Factor (TEF) has been used for the assessment of risk and regulatory control for these compounds. The TEF methodology is a relative potency scheme determined from the toxicity of each congener relative to that of TCDD on the basis of available *in vivo* and *in vitro* data. This allows for the estimation of the potential dioxin-like activity of a mixture of DLCs that are found in environment (74, 75), as shown in the following equation.

$$TEQ = \sum_{ni}(PCDD_i \times TEF_i)_n + \sum_{ni}(PCDF_i \times TEF_i)_n + \sum_{ni}(PCB_i \times TEF_i)_n$$

where i= the individual congener and its respective TEF, and n = all congeners within each class of DLCs.

Similar to the studies on the connection between ROS and TCDD, several studies reported the induction of ROS in hepatic tissues of rats exposed to PHAHs. Hassoun

found the production of superoxide anion, lipid peroxidation and DNA SSBs in liver DNA of rats after exposure to various mixtures of TCDD and two of its congeners, PeCDF and PCB 126 for 13 weeks (67). Other chronic animal studies demonstrated the involvement of ROS in the toxicity and carcinogenicity of PHAHs (77).

Recently, in an effort to evaluate the TEF methodology for the chronic toxicity and carcinogenicity of DLCs and structurally related polychlorinated biphenyls (PCBs), the National Toxicology Program (NTP) conducted a series of 2-year bioassays in female Harlan *Sprague-Dawley* rats exposed to DLCs (such as TCDD, PCB 126, and PeCDF and non-dioxin-like compounds (NDLCs) PCB153). The toxic effects of the ternary mixture of TCDD, PCB126 and PeCDF and the binary mixture of PCB126 and PCB153 were also evaluated in these studies. Besides inducing a high incidence of hepatic tumors in female rats, chronic inflammation in liver tissues was also observed in the exposed animals. Since the DNA damage caused by ROS has been implicated in a myriad of diseases, it is reasonable to **hypothesize that ROS-induced DNA damage is an important mode of action for carcinogensis of these PHAHs, which can be estimated by TEF approach, as shown in figure 1.5.**
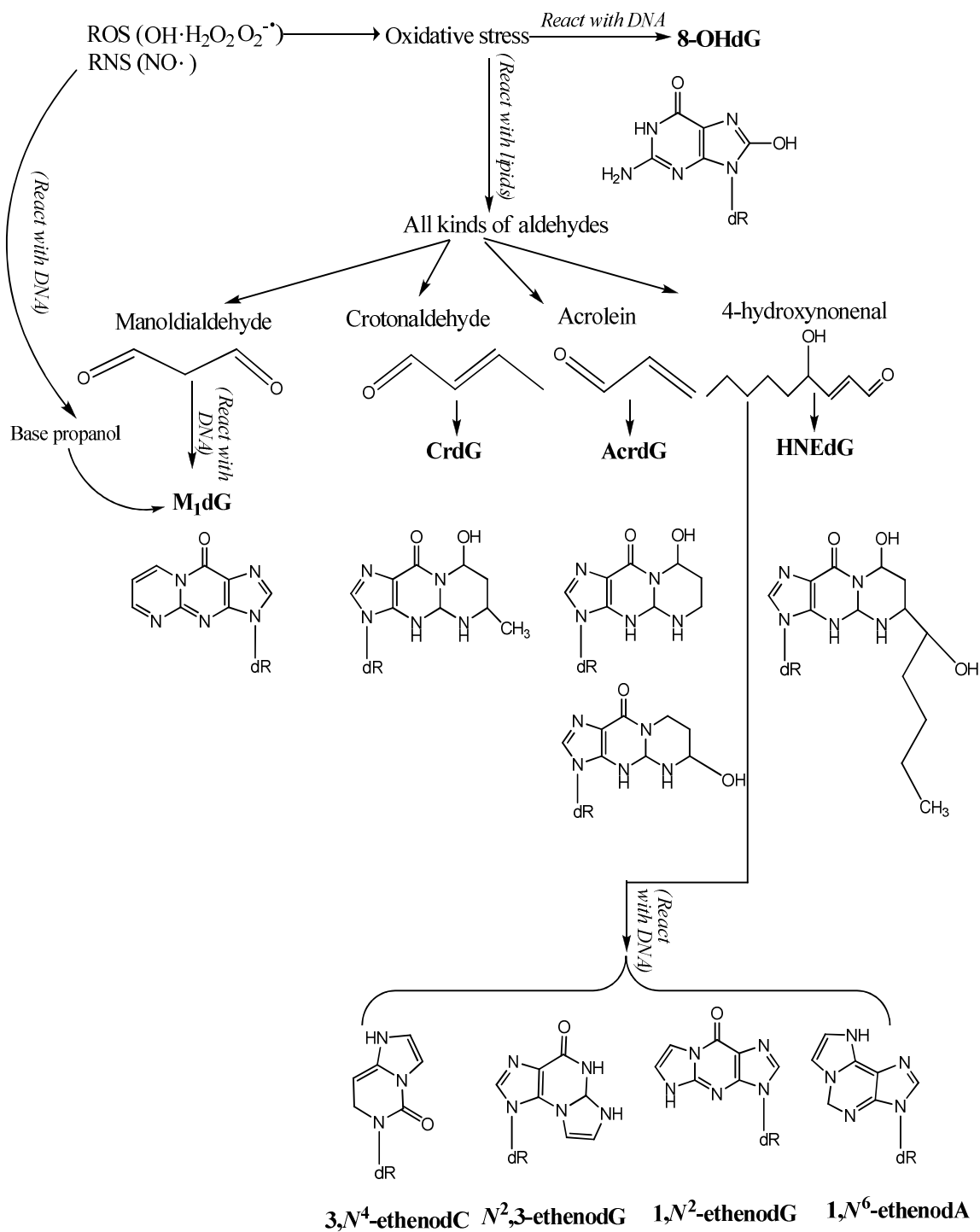
Figure1.1 Major DNA adducts induced by reactive oxygen species (ROS)
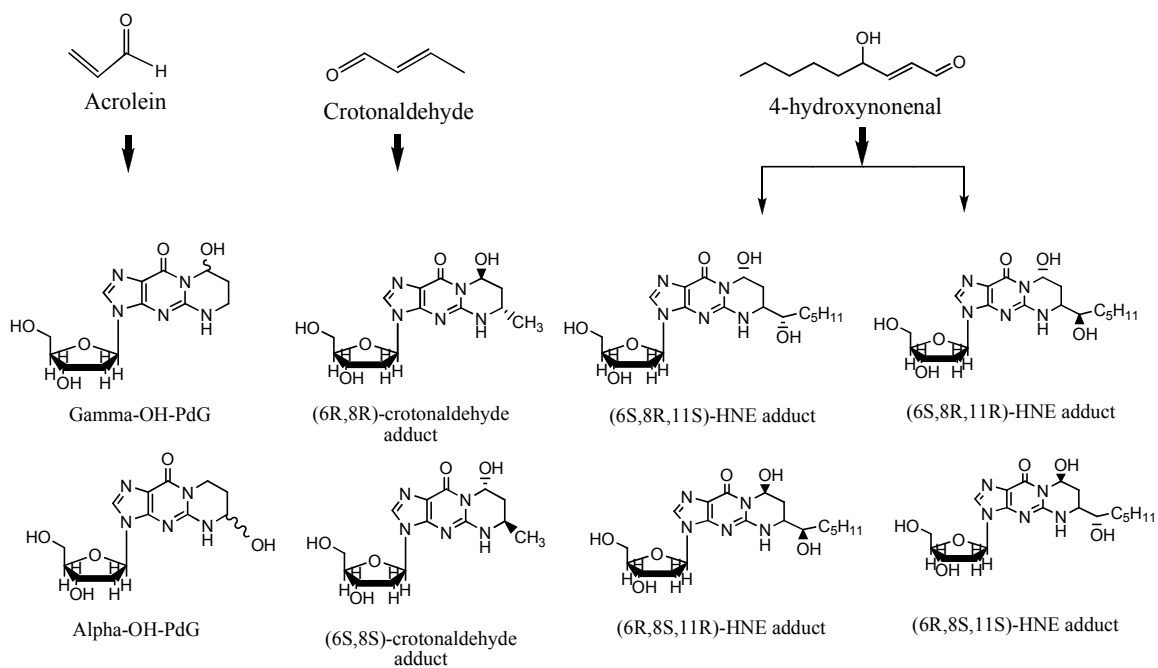
Figure 1.2 Major propano-derived adducts induced by lipid peroxidation (LPO)
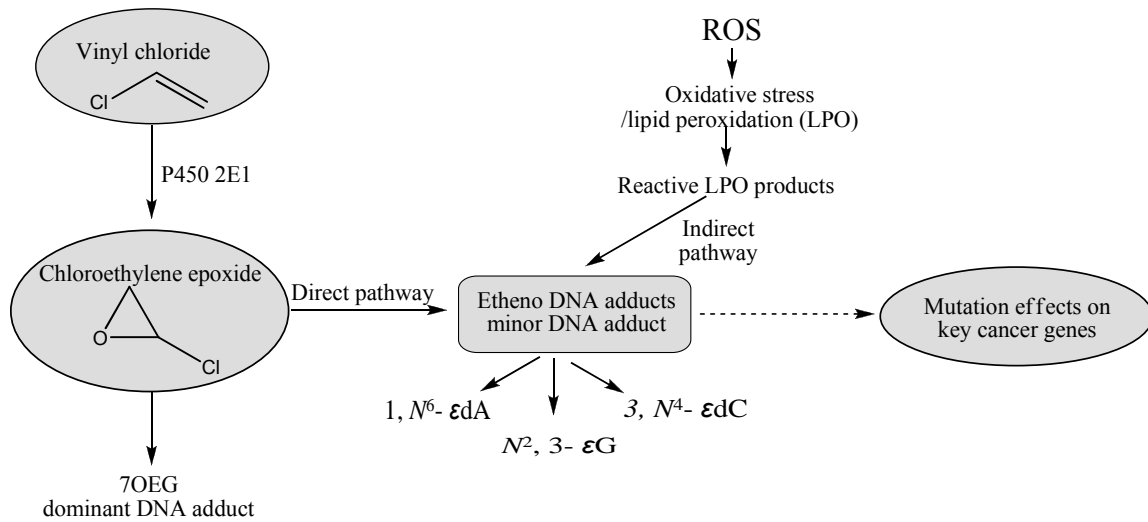
Figure 1.3 Etheno DNA adduct formation in VC exposed animals
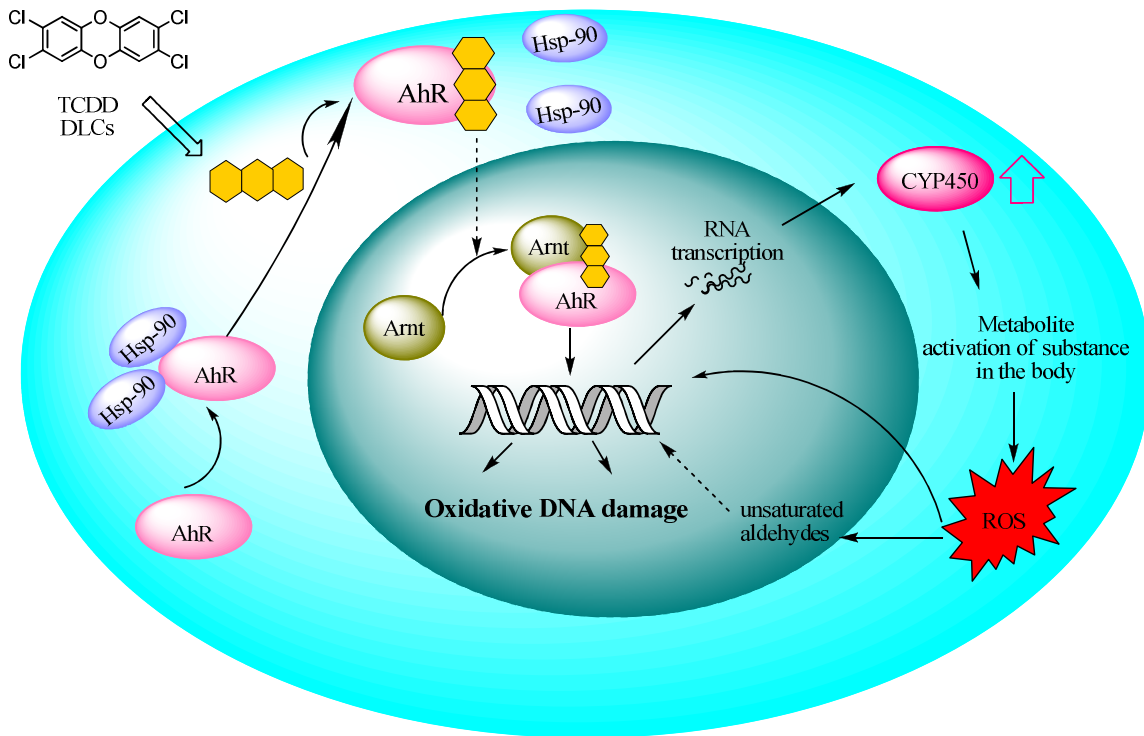
Figure 1.4 Oxidative DNA damage induced by dioxin-like compounds (DLCs)
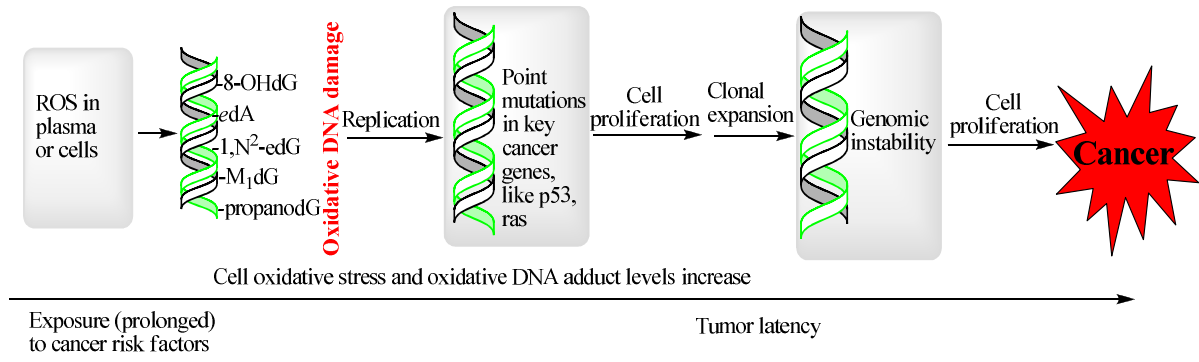
Figure 1.5 Hypothetical schemes for the role of cumulative oxidative stress in carcinogenesis

Table 1.1 Single-base changes induced by ROS related DNA adducts in the site-directed mutagenesis experiments

ROS-induced DNA lesions	*In vitro*	In *E. coli*	In mammalian cells
1, N^6-εdA	A>G, A>T, A>C (2)	A>G, A>T, A>C (3, 4)	A>G, A>T, A>C (4)
3, N^4-εdC		C>T, C>A (3)	C>T, C>A (3)
1, N^2-εdG		G>T, G>A (5)	G>T (5)
N^2, 3-εG	G>A (6)	G>A (7)	
8-OHdG	G>T (8)	G>T (8)	G>T (8)
MDA induced adducts	G>T, G>A (8)		G>T, G>A (9)
AcrdG		G>T (γ) (10)	G>T, G>A, G>C ($\gamma+\alpha$) (10)
CrdG			G>T, G>A, G>C (10)
HNEdG			G>T (10)

Table 1.2 Reported endogenous amounts of ROS-induced DNA adducts

DNA adduct	Background level
8-OHdG (24)	$20\text{-}200/10^8$ dNs in human lymphocytes
N^2, 3-εG (24)	$1.5\text{-}17.5/10^8$ dNs in human liver and unexposed rats
1, N^6-εdA (24)	ND-$0.7/10^8$ dNs in rats, mouse and human tissues
3, N^4-εdC (24)	ND-$2.7/10^8$ dNs in animals and human tissues
1, N^2-εdG (79)	$2.0\text{-}3.9/10^8$ dNs in mice (liver, spleen and kidney)
M_1dG (24)	$0.8\text{-}1.8/10^8$ dNs in animals, $0.2\text{-}110/10^8$ dNs in human
AcrdG (24, 33, 34, 79)	$1.4\text{-}23.3/10^8$ dNs in rat tissue (brain, lung, kidney, colon, prostate, mammary, leukocytes); $0.25\text{-}50/10^8$ dNs in human tissue (liver, breast, leukocytes, brain, gingival)
CrdG (24, 79)	$0.2\text{-}9.9/10^8$ dNs in rat tissue (brain, lung, kidney, colon, prostate, mammary, leukocytes); ND-$7.5/10^8$ dNs in human tissue (liver, lung and gingival)
HNEdG (24, 35, 80)	ND-$15.8/10^8$ dNs in rats (liver, kidney, lung, colon, forestomach), $2\text{-}60/10^8$ dNs in human brain and colon tissue

Table 1.3 Reported repair pathways for ROS-induced DNA adducts

DNA lesions	Bacterial enzyme/system	Mammalian enzyme/system
1, N^6-εdA	AlkA (38), AlkB	ANPG/Aag (39) (BER), ABH2 (43) (direct reversal pathway)
3, N^4-εdC	AlkB (81)	mismatch-specific thymine-DNA glycosylase (82) (BER)
1, N^2-εdG		ANPG (39) (BER)
N^2, 3-εG	E.Coli 3-methyladenine DNA glycosylase (39) (BER)	
8-OHdG	MYH, MMR	8-oxoguanine DNA glycosylase (42) (OGG1), hMYH, hMPG, hNEIL1 (BER), MMR, NER
M_1dG	NER (10)	
AcrdG	NER (10)	
CrdG	NER (10)	
HNEdG	NER (44), HR (44)	NER (10)

G. REFERENCES

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CHAPTER II

DETECTION OF ROS-INDUCED DNA ADDUCTS BY LC-MS/MS

A. INTRODUCTION

Oxidative stress is a common state in pathophysiology, where the number of reactive radicals or molecules being formed exceeds those being detoxified. It is regarded as playing an important role in many diseases including cancer, neurodegeneration and aging (1). Reactive oxygen species induced by oxidative stress can react with DNA directly and form various DNA lesions. Among them, the most studied is 8-hydroxyl-2′-deoxyguanosine (8-OHdG). Persistent oxidative stress can also induce excessive lipid peroxidation products (LPO), especially unsaturated aldehydes, which are particularly reactive in forming exocyclic DNA adducts (2). The primary exocylic adducts are a five-membered ring (etheno adducts) or a six-membered ring (propano adducts) attached to DNA bases. The major etheno adducts include 1, N^6-etheno-2'-deoxyadenosine (1, N^6-εdA), 3, N^4-etheno-2'-deoxycytidine (3, N^4-εdC), 1, N^2-etheno-2'-deoxyguanosine (1, N^2-εdG) and N^2, 3εG. The dominant propano adducts include acrolein, crotonaldehyde and 4-HNE-derived dG adducts, assigned as AcrdG, CrdG, and HNEdG respectively. Based on the direction of the Michael addition of those α, β-unsaturated aldehydes with β substituents, various stereoisomers of pronano adducts exist (3). Those ROS-induced adducts can cause base-pair substitution mutations and have been applied as valuable

biomarkers for assessing oxidative stress-derived DNA damage (2-3). With the development of sensitive and robust detection techniques, growing evidence supports that these DNA adducts are significantly induced in patients with various chronic inflammation diseases including cancer (2, 4-9).

To date, many different methods have been developed to detect ROS-induced DNA adducts. As one of the most studied adducts, 8-OHdG was measured in animal or human tissues by multiple techniques, including LC-ECD (electrochemical detector), GC-MS, immunoassay, ^{32}P-postlabeling and LC-MS/MS (8). However, lack of consistency was observed in the results obtained by different methods, sometimes by up to two or three orders of magnitude. Therefore, the European Standards Committee on Oxidative DNA Damage (ESCODD) evaluated several different methods and found that LC-ECD, instead of LC-MS/MS, was a reliable, sensitive, and precise procedure for 8-OHdG detection (10). However, only limited laboratories used LC-MS/MS and no sample purification was performed before detection in their studies. The recent rapid development of LC-MS/MS has greatly promoted its application in the detection of DNA adducts (11-13). Thus, it is still worthwhile to develop an assay for the detection of 8-OHdG by LC-MS/MS. ^{32}P-postlabeling assay has been the most widely applied technique for quantitative analysis of LPO-induced DNA adducts (14-18). The background 1, N^6-εdA and 3, N^4-εdC were first detected in tissues of control animals by an ultrasensitive, specific immunoaffinity ^{32}P-postlabeling method (14). The adduct concentrations measured by this method were found to be in good agreement with those determined by a radioimmunoassay. The small DNA amount consumption, ~10μg, made this method

especially valuable for epidemiology studies (14). At present, most propano adducts in animal tissues are detected by a HPLC-^{32}P-postlabelling method (15, 16). With LC sample purification, this assay can differentiate the stereoisomers of propano adducts. In general, the amount of DNA required was more than 100 μg. Recently, several studies reported the detection of propano adducts in human samples by LC-MS/MS (19-22). Using a capillary LC-MS/MS system, higher sensitivity was obtained in these studies (19-22). Because the measurement of M_1dG was affected by the competitive reaction of pH-dependent ring-opened formation of N^2-(3-oxo-1-propenyl)-deoxyguanosine anion (N^2OPdG$^-$), most studies chose M_1G as the target molecule for assay development (23, 24). ^{32}P-postlabeling was the first method employed for the detection and quantitation of M_1G using 10 μg DNA (17), followed by GC/MS with electron capture negative chemical ionization detection and LC-MS/MS with chemical derivatization (17, 25-29). However, most of these assays were time-consuming and required extensive sample manipulation by highly skilled personnel.

In this study, a series of assays were developed for the detection of ROS-induced DNA adducts. The general strategy for assay development is shown in figure 2.1. Based on the physicochemical properties of these DNA adducts, enzymatic hydrolysis, LC sample purification and MS detection were optimized. The processing time of 8-OHdG was minimized and antioxidant, 2, 2, 6, 6-tetramethyl-piperidinoxyl (TEMPO, 20mM final concentration) was added to samples to minimize artifactual formation of 8-OHdG. A nanoUPLC-MS/MS system with column switching was applied to measure all LPO-induced DNA adducts. These assays will help us explore the comprehensive spectra of

oxidative DNA damage in animals with chronic inflammatory diseases after chemical exposure in the future.

B. MATERIALS AND METHODS

Chemicals

Nucleic acid purification grade lysis buffer, protein precipitation solution and Proteinase K were purchased from Gentra Systems (Minneapolis, MN). TEMPO was obtained from Acros (Morris Plains, NJ). Other chemical reagents including 1, N^6-εdA and 8-OHdG standards were from Sigma-Aldrich Chemical Company (St Louis, MO). HPLC grade water, methanol and other chemical solutions were purchased from Thermo Fisher Scientific Company (Raleigh, NC). [$^{15}N_5$]-8oxodG standard, [$^{15}N_5$]-dG, [$^{15}N_3$]-2′-Deoxycytidine, and [$^{13}C_{10}$]-2′-deoxyguanosine was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Instrumentation

HPLC purification of the synthesized standards was performed on an Agilent (Agilent Technologies, Palo Alto, CA) 1100 series with a Hewlett-Packard 1040A diode array detector. Standard characterization by full scan of tandem mass spectra and the detection of 8-OHdG were done on a Finnigan Quantum triple-quandrupole mass spectrometer (ThermoFinnigan, San Jose, CA) connected to Waters UPLC system (Waters, Milford, Massachusetts). All LPO-induced adducts were measured by a

nanoUPLC-MS/MS system, comprised of a 10 kpsi nanoacquity Ultra performance LC system and a TSQ-Quantum ultra triple quad mass analyzer. An Agilent 1200 Series analytical fraction collector system was used for the LC sample purification of ROS-induced DNA adducts.

Animal exposure

The livers of Shasta strain rainbow trout (*O. mykiss*) exposed to DBP were from OSU Sinnhuber Aquatic Research Laboratory (SARL). The animal protocols were described in detail previously (88). Trout fry (1.5g) were fed standardized OTD with different levels of DBP incorporated. On days 29, fish were sampled from tank for analysis of DNA adducts.

Male wide type and XPA null ($XPA^{-/-}$) mice were dosed with olive oil (10ml/kg) or carbon tetrachloride (1600 mg/kg) by intraperitoneal injection for 13 days. After the treatment, these animals were sacrificed and livers were harvested.

The animal protocol for PHAHs treated female *Sprague-Dawley* rats (Harlan *Sprague-Dawley* Inc., Indianapolis, IN) was described in detail in the technical reports from NTP (89). Briefly, female *Sprague-Dawley* rats received the corn oil:acetone (99:1) vehicle at the age of aaproximately 8 weeks old for 53 weeks. After exposure, these animals were sacrificed by carbon dioxide asphyxiation.

DNA isolation

The DNA isolation protocol was slightly modified from Jeong's study (90). Briefly, ~300 mg liver DNA was thawed and homogenized in ice-cold PBS solution. After centrifugation for 15 min at 1000g and washing by PBS buffer, the nuclear fraction was collected and reconsitituted in 6 ml lysis buffer. Protein was removed by incubation with proteinase K (400 U/ml, 60 μl) overnight at 4 °C and extracted twice with 6ml 70% phenol solution. The nuclear acid was precipated from the sample by adding 300 μl 3 M NaCl and 12 ml cold ethanol, followed by centrifugation and rinsing by 70% ethanol. After reconstitution in PBS buffer, the samples were incubated with RNase A (0.8 KeU/ml) and RNase T1 (3 mU/ml) for 1h at 37 °C. Finally, the DNA was precipated from the sample by the sequential addition of 100 μl 3 M NaCl and 4 ml cold ethanol, and rinsed with 70% ethenal after centrifugation. After the sample was reconstituted in HPLC water, the concentration and purity of the DNA was measured by UV.

Standard synthesis

$^{15}N_5$-1, N^6-εdA standard was synthesized and characterized as described by Ham et al. (30). $^{15}N_5$-2′-deoxyadenosine (15 mg) and $ClCH_2CHO$ (8 M) were added in a solution of ammonium formate (420 mg) in water (7 ml) and ethanol (3 ml). The contents were stirred at 40 °C for 7 h followed by cooling to room temperature and neutralization to pH 7 with sodium bicarbonate. The solvent was evaporated at room temperature using argon gas. The product was dissolved in chloroform/methanol (4:1, 10 ml) and purified on silica gel. $^{15}N_5$-εdA was eluted by a mixture of chloroform (80%) and methanol (20%),

followed by purification by HPLC on a C18 column. The purity of the final product was verified by UV, NMR and mass spectrometry.

1, N^2-εdG and $^{13}C_{10}$-1, N^2-εdG were synthesized based on the report of Kusmierek et al. (31). Chloroacetaldehyde (57 μmol) was added into the stirred solution of N, N′-dimethylformamide (166 μl) with 10 mg [$^{13}C_{10}$]-2′-deoxyguanosine or 2'-deoxyguanosine (34 μmol) and 7 mg K_2CO_3 (50 μmol). After reacting for 3 h at room temperature, K_2CO_3 (33.3 μmol) and chloroacetaldehyde (33 μmol) were added and stirred for an additional 12 h. The product was purified by LC (C18 column, 250×4.6 mm; 4μ). Solvent A was methanol; solvent B was 0.5 M NH_4HCO_3 in water. The flow rate was 1000 μl/min. The elution time of 1, N^2-εdG and $^{13}C_{10}$-1, N^2-εdG was verified by UV and the purity was determined by mass spectrometry and NMR.

Malondialdehyde (MDA) modified ^{15}N- or natural abundance (NA-) DNA was made based on Jeong's study (28). Briefly, MDA was prepared from tetramethoxypropane in 1 M HCl by hydrolysis at 45 ˚C for 45 min. ^{15}N-DNA was prepared using *E. coli* cultured in minimum salt medium containing ($^{15}NH_4)_2SO_4$ as a single nitrogen source for bacterial growth. 3 mg ^{15}N- or NA-DNA was incubated for 2 h in cold room (4 ˚C) with 6 ml of an aqueous solution containing 50 mM MDA as IS or AS DNA. Excess MDA was removed by precipitating DNA using ethanol and water. $^{15}N_5$ or NA-M_1dG in DNA was determined by measuring M_1G concentration using HPLC with fluorescence detection (Applied Biosystems, Foster City, CA) after acid hydrolysis of DNA.

The standards for AcrdG, CrdG and 4-HNEdG and their $^{15}N_5$ labeled internal standards were synthesized based on previous studies (21, 32, 33). In brief, 1 ml of 400 mM acrolein, crotonaldehyde or 4-HNE were allowed to react with dG or $^{15}N_5$-dG (50mg) in 10 ml of 0.02 M phosphate buffer (pH = 7) at 37 ˚C overnight. Then 200 μl of 4 M acrolein, crotonaldehyde or 4-HNE was added into the sample and incubated at 37 ˚C overnight followed by two extractions with 10 ml chloroform and solvent evaporation using argon gas. The products were further purified on C18 column (150 mm × 4.6 mm, 5 μm) by HPLC using PDA detector with separate collection of the stereoisomer. The product peak was verified by UV spectrometry and mass spectrometry.

Enzymatic digestion and HPLC purification

Three different enzymatic systems were evaluated for 1, N^6-εdA detection (30, 34, 35). Method A (30): 100 μg DNA was incubated in Tris buffer (pH 7.0, 80 mM Tris-Hcl, 20 mM $MgCl_2$) with DNaseI (200 U) for 10 min at 37 ˚C. Alkaline phosphatase I (AP, 10 U) and phosphodiesterase I (PDE, 0.013 U) were added into the sample for additional 1 h incubation at 37 ˚C. Method B (34): 100 μg DNA was incubated at 37 ˚C for 3 h in sodium acetate buffer I (sodium acetate 30 mM, pH 5.6, 0.2 mM zinc chloride) with 5 μg of nuclease P1 (NP1), followed by an additional 6 h incubation with 520 μl of sodium acetate buffer II (sodium acetate 30mM, pH 8.1), 20 U of AP and 0.01 U of PDE. Method C (35): micrococcal nuclease (2 U) and spleen phosphodiesterase (0.2 U) were added into 40 mM sodium succinate buffer (20 mM calcium chloride, pH 6) to digest 100 μg DNA. After a 4 h incubation at 37 ˚C, NP1 (5 μg) was added for an additional 2 h incubation.

After DNA hydrolysis, the samples were purified on an Atlantis T3 column (4.5×150 mm, 5 μ). Solvent A was 10 mM ammonium acetate in water; B was 100% methanol. The initial condition was 5% B at a flow rate of 1 ml/min for 10 min. Then solvent B increased to 10% in the next 5 min, 20% in 10 min and 80% in 13 min and return to the initial condition 5% in the last 5 min.

50 μg DNA was processed with TEMPO (5μl, 1.5mM) added at the beginning of 8-OHdG assay. The same enzymatic hydrolysis as 1, N^6-εdA was applied to digest DNA. A short LC purification method was developed especially for 8-OHdG using the same LC columns and mobile phases as 1, N^6-εdA. The gradient program was as follows: solvent B was 15% for the initial 5 min, increased to to 30% in 20 min and 80% in 10 min before returning to 15% in 5 min. The LC flow rate was 0.6 ml/min.

Two digestion systems were evaluated for the measurement of propano adducts. System A consisted of DNase I, PDE and AP in Tris buffer (30). The incubation time was the same as method A for 1, N^6-εdA. System B was composed of DNase I, NP, PDE and AP in sodium acetate buffer (34, 36). The same optimized incubation time for 1, N^6-εdA detection was applied for these adducts. 200U DNase I was added into the sample with NP1 at the beginning of the digestion. Calf thymus DNA (ctDNA) treated by acrolein, crotonaldehyde or 4-HNE was digested by both of these systems. CrdG and 4-HNEdG were processed together with 10 mM ammonium acetate in water as solvent A and methanol as solvent B. With LC flow rate of 0.7 ml/min, solvent A was 65% for the initial 5 min, decreased to 30% in 15 min and kept stable for 10 min before returning to

65% in 5 min. For simultaneous purification of AcrdG, M_1dG and 1, N^2-εdG, the mobile phases were 5 mM ammonium formate and 0.1% formic acid in water (pH 3.5) as solvent A and methanol as solvent B. The flow rate was 0.6 µl/min. Solvent B was 10% for the initial 8 min, increased to 30% in 12 min and kept stable for 10 min before returning to 10% in 5 min.

LC-MS/MS detection

Three LC-MS/MS systems were evaluated for 1, N^6-εdA detection, UPLC-MS/MS, capillary LC-MS/MS and nanoUPLC-MS/MS. In nano UPLC-MS/MS, a nanoacquity symmetry trap column (180µm×20mm, 5 µm), was connected with a nanoUPLC BEH C18 analytical column (1.0mm×100mm, 1.7µm) through a control valve. UPLC and capillary LC-MS/MS systems consisted of an Aquity UPLC system or Agilent 1200 capillary system and a TSQ triple quadrupole mass spectrometer. In the UPLC system, 0.1% formic acid in water was solvent A and 0.1% formic acid in methanol was solvent B. The flow rate was 200µl/min. A HSS T3 UPLC (1.8 µm, 2.1×100 mm) column was used to purify the samples before MS detection. In capillary system, the separation was performed on a ZORBAX SB-C18 column (100mm×0.5mm, 3 µm) with the flow rate of 7µl/min. Solvent A and B were 5mM ammonium formate in water and methanol respectively. The same collision energy and transitions were applied in all the three systems. 1, N^6-εdA was detected under multiple reaction monitoring (MRM) in the positive ESI mode, m/z 276.0 → 160.0 for 1, N^6-εdA and m/z 281.0 → 165.0 for $^{15}N_5$-1, N^6-εdA. The capillary temperature was set as 285 ˚C. The spray voltage was 2000 V in nano and capillary system, and 2800 V in UPLC system. The collision energy (CE) was

16 eV. The sheath and auxillary gas pressure was 35 and 30 Torr respectively in UPLC systems, but none of them was used in capillary or nano system.

8-OHdG was detected by UPLC-MS/MS on a T3 HSS column (2.1×100mm, 1.7 μ) with 0.1% acetic acid in water as solvent A and methanol as solvent B at flow rate of 200μl/min. The gradient program was as follows: solvent B was increased from 1% to 5% in 2 min, held at 5% for 10 min, and increased to 50% in 2 min. m/z 284.1 → 168.0 and m/z 289.1 → 173.0 were monitored for 8-OHdG and [^{15}N$_5$]8-OHdG respectively with CE 12 eV. The spray voltage was 3000 V. The vaporizer temperature was 250 ˚C. The sheath and auxillary gas pressure was 35 and 30 Torr respectively. The capillary temperature was 285 ˚C.

NanoLC-MS/MS was used to detect all LPO-induced DNA adducts. For the detection of 1, N^2-εdG, M$_1$dG, CrdG, 4-HNEdG and AcrdG, solvent A and B were 0.1% formic acid in water and acetonitrile respectively. The sample was first loaded on the trap column by 99% A for 3 min with flow rate of 5μl/min. For 1, N^2-εdG, M$_1$dG, and AcrdG detection, solvent B was 5% for the initial 1 min, followed by a increase to 30% in 10 min before returning to 5% in 15 min. For CrdG detection, solvent B was 10% for the initial 5 min, increased to 50% in 10 min and kept stable for 5 min. For 4-HNEdG detection, solvent B was 15% for the initial 2 min, increased to 70% in 10 min and returning to 15% in 10 min. The flow rate was 1.0 μl/min for the detection of all above adducts. Similar MS parameters were used to detect 1, N^2-εdG, M$_1$dG, AcrdG, CrdG and 4-HNEdG except different transitions were observed by MRM. The capillary temperature

was set as 285 °C. The spray voltage was 1500 V. Neither nebulizing nor drying gas was used in this system. Two transitions were monitored for the detection of 1, N^2-εdG, M$_1$dG and 4-HNEdG respectively with CE 16 eV, m/z 304.0 → 188.0 and 308.0 → 193.0 for M$_1$dG and [^{15}N$_5$]-M$_1$dG, m/z 292 → 176.0 and 302 → 181.0 for 1, N^2-εdG and [^{13}C$_{10}$]-1, N^2-εdG, and m/z 424.0 → 308 and 429.0 → 313.0 for 4-HNEdG and [^{15}N$_5$]-4-HNEdG. Three and four transitions were monitored for CrdG and AcrdG detection respectively, m/z 338 → 222 with CE 12 eV and m/z 338 → 178 with CE 32 eV for CrdG, 343 → 227 with CE 12 eV for [^{15}N$_5$]-CrdG, m/z 324 → 208 with CE 12 eV and 324 → 164 with CE 32 eV for AcrdG, 329 → 213 with CE 12 eV and m/z 329 → 190 with CE 32 eV for [^{15}N$_5$]-AcrdG.

C. RESULTS

Enzymatic hydrolysis and HPLC purification

In comparing method A, B and C for the detection of 1, N^6-εdA, less interference was found in system B and C than A. Because of the convenience of sample processing for method B, it was selected for further optimization. By evaluating the concentrations of dA and 1, N^6-εdA in ctDNA, the final incubation time was adjusted to be 2 hours in method B. Using the optimized incubation time, similar amounts of 1, N^6-εdA were detected with 0.0118 U and 1.18 U PDE, which indicated that 0.0118 U PDE was enough for 100 μg DNA digestion, as shown in figure 2.2.

Higher amounts of AcrdG were observed in the acrolein treated ctDNA samples digested by system B than A and C, although no difference was found for the amount of CrdG in the crotaldehyde treated samples, as shown in figure 2.3. Background noise appeared for the detection of 4-HNEdG and M_1dG in control and 4-HNE or MDA treated ctDNA by system A. Thus, system B was selected for the assay development of propano adducts, 1, N^2-εdG and M_1dG. 200 U DNase I was added into system B at the beginning of digestion, because some studies mentioned that it was necessary for the detection of bulky DNA adducts (36). 2′-Deoxyadenosine (dA) co-eluted with AcrdG on C18 columns when 5 mM ammonium formate in water was used as solvent A and methanol as B. With 0.1% formic acid added in 5mM ammonium formate aqueous solution as solvent A, complete separation of dA and AcrdG was achieved, as shown in figure 2.4. The peak of M_1dG appeared after that of 1, N^2-εdG determined by LC-MS/MS using MDA-treated DNA. The concentration of M_1dG in MDA-treated ctDNA was below the LOD of the UV detector of the fraction collector. The typical chromatograms for LC purification of 1, N^2-εdG, AcrdG, 4-HNEdG and CrdG are shown in figure 2.4. The fractions containing 1, N^6-εdA and 8-OHdG were collected based on retention time of standards on fraction collector, as shown in figure 2.5.

LC-MS/MS detection and assay validation

The dynamic range of nanoUPLC system for 1, N^6-εdA detection was evaluated by standards, as shown in figure 2.6. The limit of quantification (LOQ) was determined to be 200 atmol on-column. Using the same standards with concentrations from 1.6 to 106 nM, the sensitivity of three LC-MS/MS systems was determined (the slope of the linear

dynamic curve), as shown in figure 2.7. The sensitivity of nanoUPLC system was more than 20 times higher than the UPLC system, followed by the capillary system which had 3 to 4 times higher sensitivity than the UPLC system. After LC purification, no significant matrix effects were observed with the nanoUPLC system, as indicated by figure 2.6. Better results were also obtained for the detection of 1, N^6-εdA using the nanoUPLC than the UPLC system when evaluating rat liver DNA exposed to ($^{13}C_2$)-vinyl chloride (VC), as depicted in figure 2.8. Transitions m/z 276 → 160, 278 → 162 and 281 → 165 were used to monitor endogenous NA-1, N^6-εdA (lipid peroxidation induced 1, N^6-εdA), exogenous [$^{13}C_2$]- 1, N^6-εdA (VC induced 1, N^6-εdA) and internal standard [$^{15}N_5$]- 1, N^6-εdA respectively.

The typical chromatograms of 8-OHdG, 1, N^2-εdG and propano adducts in ctDNA and their calibration curves are shown in figure 2.9 A~E. Well-shaped peaks appeared in each monitored transition of these adducts. With $R^2 \geq 0.98$, all of these calibration curves indicated a good linear dynamic range (from sub-femto mole to several hundred femto mole) for the detection of these adducts by nanoUPLC-MS/MS system. The LOQ for propano adducts was 50 atmol on-column, which was determined by injecting standards with S/N higher than 10. Using this battery of assays, the background concentrations of these adducts in control liver DNA of female *Sprague-Dawley* rats (61 weeks old) were determined (table 2.1). 8-OHdG had the highest concentration in these samples, followed subsequently by AcrdG, M_1dG, 1, N^2-εdG, 1, N^6-εdA, 4-HNEdG and CrdG. Besides, M_1dG, 1, N^2-εdG and propano adducts were also measured in liver DNA from mice exposed to a single dose carbon tetrachloride (1600 mg/kg in olive oil) and trout liver

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DNA exposed by dibenzo[α,l]pyrene (DBP). The results are shown in figure 2.10A~D. After i.p. exposure to carbon tetrachloride for 13 days, significant increases of M_1dG, 1, N^2-dG and AcrdG were observed in the liver DNA of wild type mice (2 to 3 fold increase in the exposed compared with the control), as shown in figure 2.10A. With the same chemical treatment, M_1dG, 1, N^2-εdG and AcrdG were significantly induced in liver DNA of exposed XPA$^{-/-}$ mice compared with the control, as depicted in figure 2.10B. Three- to four-fold increases of M_1dG, 1, N^2-εdG and AcrdG were present in the liver DNA from XPA$^{-/-}$ mice exposed to carbon tetrachloride, compared with exposed wide type mice, suggesting that NER is a dominant repair pathway for these DNA adducts. After 28 days exposure, no significant increases of 1, N^2-εdG, M_1dG, AcrdG, 4-HNEdG and CrdG were observed in the liver of trout treated by 80 ppm DBP, compared with the 0.45 ppm treated group, as indicated in figure 2.10C and 2.10D.

D. DISCUSSION

With background adducts usually around $1/10^6$ guanine, 8-OHdG has been detected by many different techniques, including immunoaffinity assay, LC-ECD, LC-MS/MS, ^{32}P-postlabelling, GC-MS and formamidopyrimidine DNA glycosylase (FPG) based methods (10, 37-51). The main problem for 8-OHdG detection is artifact formation from the oxidation of unmodified guanine bases present in DNA. This may explain why there is a great variation in the amounts of 8-OHdG *in vivo* reported by different methods. GC-MS method is often cited as overestimating the concentration of 8-OHdG in DNA samples due to the harsh sample processing, such as severe acidic conditions for DNA

hydrolysis and a lengthy derivatization step (39, 43, 50). ^{32}P-postlabelling has the advantage that sub-milligram DNA samples are required, which provides unique advantages in biomonitoring studies of human populations (10, 39). However, it has also been suggested that this method may be prone to artefactual production as result of radiolysis of guanine residues by [^{32}P]-ATP. The main advantage of the immunoaffinity method is the specificity of the antibody for recognizing 8-OHdG in DNA, which also determines its limits in cross-reactivity of antibody with structurally related analytes present in complex mixtures (10, 39, 43, 47, 48). FPG is an enzyme which recognizes 8-OHG, as well as formamidopyrimidines (ring-opened purines resulting from oxidation). 8-OHdG amounts are determined by measuring the DNA breaks after application of FPG, which convert the oxidized base to a DNA break. This method emerged in the mid-1990s and tended to report the lowest 8-OHdG values compared with LC-ECD and GC-MS methods (10). It could detect dose response curves of 8-OHdG for HeLa cells treated with visible light in the presence of phtosensitizer (10, 37, 39). Based on ESCOD's studies, LC-MS/MS methods have not reached an acceptable standard for 8-OHdG detection with direct injection after enzyme digestion. However, because of the rapid development of sensitive LC-ESIMS/MS, several studies began to explore its application in 8-OHdG detection (40-51). Sample purification before MS detection was regarded as a key step for the detection of DNA adducts by LC-ESIMS/MS (11, 12). The enormous amounts of dT and dG in the enzymatic hydrolytes can cause ion suppression, artifact formation and poor sensitivity, especially when they co-elute with 8-OHdG from the C18 column using acetic acid or formic acid in water as phase A. Traditional C18 columns, solid phase extraction (SPE) and immunoaffinity columns were all evaluated as

purification techniques for 8-OHdG detection by LC-MS/MS (40-51). With LC purification, decent peaks of 8-OHdG were observed by LC-MS/MS and the LOQ was reach several fmol on-column in the present and other published studies (10, 34, 48, 49). However, comprehensive comparison between LC-MS/MS assay with LC-ECD with sample purification before injection is still necessary. Besides the reported artifact formation during DNA isolation by ESCODD (37), Chao et al. found the production of artifacts during vacuum drying, which was supported by other reports (44, 49-51). Their studies indicated that as a concentration process, both vacuum and freeze drying for intact or hydrolyzed DNA could introduce artifact formation. With SPE purification before column switching LC-MS/MS detection, higher 8-OHdG was observed than the one without SPE, which indicated that excessive sample processing also contributed to the formation of artifact. In our studies, the further oxidation of $[^{15}N_5]$8-OHdG in samples was observed when offline LC sample purification was applied before LC-MS/MS detection, which made the quantification of 8-OHdG even more complicated. Besides, background 8-OHdG levels, similar to other reports (48, 49) were detected in this study. These results suggest that online column switching may work well for the detection of 8-OHdG.

1, N^6-εdA and 3, N^4-εdC have been measured in numerous animal and human studies, especially in chronic inflammatory diseases, such as chronic pancreatitis and hepatitis, following development of an immunoaffinity-^{32}P-postlabeling method (2, 14, 52, 53). With low DNA amounts (~10 μg), the sensitivity of this assay can reach several adducts per 10^{10} unmodified nucleosides. The whole assay involved immunoaffinity

column purification, ^{32}P-postlabelling procedure and thin-layer chromatographic separation. The mean 1, N^6-εdA and 3, N^4-εdC levels per 10^8 parent nucleotides in normal human liver were determined to be 1.9 ± 0.5 and 2.8 ± 1.0 respectively by this assay (53). Although it has excellent sensitivity, this assay also has shortcomings. The accuracy of the measurements was not well defined due to the lack of an authentic internal standard and no chemical structural information is provided. Compared with ^{32}P-postlabelling method, mass spectrometry is a powerful technique with the particular advantage in providing the information on chemical structure of adducts. Using isotopically labeled internal standards, the entire sample processing scheme can be monitored to better assess the accuracy of the assay. Using various sample purification techniques, GC-MS/MS or LC-MS/MS has been applied to detect etheno adducts, including 1, N^6-εdA or εA, 3, N^4-εdC or εC, N^2, 3εG and 1, N^2-εdG or 1, N^2-εG (54-63). There were several steps involved in the detection of etheno adducts by GC-MS, including DNA hydrolysis, adduct derivatization, and adduct detection with selective ion monitoring (SIM) (54-57). Using GC/NICI (negative ion chemical ionization) /MS and C18 solid phase extraction, Chen et al. measured ethenoA in ctDNA. The detection limits for the entire assay could reach 6.3 fmol after derivatization by pentafluorobenzylation (PFB) (54). Ham and Morinello successfully applied the similar technique in the measurement of 1, N^2-εG and N^2, 3εG in rodent DNA. Using immunoaffinity columns as the dominant purification technique, the limit of detection was determined to be 5 N^2, 3εG adducts/10^8 normal dG nucleosides in 150 μg DNA and 15 fmol 1, N^2-εG in 250 μg DNA respectively (55, 56). Besides, they also demonstrated that neutral thermal hydrolysis and HCl hydrolysis gave higher detection limits than enzymatic hydrolysis (55). Because of the development of

electrospray interfaces and the lack of a need for chemical derivatization, LC-MS/MS has become more popular as a detection technique for etheno adducts (30, 34, 49, 57-66). Several groups have successfully developed LC-MS/MS assay for the detection of etheno adducts with diverse sample enrichment techniques (30, 34, 49, 57-66). With on-line sample processing, Churchwell et al. detected background 1, N^6-εdA and 3, N^4-εdC in commercially available ctDNA by LC-MS/MS, with the mean value of 5.8 ± 3.7 and 9.7 ± 8.2 adduct/10^8 unmodified nucleosides respectively (66). Their corresponding LOD was 10 fg and 20 fg on-column. Ham et al. applied offline immunoaffinity-LC-MS/MS in the detection of 1, N^6-εdA in mice. The mean number of adducts in ctDNA was determined to be 2.0 ± 0.9 adduct/10^8 dA (30). Chen et al. developed a LC-MS/MS assay with SPE to measure εA in human urine with LOD 2pg on-column (57). Compared with other etheno adducts, more LC-MS/MS assays have been developed for the detection of 1, N^2-εdG or 1, N^2-εG (34, 49, 58-63), which could be divided into online and offline LC-MS/MS assays. Based on the different optimal collision energy between 1, N^2-εG and N^2,3-εG in MS/MS systems (the CE of 1, N^2-εG and N^2, 3-εG was 28 and 36 mV respectively), Müller successfully developed a LC-MS/MS assay to measure 1, N^2-εG in 2-chlorooxirane treated ctDNA with HCl hydrolysis (61). With LOD 5.8 fmol on column, this assay was applied to measure 1, N^2-εG in a human study (62). Using a similar technique, Gonzalez-Reche et al. identified both 1, N^2-εG and N^2, 3-εG in human urine (63). Recently the development of microflow rate LC system and nanospray interface improved the sensitivity of LC-MS/MS; the LOD of 1, N^2-εdG could reach attomole-level in this study and others (59). As shown in figure 2.6 and 2.9B, sub-femto mole LOD was provided by this nanoUPLC-MS/MS system with nanospray interface installed.

Background 1, N^6-εdA and 1, N^2-εdG can be consistently detected. Because of the instability of the glycosyl bond in N^2, 3-εdG, the direct detection of this adduct by LC-MS/MS was impeded (34, 49, 64).

M_1G has been measured by MS, ^{32}P-postlabeling or immunochemical techniques (25-29, 67). Each technique offers advantages and disadvantages based on a combination of sensitivity and specificity. ^{32}P-Postlabeling was first employed for the detection and quantitation of M_1G (17). Increased M_1G was reported in the kidneys of Syrian hamster treated with estradiol, in white blood cells from women fed a diet high in sunflower oil and in normal breast tissues of women with breast cancer using this technique (68-70). Marnett's laboratory employed GC-MS with electron capture negative chemical ionization detection for quantitation of M_1G after immunoaffinity column purification and derivatization with pentafluorobenzyl bromide (17). The LOD of the assay was approximately 1 M_1G per 10^8 nucleotides with 1mg of DNA. Using 500 ug MDA-treated ctDNA, Hakala evaluated the application of LC-ESIMS/MS for the measurement of M_1G. Besides, several studies developed assays to measure M_1dG indirectly and directly (26-29, 67). Jeong et al. developed a novel method for analysis of M_1dG by aldehyde reactive probe (ARP) labeling and LC-MS/MS with SPE purification (28). Both ring-open and ring-closed forms of M_1dG were conjugated with a stable ARP group before detection, with an LOD of 2 M_1G adducts/10^8 guanines. Both LC-APCIMS/MS and LC-ESIMS/MS were evaluated to measure the 5, 6-dihydro derivative of M_1dG in urine after immunoaffinity and SPE column purification (26, 27). However, analysis of urine samples from control rats or rats treated with CCl_4 indicated that the levels of M_1dG were

below the LOD of LC-MS/MS system. To date, only one study reported the direct measurements of M_1dG by accelerator mass spectrometry in rat urine (29). In our studies, because of the high sensitivity of nanoLC-MS/MS system, M_1dG was detected directly after LC purification in control and chemically treated animal samples, as shown in figure 2.9D, 2.10 and table 2.1. Considering the acid stability of M_1dG (23, 24), 0.1% formic acid in water was used as phase A of LC purification and the reconstituting the solution after drying by speed vacuum. The background M_1dG in control mice liver DNA was 4.9 $\pm$ 0.82 and 6.3 $\pm$ 1.8 adduct/10^8 dG, which was comparable to the background level in control rat liver determined by Jeong (28). However, further comparison between these two assays is still necessary.

^{32}P-Postlabeling with LC purification was first developed for the detection of propano adducts (71). The whole process included four steps of LC purification, the postlabeling and enzymatic digestion steps with 300 μg DNA. Using this assay, Chung et al. successfully measured AcrdG and CrdG in the tissues of carcinogen-treated rodents and background lesions in DNA from humans and untreated rodents. Different patterns of adduct distribution were found in different organs of rodents (71, 72). The average AcrdG and CrdG values were from 0.55 to 3.7 adduct/10^7 guanine, and from 0.09 to 3.97 adduct/10^7 guanine in control rats respectively. Because of incomplete separation of some AcrdG isomers from normal bases, only some of them were monitored in their studies. A similar assay was developed by Wacker et al. to measure 4-HNEdG (18). After enzymatic hydrolysis, the sample was subsequently reacted with [γ-^{32}P] ATP to give the respective 3'-5'-bisphosphates, which were two-directionally separated on TLC and

quantitated by autoradiography. The concentration of adducts varied from not detected to 158 adduct/10^9 normal nucleotides in control rats (18). However, ^{32}P-postlabeling is labor intensive and low-throughput, requires handling large quantities of radioactivity and lacking information on chemical structure (73). These disadvantages indirectly promote the application of LC-ESIMS/MS techniques in the detection of these adducts (19-22), especially, with the advent of the low flow rate LC and electrospray interfaces of LC-MS/MS, which provid outstanding sensitivity (low-femotomole to attomole detection limits) for the measurement of DNA adducts. By capillary LC-ESIMS/MS with SPE purification, AcrdG, CrdG and 4-HNEdG were successfully measured in 0.1~1.5mg DNA of human brain, liver and lung tissues (19-22). Although large quantity of DNA was used, it was still difficult to detect the endogenous CrdG in some samples (20). The complete separation of AcrdG and unmodified nucleosides was still a problem, although multiple enzyme systems or SPE columns were evaluated for its detection (19). Using up to 1mg DNA and long incubation time (overnight) for enzymatic digestion, Zhang et al. measured different isomers of AcrdG in human lung samples. However, whether long time or high temperature incubation could induce artifact formation was not investigated (19). Similar problems exist for the assay developed by Liu for measurement of 4-HNEdG (21). In our studies, 2-hour incubation at 37°C was applied instead of 100°C (19) for a half hour or 37°C for 24 hours (21). Besides, in order to get complete separation of AcrdG from unmodified nucleosides, 0.1% formic acid was added into 5mM ammonium formate aqueous solution as phase A. With slightly acid (pH = 3~4) mobile phase, all peaks of AcrdG isomers merged into a single peak and the baseline separation of AcrdG with unmodified nucleosides was achieved. Moreover, recovery studies found that similar

results were obtained when acid mobile phase was applied rather than neutral phase (5mM ammonium acetate in water) using pure AcrdG standards. Considering the lipophilicity of CrdG and 4-HNEdG, these two adducts were processed together using 10mM ammonium acetate in water as phase A with a short LC gradient program. By nanoLC-MS/MS, the mean number of AcrdG, CrdG and 4-HNEdG adducts was $7.06 \pm 2.12/10^8$ dG, $1.79 \pm 0.34/10^9$ dG and $2.70 \pm 0.96/10^9$ dG respectively in 0.45 ppm DBP treated trout liver, as shown in figure 2.10. No significant difference of these adducts was found between 0.45 ppm and 80 ppm DBP exposed samples, which can be explained by the possible limited LPO induced by DBP.

Although a variety of methods have been developed for the detection and quantitation of ROS-induced DNA adducts, most of them focused solely on measurement of one or two DNA adducts. Biochemical studies have validated the diverse pathways of formation, repair and mutagenic potential of different adducts, which supports the necessity of developing a battery assays to monitor multiple DNA adducts to better understand the roles of oxidative DNA adducts in risk assessment of chemical carcinogenesis. The initial exploration was made by Doerge et al. using LC-ESIMS/MS in 1998, which discussed the feasibility of LC-ESIMS/MS application for simultaneously measuring multiple LPO-induced adducts (65). Both nucleosides and nucleotides were tested on that system under full-scan and MRM modes, but no real samples were evaluated. Recently several studies were reported for simultaneously detecting multiple DNA adducts by LC-ESIMS/MS (34, 35, 49, 58, 59, 65, 66, 74-80). Offline LC (34, 49), immunoaffinity column (66), solid phase extraction purification (76, 77, 80), online

column-switching techniques (35, 65, 66, 75) and direct injection without any pre-purification (74, 78, 79) were all evaluated for the detection of DNA adducts by LC-ESIMS/MS. One of the dominant problems for assay development of DNA adducts is efficient sample enrichment before adduct detection. With online column-switching technique, Doerge et al. developed an assay to detect 3, N^4-εdC, 1, N^6-εdA, M_1dG and 8-OHdG in 100 μg DNA simultaneously, but the interferences, which built up on the chromatographic columns, caused problems for adduct detections (66). Using similar techniques, Singh et al. developed an assay to simultaneously detect 8-OHdG and 8-OHdA in DNA (75). It was found that the separation of 8-OHdA and dG, 8-OHdG and dG or dT was crucial for avoiding artifact formation and 8-OHdG or 8-OHdA detection (75). Considering the special technique requirement and potential contamination with online column purification, some investigators applied offline LC or SPE purification (35, 49, 76, 77, 80). Using neutral thermal or enzymatic hydrolysis of ethylene oxide (EO) treated DNA after offline LC purification, five different 2-hydroxyethyl-DNA adducts formed by ethylene oxide exposure were detected by LC-ESIMS/MS under MRM mode (76). With limits of detection in the range of 0.5 to 25 fmol for each individual adduct, this study illustrated the feasibility of this assay in the detection of endogenous/background EO-induced adducts in a variety of DNA samples. Nevertheless, artifactual peaks for N1-HEdA and O^6-HEdG were observed in this study, which probably originated from cross-contamination with different fractions of the LC purification. Taghizadeh et al. successfully measured ROS-induced DNA adducts in rodents after collecting them individually on offline LC columns (49). Besides MRM, neutral loss monitor (NLM) was also applied to simultaneously measure multiple

endogenous DNA adducts (78-80). However, without isotopically labeled internal standards, the robustness and reliability of the assay is uncertain. The ^{32}P-postlabeling method was another technique evaluated for simultaneous quantification of exocyclic DNA adducts (15, 16). After LC purification to remove unmodified nucleotides, DNA adducts were labeled and detected. But unlike immunoaffinity purification, residual levels of unmodified bases in the samples were hundreds of times higher than the target DNA adducts after purification by C18 or SPE columns, which have the capacity for removing 99.9% of unmodified bases from the samples. Without relevant chemical structure information, ^{32}P-postlabeling needs to be further validated. In the present study, different enzymatic hydrolysis, LC purification and LC-MS/MS methods were evaluated for the detection of ROS-induced DNA adducts based on the special physiochemical properties of different DNA adducts. Considering the stability and sample purification of M$_1$dG, 1, N^2-εdG and AcrdG in acid mobile phase, these three adducts were combined to process together. The lipophilicity of CrdG and 4-HNEdG made it feasible to simultaneously purify them as well. Due to the artifact formation during sample processing for 8-OHdG detection and background interference for 1, N^6-εdA detection, these two adducts were processed individually.

The major problem encountered in DNA adduct detection was the detection, structural identification and quantitation in animal and human samples where they are present in only minute amounts (1 modified base out of 10^6-10^{11} parent bases). Therefore, sensitive detection techniques become another key factor for successful assay development. Since the introduction MS in this area, several excellent reviews have been

written (11, 12, 81, 82, 37). Chiarelli et al. first comprehensively reviewed the application of mass spectrometry in the analysis of carcinogen-DNA adducts in the form of modified nucleosides in 1992 (81). As a technique capable of elucidating the chemical structure of DNA adducts, mass spectrometry provided an incomparable advantage over the other techniques, such as providing specific chemical structure proof for validating the existence of DNA adducts *in vivo*. However, because of the limited development of the sensitivity and hyphenated techniques for chromatography and mass spectrometers, very few adducts could be measured from *in vivo* sources (81, 82). GC-MS was the dominant MS technique for quantitative analysis of DNA adducts in biological samples after chemical derivatization during that period (81, 82). With the development of ESI interfaces and their successful application in LC-MS/MS, LC-ESIMS/MS became a dominant technique for the detection of DNA adducts since 1990's (12, 13). Without a requirement for chemical derivatization and providing enough sensitivity for the detection of trace level DNA adducts, this technique has been applied in the measurement of various carcinogens induced DNA adducts, such as alkylating agents, aromatic carcinogens, arylamine carcinogens and even endogenous oxidative carcinogens (12, 13). Because the best performance of ESI interface was found at flow-rate around 0.5-5μl min^{-1}, recent studies began to explore the application of micro- or nanobore LC columns in the detection DNA adducts combined with micro- or nanoESI interfaces (19-22, 59, 77, 83-85). The advantages of low flow-rate LCMS systems over the traditional ones have been studied by several groups (85-87). Vanhoutte and his colleagues found that the mass sensitivity could be improved by a factor of 3300 by using nanoflow ES LC/MS, compared with conventional ES LC/MS system by analyzing a reaction mixture of 2´-

deoxyguanosine 5´-monophosphate with bisphenol A diglycidyl ether (86). Hoes et al. reported that the absolute detection limit could be improved by a factor 10 using nano LC-MS than capillary LC-MS (87). Furthermore, several studies reported the application of capillary or nanoLC-ESIMS/MS in detection of various DNA adducts (59, 77, 83-85). Embrechts et al. reported that the LOD of equilenin-2´-deoxyguanosine adduct could reach 197 fg in MRM mode by nanoLC-ESIMS/MS (83). Additionally, Chen and his colleagues developed a sensitive assay for detection of crotonaldehyde and acrolein derived dG adducts by nanoLC-ESIMS/MS after SPE extraction of enzyme digestion hydrolytes, which was successfully applied to the samples from human placenta and leukocytes (77). All these studies supported the potential application of nanoLC-MS/MS in the detection of trace level DNA adducts in animal or human samples.

100~200ug DNA

Enzymatic
hydrolysis   *optimization I*

Nucleosides+DNA adducts

Microcon 10 filtration

Concentration by
speedvacuum

T3 HPLC column   *optimization II*
purification

Concentrate the fraction containing the
target DNA adducts by speedvacuum

LCMSMS detection method   *optimization III*
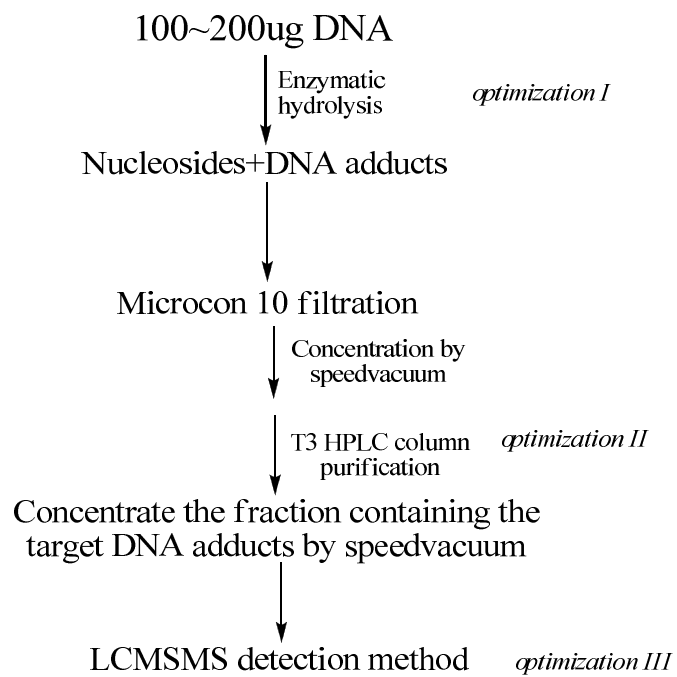
Figure 2.1 Mass spectrometry scheme for analysis of DNA adducts

Student t-test, ns, no significant difference was found between these two groups, p>0.05, *n*=3
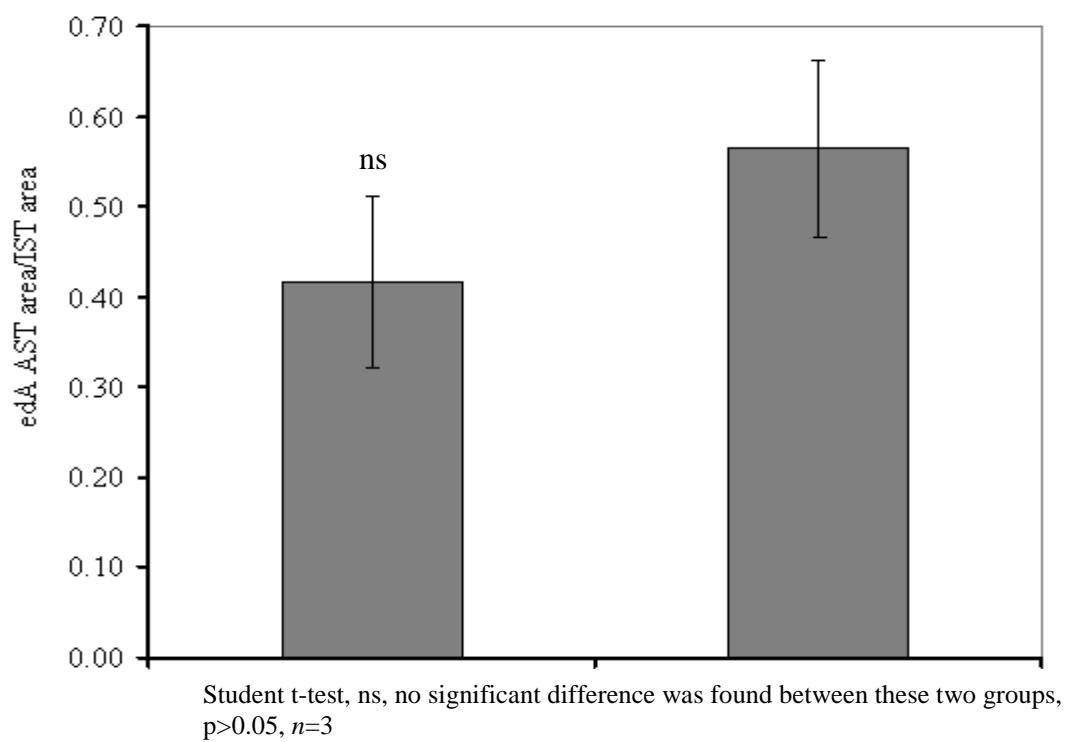
Figure 2.2 1, N^6-εdA level using the orginal method B (PD) and the optimized method B (OPD)
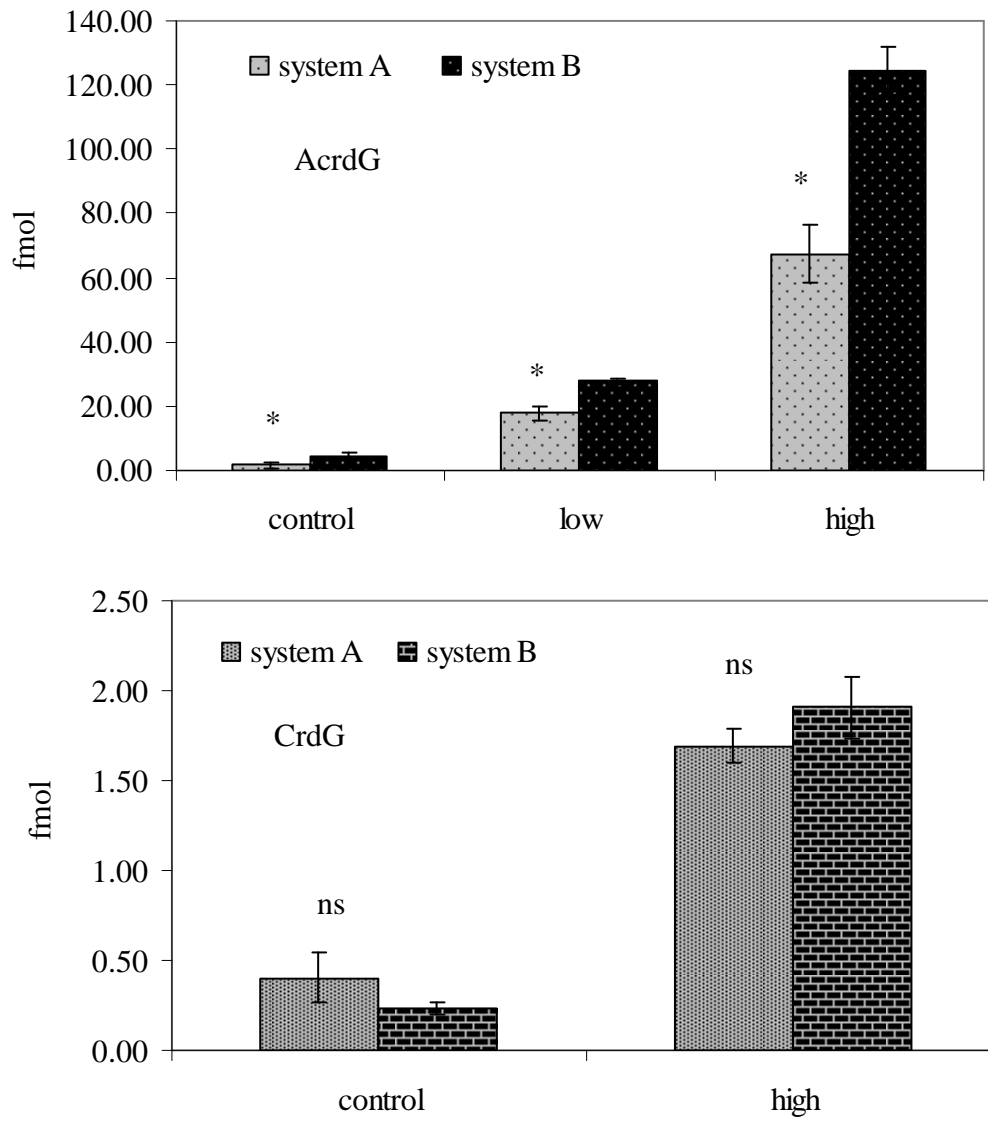
Student t-test, ns, no significant difference between system A and B; *, significant difference between system A and B, p<0.05, *n*=4; ns, no significant differerence between system A and B

Figure 2.3 The optimization of enzyme digestion for the measurement of AcrdG and CrdG
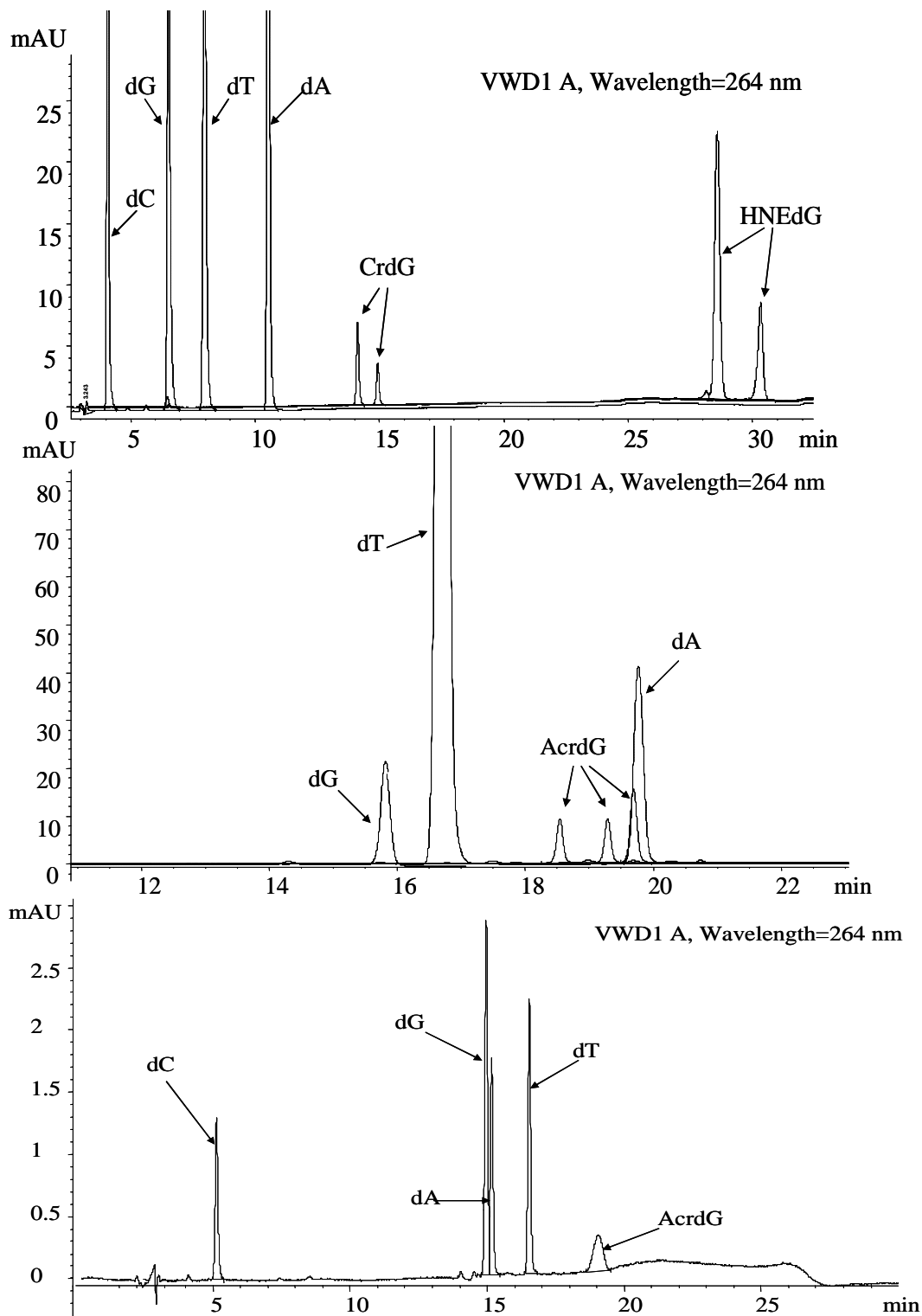
Figure 2.4 The typical chromatograms for LC purification of CrdG, 4-HNEdG, AcrdG and 1, N^2-εdG by UV detector
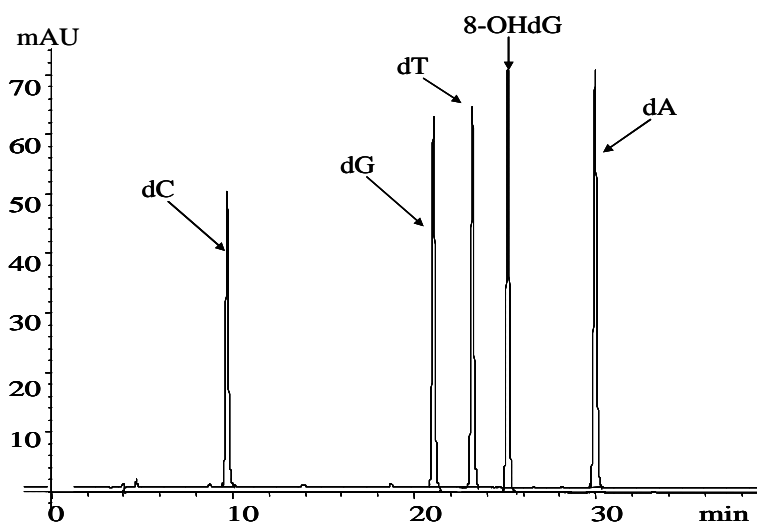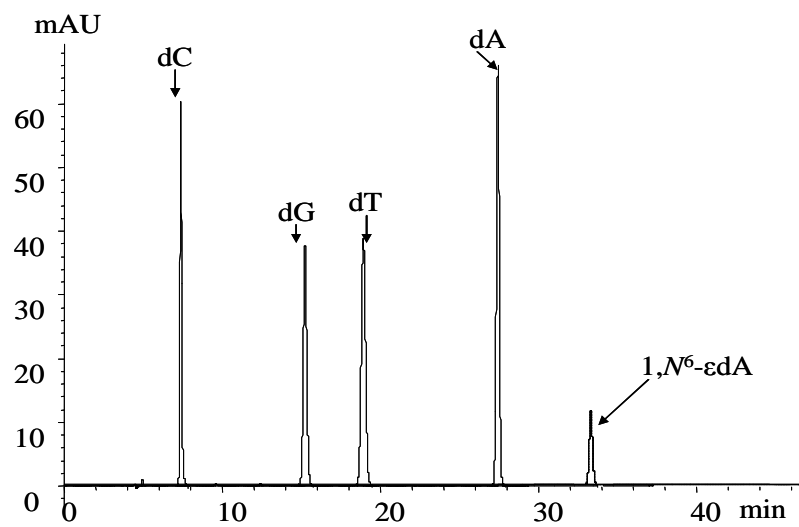
Figure 2.5 The chromatogram of LC purification for 1,N^6-εdA and 8-OHdG by UV detector
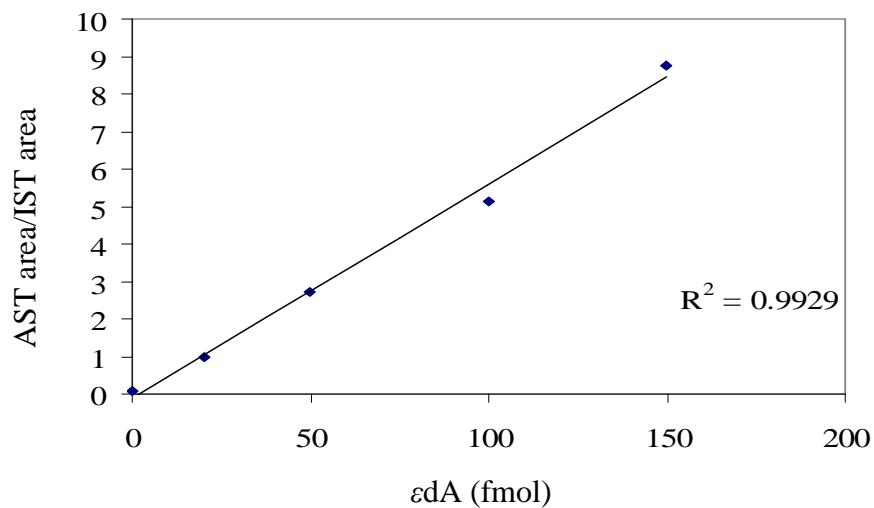
A: LC-MS/MS calibration curve for 1, N^6-εdA obtained using ctDNA with spiked εdA , n=3
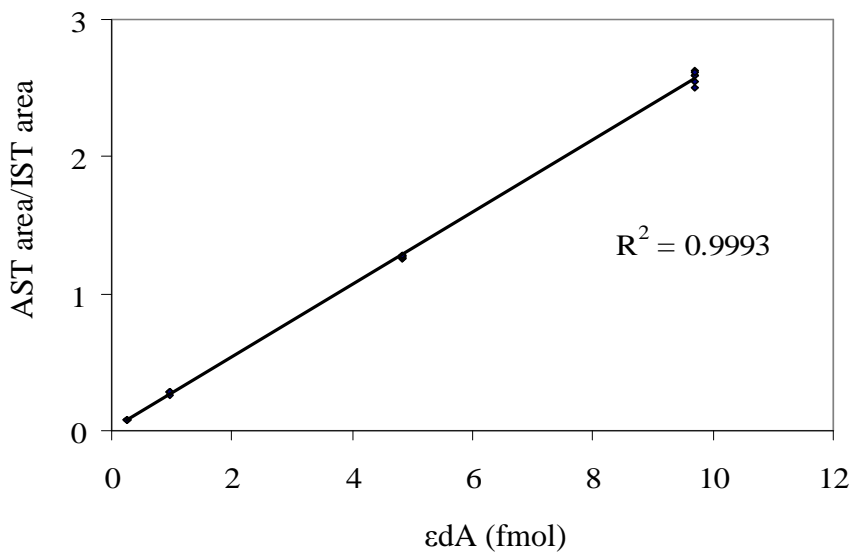
B: nanoLC-MS/MS calibration curve and reproducibility for 1, N^6-εdA, n=6

Figure 2.6 Assay validations for 1, N^6-εdA measurement
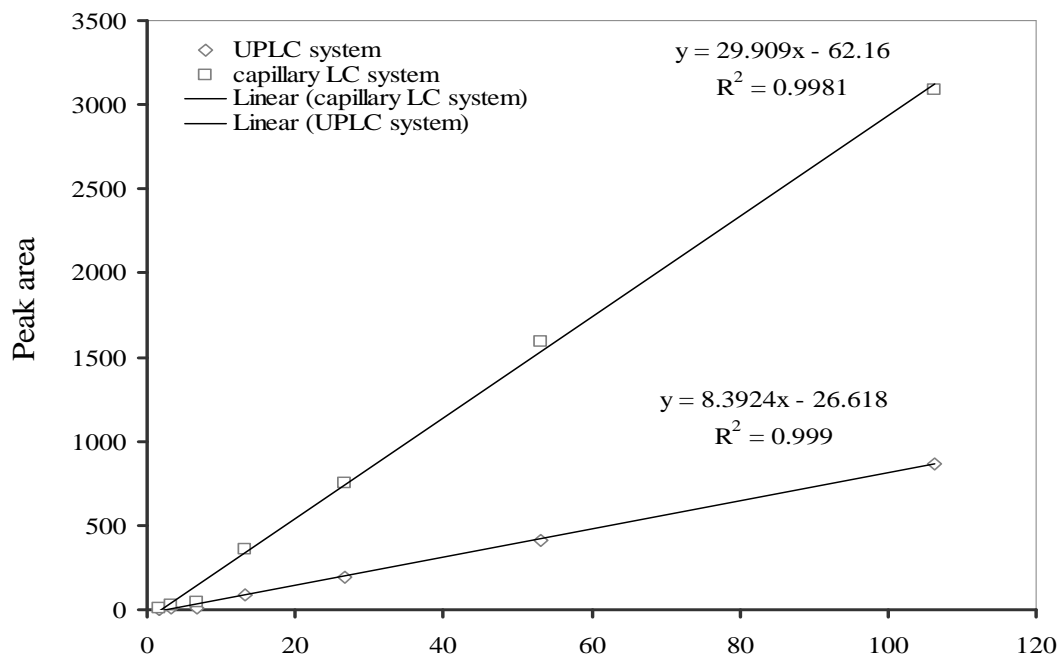
A: Comparison between UPLC-MS/MS and capillary LC-MS/MS
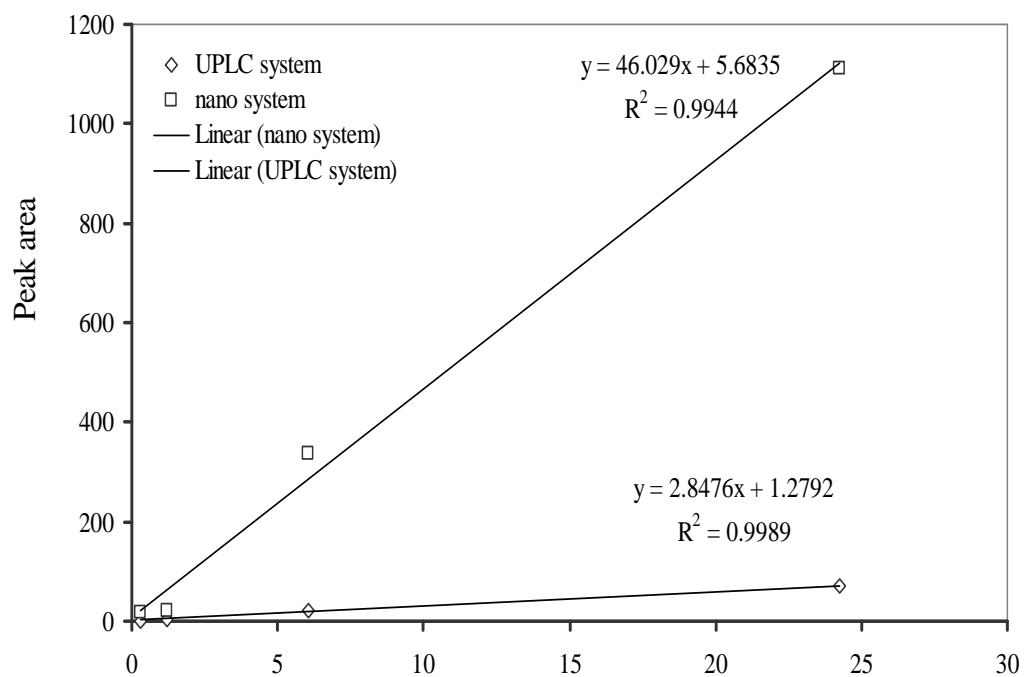
B: comparison between UPLC-MS/MS and nanoUPLC-MS/MS

Figure 2.7 Instrument sensitivity comparisons for 1, N^6-εdA detection
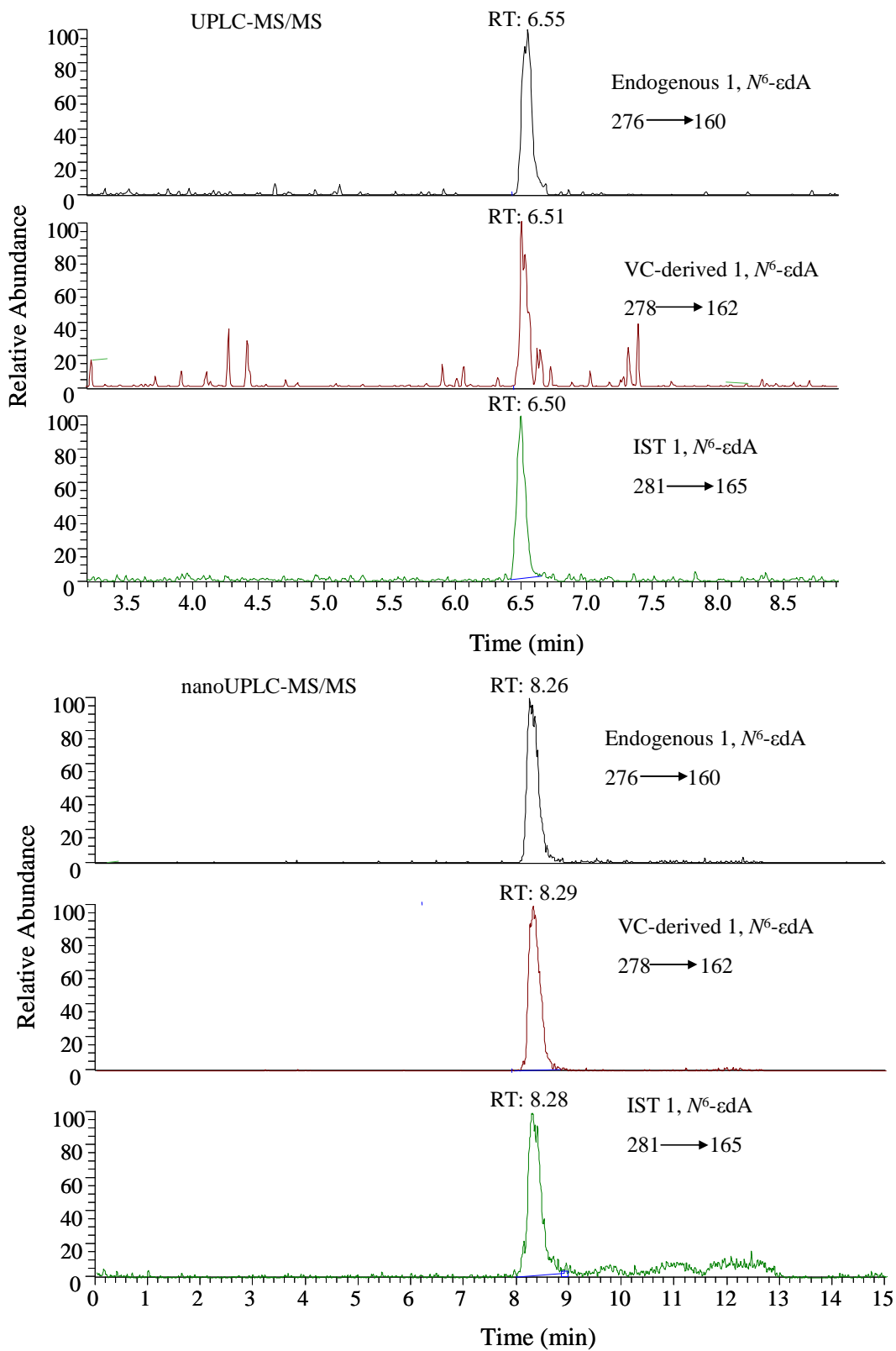
Figure 2.8 Instrument sensitivity comparisons for 1, N^6-εdA detection ($^{13}C_2$)-VC exposed adult rat liver DNA (UPLC vs nano)
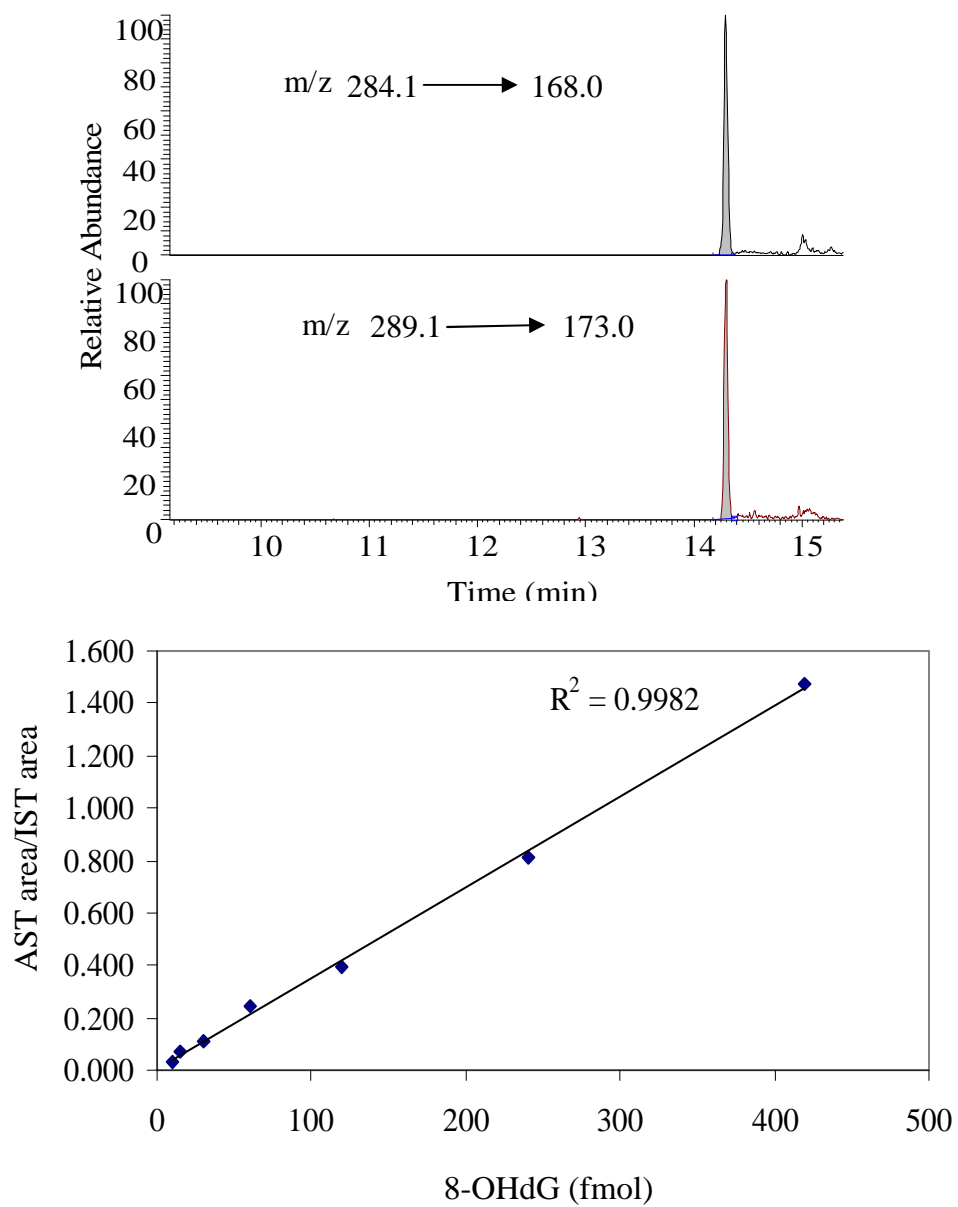
Figure 2.9A The typical chromatogram for ctDNA samples and calibration curve for 8-OHdG by UPLC-MS/MS
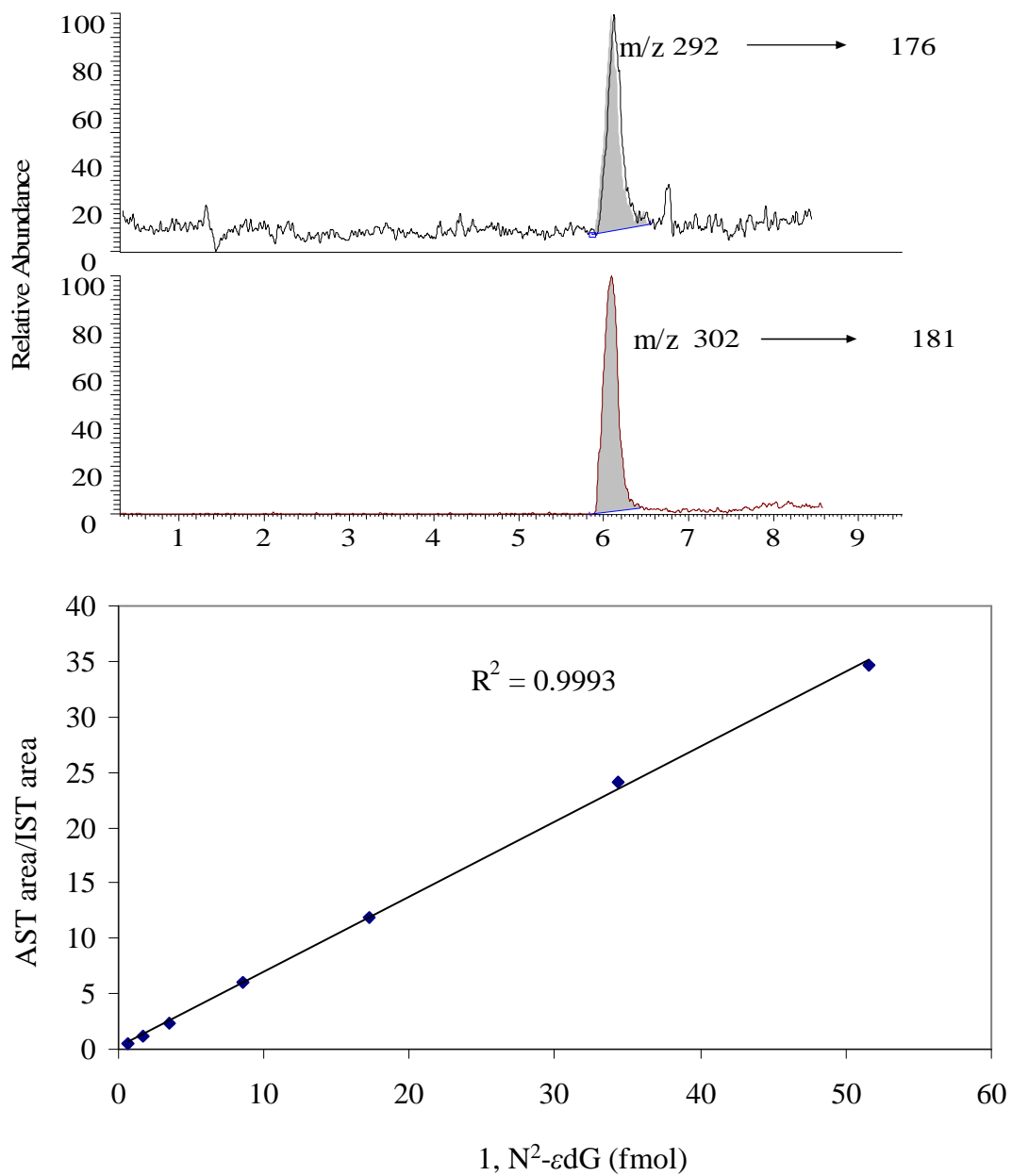
Figure 2.9B The typical chromatogram for 1, N^2-εdG in ctDNA and calibration curve by nanoUPLC-MS/MS
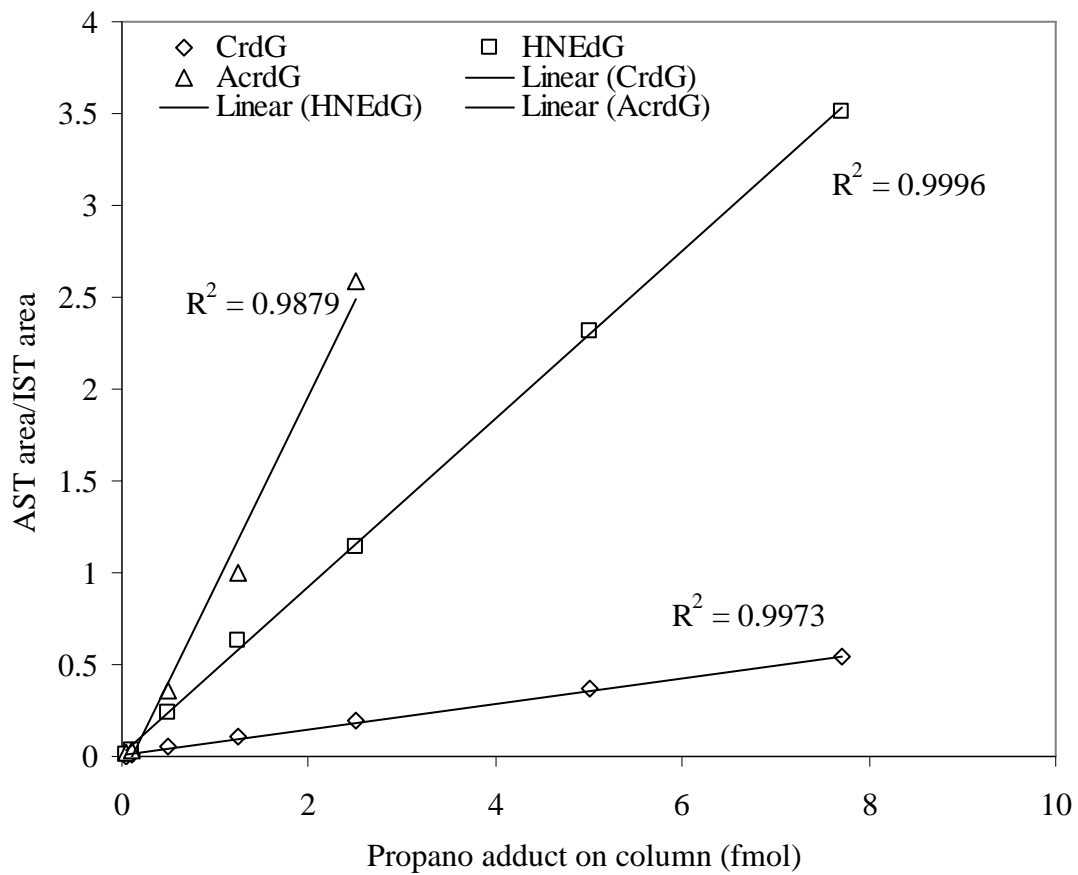
Figure 2.9C The calibration curves of propano adducts by nanoLC-MS/MS
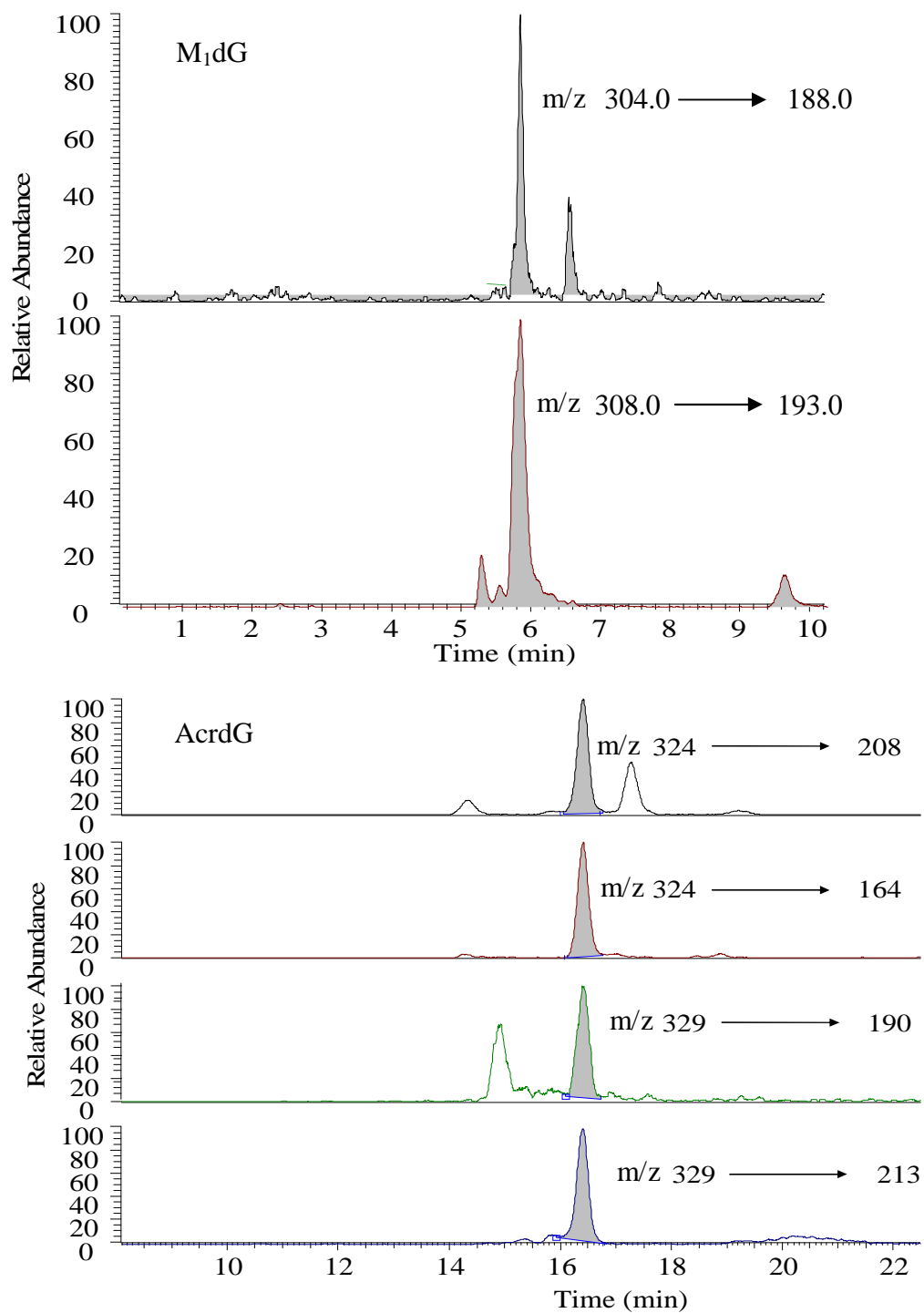
Figure 2.9D The typical chromatogram for M₁dG and AcrdG in ctDNA by nanoUPLC-MS/MS
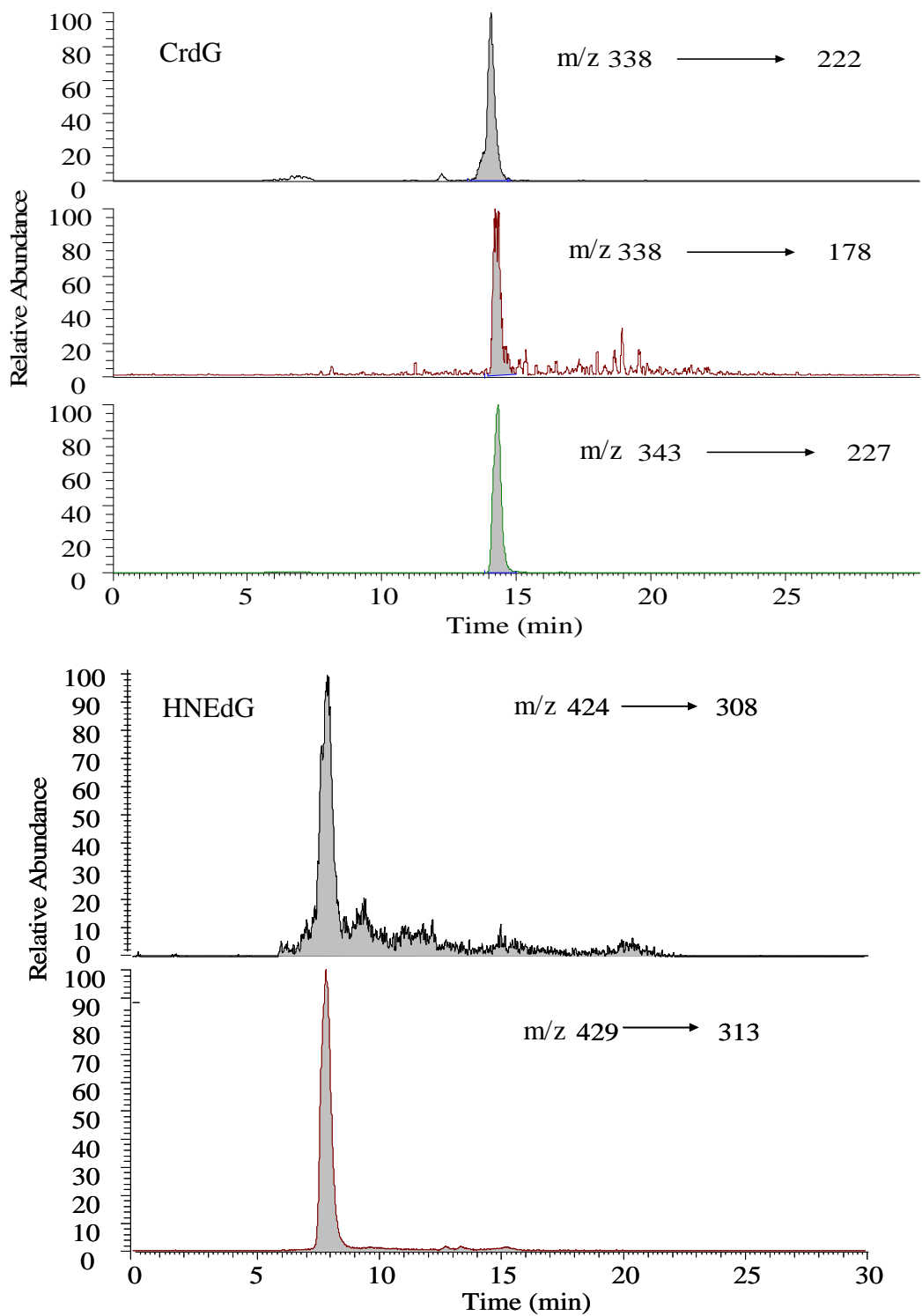
Figure 2.9E The typical chromatogram for CrdG and HNEdG in ctDNA by nanoUPLC-MS/MS

Table 2.1 ROS-induced DNA adducts in control liver DNA of adult female *Sprague-Dawley* rats (61 weeks old)

DNA adduct	8-OHdG $(1/10^6 dG)$	1, N^6-εdA $(1/10^8 dA)$	1, N^2-εdG $(1/10^8 dG)$	$M_1 dG$ $(1/10^8 dG)$	AcrdG $(1/10^7 dG)$	CrdG $(1/10^9 dG)$	4-HNEdG $(1/10^9 dG)$
n	22	31	10	5	9	9	5
Median	4.6±1.7	1.3±0.69	3.0±1.3	4.7±2.2	1.2±0.4	2.4±0.8	9.0±2.2
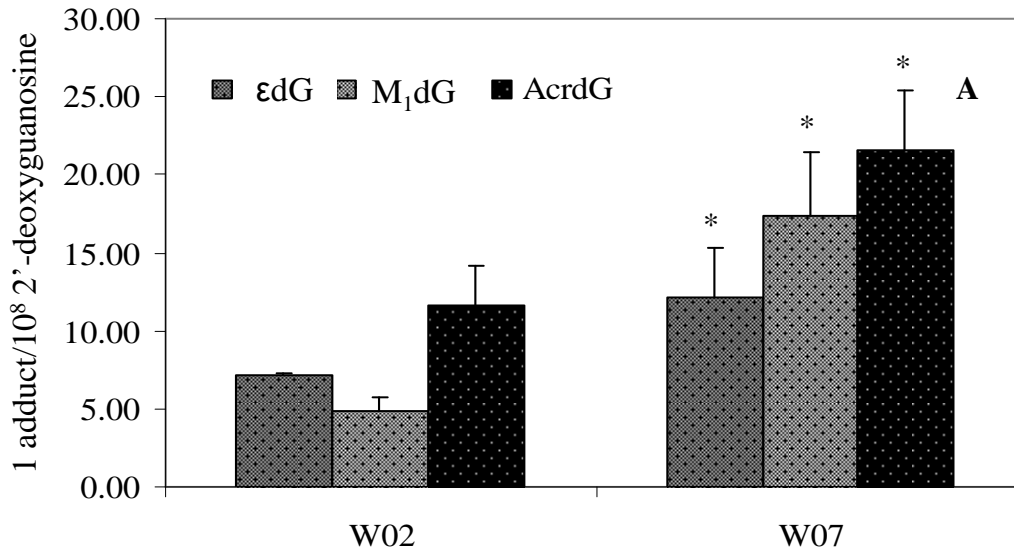
W02: control wild type mice; W07: wild type mice exposed to CCl₄

2.10A. 1, N^2-εdG, M₁dG and AcrdG in wild type C57BL/6J mice exposed to CCl₄ (1600 mg/kg in olive oil) or olive oil (10 ml/kg) by intraperitoneal injection for 13 days
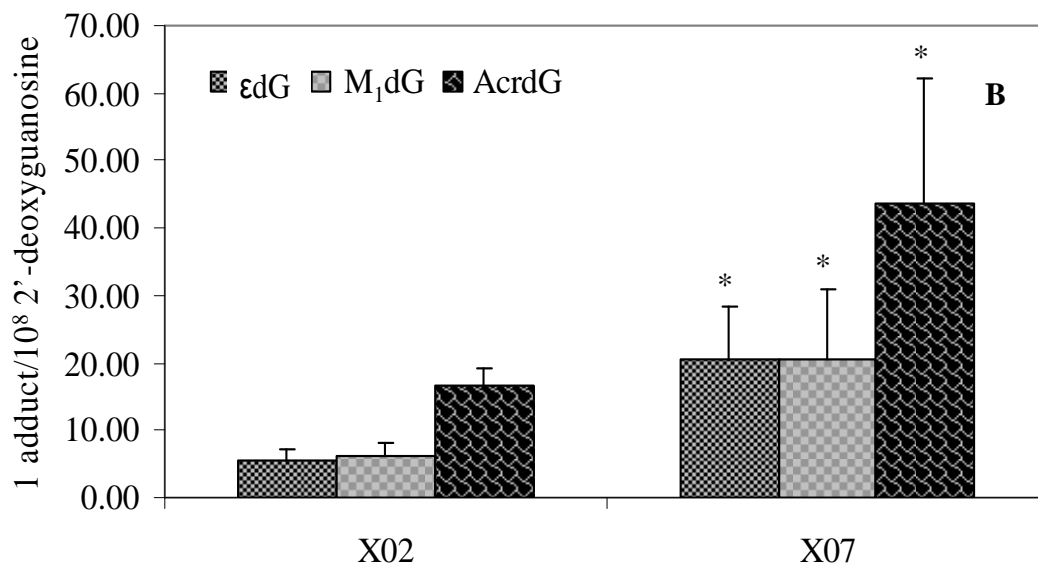
X02: control XPA⁻ᐟ⁻ mice (olive oil 10 ml/kg); X07: XPA-/- mice exposed to CCl₄ 1600 mg/kg for 13 days

;

2.10B. 1, N^2-εdG, M₁dG and AcrdG in XPA⁻ᐟ⁻ C57BL/6J mice exposed to CCl₄ 1600 mg/kg in olive oil by intraperitoneal injection for 13 days
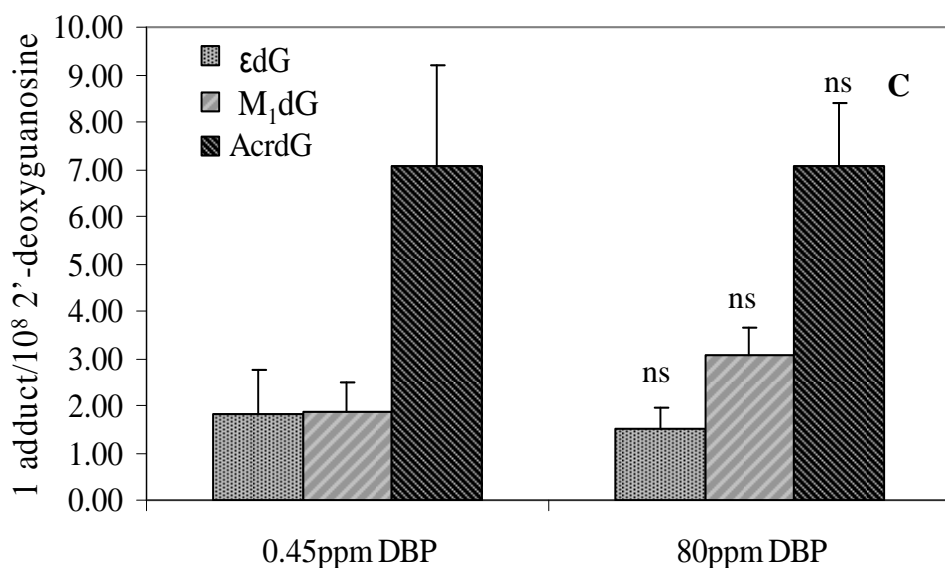
2.10C 1, N^2-εdG, M_1dG and AcrdG in the liver of Shasta strain rainbow trout exposed to dibenzo[*a,l*]pyrene (DBP) for 29 days
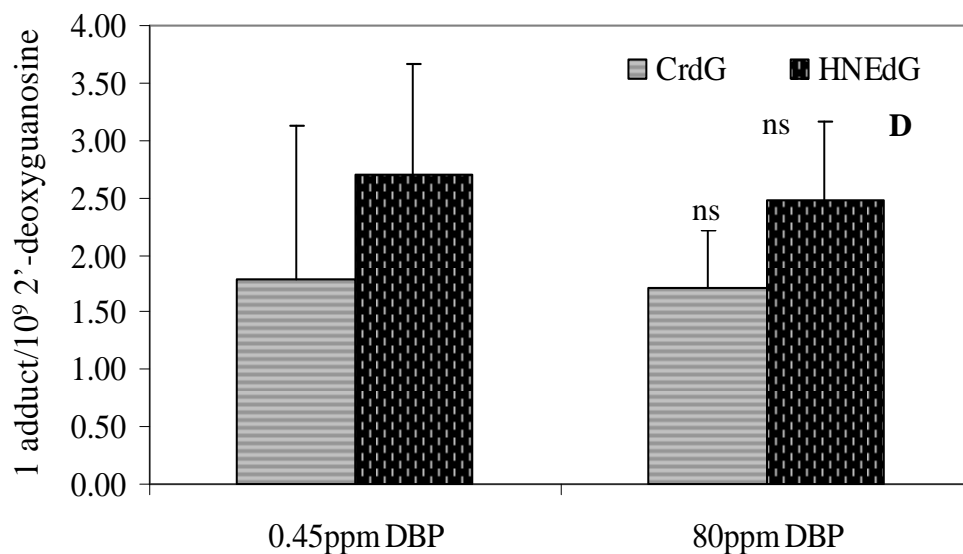
2.10D CrdG and HNEdG in the liver of Shasta strain rainbow trout exposed to dibenzo[*a,l*]pyrene (DBP) for 29 days

p values (two-sample t-test) for comparison between treated and control groups, *, p<0.05, ns, no significance difference, p>0.05, *n*=3~5

Figure 2.10 1, N^2-εdG, M_1dG, AcrdG, CrdG and HNEdG in carbon tetrachloride treated mice liver DNA or dibenzo[*a,l*]pyrene (DBP) treated trout liver DNA

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CHAPTER III

ENDOGENOUS AND EXOGENOUS 1, N^6-ETHENO-2´-DEOXYADENOSINE IN WEANLING AND ADULT SPRAGUE-DAWLEY RATS EXPOSED TO VINYL CHLORIDE BY INHALATION

A. INTRODUCTION

Vinyl chloride (VC) is a colorless organic gas used almost exclusively by the plastic industry to produce polyvinyl chloride and copolymers, which are ubiquitous materials in many consumer and industrial products. As one of the highest volume chemicals produced in the United States, the annual US production of VC increased from approximately 6 to 9 billion pounds in the late 1970s and to approximately 15 billion pounds in the mid 1990s (1). Among chemicals produced in the United States, VC ranked 18th in production volume in 1995 (1). The reported production capacity for vinyl chloride was 17.6 billion pounds (8.0 billion kilograms) in 2000 (1). Besides anthropogenic sources, VC is also found in landfill leachates and in groundwater of sites polluted with halogenated hydrocarbons (2).

VC is a known human carcinogen with its primary target being hepatic endothelial cells, resulting in the induction of angiosarcoma (2, 3). As a multisite carcinogen, this chemical has also induced mammary gland carcinomas, nephroblastomas, lung adenomas,

and esthesioneuroblastomas in animal studies (3). VC is predominantly metabolized by cytochrome P450 2E1 to chloroetheylene oxide. This reactive compound is a highly unstable epoxide that reacts with DNA and is thought to be the ultimate mutagen and carcinogen (4). VC is mutagenic in several systems (5), causing base-pair substitution mutations, which result from exocyclic DNA adducts (6, 7). Several mutations in key oncogenes and tumor suppressor genes have been reported in neoplasms of humans and animals exposed to VC. The mutations were consistent with the promutagenic properties of the VC-induced DNA adducts (8~14). Several DNA adducts are formed by VC, including the dominant adduct, 7-oxoethyl-guanine (7OEG), and the minor adducts, 1, N^6-ethenodeoxyadenosine (1, N^6-εdA), N^2, 3-ethenoguanine (N^2, 3-εG) and 3, N^4-ethenodeoxycytidine *(15-18)*. These adducts can also be formed by lipid peroxidation induced by oxidative stress, which contributes to their endogenous presence (9-22). Several lipid peroxidation products play an important role in this process, including trans-4-hydroxy-2-nonenal.

The connection between 1, N^6-εA and the genotoxic effects of VC was first proposed by Barbin et al. in 1975 (15). He found that εA was formed *in vitro* by reaction of VC and mouse liver microsomes with adenosine. In the following years, several rodent studies (16~18) confirmed the importance of this adduct for VC genotoxic effects. The promutagenic properties and the repair pathways for this adduct were also widely studied in order to better evaluate the role of this DNA adduct on the mutagenic effects of VC *in vivo* (23~30). As one of the promutagenic etheno nucleosides, 1, N^6-εdA can induce A→G transitions, as well as A→T and A→C tranversions. Similar genetic changes were

found in p53 and H-ras genes in animal and human angiosarcoma DNA arising from VC exposure (7~9, 11). It was also discovered that more than one DNA repair enzyme was capable of removing such DNA lesions in different organisms (31~36). Alkyl-N-purine DNA glycosylase, was thought to be the primary repair pathway, however, it was reported recently that *E. coli* mismatch-specific uracil-DNA glycosylase and AlkB can also remove 1, N^6-εdA with efficiency (34~36).

With the development of ultrasensitive detection methods of 1, N^6-εdA, more molecular dosimetry studies have appeared (18, 37~42). Çiroussel et al. (37) determined 1, N^6-εdA concentrations in hepatic DNA of seven-day-old and 13-week-old BD VI rats exposed by inhalation to 500 ppm VC using HPLC and competitive radioimmunoassay. The limit of detection for this adduct was 3×10^{-8} 1, N^6-εdA/dA (± 15%, SD; n=27). It was observed that 1, N^6-εdA adducts in liver were not significantly higher than those in lung and the brain. Guichard et al. (38) developed a ^{32}P-postlabeling method to detect 1, N^6-εdA in VC exposed adult *Sprague-Dawley* rats. An exposure-dependent increase of 1, N^6-εdA was observed in liver DNA of these animals after 8 weeks of exposure. But this trend was not observed in lung, kidney nor circulating lymphocytes. Although accumulation of 1, N^6-εdA in liver DNA of VC-exposed preweanling *Sprague-Dawley* rats was found (39), it was also observed that liver, the target organ of VC carcinogenesis, did not show higher amounts than other tissues in the exposed rodents (7). Although a slight decrease (from 2.1 to 1.4 mol/10^7 mol of parent base) of 1, N^6-εdA was observed in liver DNA of preweanling rats 3 days after exposure to VC, the adduct remained ~1 mol/10^7 mol of parent base over the following two weeks. Because of the existence of endogenous 1, N^6-εdA, it is difficult to study exposure-related adducts.

The primary focus of this chapter is on the relationship between endogenously formed 1, N^6-εdA and exogenous 1, N^6-εdA arising from inhalation exposure to VC, as well as accurately determining its rate of repair in rats exposed to ($^{13}C_2$)-VC. In order to evaluate the formation of endogenous and exogenous 1, N^6-εdA in rodents after VC exposure, an inhalation rat study was designed that utilized exposure to stable isotope labeled ($^{13}C_2$)-VC, as shown in figure 3.1. In this study, a sensitive nanoUPLC-MS/MS assay was developed to detect both endogenous NA- and exogenous ($^{13}C_2$)-1, N^6-εdA in the genomic DNA. Adult and weanling *Sprague-Dawley* rats were exposed to 1100 ppm ($^{13}C_2$)-VC for 1 week (6h/day, 5 days/week). The weanling animals were sacrificed immediately after the last exposure. The adults were sacrificed immediately, 2, 4, or 8 weeks after exposure to evaluate the persistence of ($^{13}C_2$)-1, N^6-εdA. Both age and organ specificity for 1, N^6-εdA metabolism was evaluated in VC exposed rats.

B. MATERIALS AND METHODS

Materials

VC (99% chemical purity) was purchased from Supelco (Bellefonte, PA). ($^{13}C_2$)-VC (98% chemical purity; 99% isotopic purity) was obtained from Cambridge Isotope Laboratories (Andover, MA). Nucleic acid purification grade lysis buffer, protein precipitation solution and Proteinase K were purchased from Gentra Systems (Minneapolis, MN). Other chemical reagents including 1, N^6-εdA standards were from Sigma-Aldrich Chemical Company (St Louis, MO) and used, without further purification.

HPLC grade water, methanol and other similar chemical solutions were purchased from ThermoFisher Scientific Company (Raleigh, NC). (^{15}N$_5$)-1, N^6-εdA standard was synthesized and characterized as described by Ham et al. (32).

Animal exposures

The detailed animal protocols were described in Mutlu's studies (56). The animal exposure studies were conducted at the US Environmental Protection Agency, in Research Triangle Park, NC, and approved by their Institutional Animal Cares and Use Committee. The experimental design is shown in figure 3.1. For the adult studies, a total of 16 adult *Sprague-Dawley* male rats (10 weeks old) were exposed to 1100 ppm (^{13}C$_2$)-VC. Adult rats were sacrificed immediately, 2, 4 and 8 weeks after 1-week (6 hours/day, 5 days) (^{13}C$_2$)-VC exposure. An additional four adult *Sprague-Dawley* male rats were exposed to 1100 ppm unlabeled vinyl chloride for 1 day (6 hours/day) to test the initial exposure conditions. A total of 8 weanling rats *Sprague-Dawley* male (21 day old) also were exposed to 1100 ppm (^{13}C$_2$)-VC for one week (6 hours/day, 5 days) and sacrificed immediately after exposure. In addition, eight weanling rats were exposed to 1100 ppm unlabeled vinyl chloride for 1 day (6 hours/day).

DNA isolation

Approximately 500 mg of tissue was thawed in 6 ml PBS solution, TEMPO, a radical scavenger was added to all solutions prior to homogenization using a Tehran homogenizer (Wheaton Instruments, Millville, NJ). After centrifugation at 1000g for 10

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min, the nuclear pellet was resuspended in 5 ml cell lysis buffer. Proteinase K (50 U) was added to the samples and incubated overnight at 4˚C. Protein precipitation solution (2 ml) was added to remove the protein, and the supernatant was decanted to a new tube. Isopropanol (6 ml) was added to these samples, followed by gentle mixing by inversion. The DNA precipitated and was pelleted by centrifuging at 2000g for 5min at 4 ˚C. The DNA was washed with 70% ice cold ethanol. The DNA was reconstituted in 1ml ice cold PBS. An RNAase mixture containing RNAase T1 and RNAase A was added to these samples followed by incubation for 30 min at 37 ˚C. The DNA was rinsed with 100% ethanol, then 70% ethanol. Finally the DNA was resuspended in HPLC grade water and quantitated by UV spectrophotometry.

1, N^6-εdA measurement in isolated DNA

The procedure consisted of enzymatic hydrolysis, HPLC purification and LC-MS/MS detection, as described in chapter 2. Briefly, the DNA in sodium acetate buffer (pH 5.6) underwent enzyme hydrolysis by the method of Pang et al. (40) with minor modification. After the addition of Nuclease P1 to 100 μg DNA and incubation at 37 ˚C for 1 h, the solution pH was adjusted with sodium acetate buffer (pH 8.1) and alkaline phosphatase (AP) and phosphodiesterase (PDE) was added with additional 1h incubation. Enzymes were removed by Microcon 10 filtration and the solvent was removed by vacuum evaporation. Samples were reconstituted in HPLC-grade water and stored at -80 ˚C until clean-up by C18 column on an Agilent 1200 Series analytical fraction collector system. For sample purification, all nucleosides were monitored at 264 nm. The relevant chromatography parameters were described in chapter 2. The column temperature was

kept as 30°C. The quantitative analysis of 1, N^6-εdA was performed using a 10 kpsi Nanoacquity Ultra Performance LC system (Waters, Milford, MA) coupled to a TSQ-Quantum Ultra triple quad mass analyzer (ThermoFinnigan, San Jose, CA). A nanospray needle with 20 μm ID capillary ending and 10μm tip was obtained from New Objective (Woburn, MA). 1, N^6-εdA was detected by multiple reaction monitoring (MRM) in the positive ESI mode, 276.0 to 160.0 for 1, N^6-εdA and 281.0 to 165.0 for $^{15}N_5$-1, N^6-εdA. The capillary temperature was set as 285 °C. The spray voltage was 2000 V. CE was 16 eV. Solvent A and B were 5mM ammonium formate in water and methanol respectively.

Statistical analysis

Statistical analyses were performed using Microsoft Excel spreadsheet analysis tools and SAS Ver. 8.02 (Cary, NC) (19). The amounts of 1, N^6-εdA were reported as average and standard deviation (mean ± SD) for each animal group. The comparison between control and exposed group, adult and weanling rats was performed by using a student's *t* test (two-sample unequal variances). Multiple comparisons of organ and time course differences in adduct levels were made by using one-way ANOVA in SAS. A significant difference was defined by $p < 0.05$ in both of these analyses.

C. RESULTS

1, N^6-εdA adducts in liver DNA of VC exposed weanling and adult rats

To determine the relationship between endogenous NA- and inhalation-derived exogenous $(^{13}C_2)$-1, N^6-εdA *in vivo*, we monitored both adducts in the liver DNA of weanling rats exposed to 1100 ppm $(^{13}C_2)$-VC. After a 1-week $(^{13}C_2)$-VC exposure by inhalation, the weanling rats were sacrificed immediately. As described in figure 3.2, no significant change was observed for hepatic NA-1, N^6-εdA between the control (1.13 ± 0.22 1, N^6-εdA per 10^8 unmodifed nucleosides) and exposed (1.42 ± 0.68 1, N^6-εdA per 10^8 unmodifed nucleosides) groups ($p > 0.5$). High amounts of $(^{13}C_2)$-1, N^6-εdA (2.8 ± 0.7 $(^{13}C_2)$-1, N^6-εdA per 10^8 unmodified nucleosides) were found in the $(^{13}C_2)$-VC exposed liver DNA, which was twice the amount of the NA-1, N^6-εdA level present in the same samples, as seen in figure 3.2 and 3.3. In addition, eight weanling rats were sacrificed after 1-day exposure to NA-VC with unexposed control animals. A two fold increase of NA-1, N^6-εdA was observed in the exposed group ($2.48 \pm 0.39/10^8$ unmodified nucleosides), compared with the control ($0.95 \pm 0.23/10^8$ unmodified nucleosides), as indicated in figure 3.4. Comparing the total amount of 1, N^6-εdA (the sum of NA- and $(^{13}C_2)$-1, N^6-εdA) in the liver DNA of weanling rats exposed to NA-VC for 1 day with $(^{13}C_2)$-VC for 1 week, it was found that more than a 4-fold increase was observed in the exposed group ($4.21 \pm 1.17/10^8$ unmodified nucleosides), compared to the control after 1-week $(^{13}C_2)$-VC exposure, as shown in figure 3.5, while only 2-fold increase was observed in the exposed group compared with the control after a 1-day NA-VC exposure as indicated in figure 3.4.

Post-exposure studies were performed in adult rats in order to better evaluate the half-life of 1, N^6-εdA. The typical chromatogram of 1, N^6-εdA in the liver of adult rats

exposed to ($^{13}C_2$)-VC for 1 week without recovery was shown in figure 3.6. Four adult rats were sacrificed immediately, or 2, 4, or 8 weeks after 1-week 1100 ppm ($^{13}C_2$)-VC exposure by inhalation. ($^{13}C_2$)-1, N^6-εdA (1.27 $\pm$ 0.15 ($^{13}C_2$)-1, N^6-εdA /10^8 unmodified nucleosides) was detected in the liver of rats sacrificed immediately after exposure, while it was below the limit of detection in all post-exposure groups, indicating rapid repair of hepatic 1, N^6-εdA after the exposure. No significant change was observed for the number of NA-1, N^6-εdA lesions between the control and post-exposure groups, as shown in figure 3.7 ($p > 0.05$). Four adult rats were sacrificed after 1-day 1100 ppm NA-VC exposure with their control. The concentrations of NA-1, N^6-εdA were 1.52 $\pm$ 0.36 $\times$ 10^{-8} in the control and 3.72 $\pm$ 0.42 $\times$ 10^{-8} in the exposed livers, as seen in figure 3.8, demonstrating a two fold increase after 1-day of exposure. The increase in total 1, N^6-εdA after 1-week ($^{13}C_2$)-VC exposure was 2.51 $\pm$ 0.12 vs 1.08 $\pm$ 0.17/10^8 unmodified nucleosides was similar to the increase observed following a 1-day NA-VC exposure in adult rats, as shown in figure 3.8 and 3.9.

1, N^6-εdA adducts in kidney and lung DNA of VC exposed weanling and adult rats

Significant changes were observed for NA-1, N^6-εdA in the lung or kidney DNA of weanling rats after 1-week exposure to 1100 ppm ($^{13}C_2$)-VC exposure by inhalation, as indicated in figure 3.2. NA-1, N^6-εdA concentrations in the control and exposed group were 1.76 $\pm$ 0.69 and 2.96 $\pm$ 0.64 adducts/10^8 unmodified nucleosides in the kidney DNA. NA-1, N^6-εdA adducts were 1.43 $\pm$ 0.15 and 3.31 $\pm$ 0.85 adducts/10^8 unmodified nucleosides in the lung DNA of the control and exposed group respectively. The amounts of ($^{13}C_2$)-1, N^6-εdA were 1.20 $\pm$ 0.33/10^8 unmodified nucleosides in the lung, and 0.81 $\pm$

$0.25/10^8$ unmodified nucleosides in the kidney in weanling rats exposed to ($^{13}C_2$)-VC, as shown in figure 3.3. In addition, the total amounts of 1, N^6-εdA in 1-week ($^{13}C_2$)-VC exposed lung and kidney DNA of weanling animals were increased 2 to 3-fold compared with the control group, which is similar to the increase of 1, N^6-εdA in the lung and kidney DNA of 1-day 1100 ppm VC exposed weanling animals, as depicted in figure 3.4 and 3.5.

One-week 1100 ppm ($^{13}C_2$)-VC exposure did not induce a significant change in the NA-1, N^6-εdA adducts in the lung and kidney of adult rats, as depicted in figure 3.10. The concentrations of NA-1, N^6-εdA were $0.97 \pm 0.11 \times 10^{-8}$ in the exposed and $0.77 \pm 0.29 \times 10^{-8}$ in the lung of the control. The concentrations of NA-1, N^6-εdA were $0.88 \pm 0.27 \times 10^{-8}$ in the exposed and $0.70 \pm 0.18 \times 10^{-8}$ in the kidney of the control. ($^{13}C_2$)-1, N^6-εdA adducts in the lung were found to be the second highest ($0.79 \pm 0.06/10^8$ unmodified nucleosides), followed by the kidney ($0.50 \pm 0.10/10^8$ unmodified nucleosides) in the adult rats exposed to ($^{13}C_2$)-VC, as shown in figure 3.3. Moreover, significant increases of NA-1, N^6-εdA adducts were found in the lung and kidney of adult rats after 1-day 1100 ppm NA-VC exposure, as shown in figure 3.8. A 2-fold increase of total 1, N^6-εdA was observed in the lung and kidney of adult animals after both 1-week ($^{13}C_2$)-VC and 1-day VC exposure, as depicted in figure 3.8 and 3.9. The total amounts of 1, N^6-εdA were 1.77 ± 0.17 and 1.38 ± 0.32 adducts/10^8 unmodified nucleosides in the lung and kidney DNA of adult animals after 1-week ($^{13}C_2$)-VC exposure without post.

D. DISCUSSION

The aim of this study was to measure the formation of both ($^{13}C_2$) and NA-1, N^6-εdA in adult and weanling rats after inhalation exposure to ($^{13}C_2$)-VC. Previously, such studies had only examined N^2, 3-εG in rats, which forms 10 times more adducts than 1, N^6-εdA in animals exposed to VC (41, 42). The quantification of 1, N^6-εdA in animal tissues was first reported using competitive radioimmunoassay to measure 1, N^6-εdA in the liver and lung DNA of preweanling rats exposed to VC (18). However, in these studies, 1, N^6-εdA was not detected in unexposed control rats; it was highest in preweanling rats and just detectable in exposed adult rodents. This problem was resolved with the advent of a sensitive immunoaffinity purification/^{32}P-postlabeling assay (43) that had a limit of detection of 5 1, N^6-εdA per 10^{10} parent bases in DNA. Considering the small quantity of DNA required (usually around 10 μg), this assay has been widely applied in many dosimetry studies (18, 22, 37-39, 43-48). However, it also has some shortcomings. The accuracy of measurements does not use a true internal standard and no information about chemical structure of adducts is provided. Compared with ^{32}P-postlabeling methods, mass spectrometry is a powerful technique because it can distinguish different carcinogen-DNA adducts, provide chemical structure information and employ an authentic internal standard. Although LC-MS has been applied in 1, N^6-εdA detection since 1999 by Doerge (49), it has not been widely used for quantitative analysis of trace endogenous DNA adducts because it is generally less sensitive than ^{32}P-postlabeling. However, through recent ESI developments such as microelectrospray and nanospray devices coupled with highly efficient separation techniques and miniaturized chromatographic columns with less than 2 μm particle size, nanoUPLC-nanoESI/MS/MS systems can provide more than 20 times better sensitivity compared to conventional LC-

MS/MS systems, as shown in chapter 2. Besides improved sensitivity, this system also brings advantages in terms of reduced solvent and sample consumption (50).

Using this new assay, the repair efficiency of $(^{13}C_2)$-1, N^6-εdA was investigated in DNA from adult rats exposed to 1100 ppm VC for 1 week and sacrificed immediately, 2, 4 or 8 weeks post-exposure. Since $(^{13}C_2)$-1, N^6-εdA was only detected in the liver, lung and kidney from the group sacrificed immediately after exposure, it was rapidly repaired in these organs. It is assumed that $(^{13}C_2)$- and NA-1, N^6-εdA are repaired in the same manner. While $(^{13}C_2)$-1, N^6-εdA was repaired so that none could be detected 2, 4 or 8 weeks after exposure, but the number of NA-1, N^6-εdA was constant in liver over time, as shown in figure 3.7. Its presence was due to continuous endogenous formation by lipid peroxidation. Several studies reported rapid repair of 1, N^6-εdA in mice. Barbin (31) studied the persistence of 1, N^6-εdA in liver and lung DNA from 6-week-old male and female C57Bl/6J mice after five daily i.p. injections of 250 nmol/g of vinyl carbamate. These animals were sacrificed at different time intervals (from 6 h to 96 h) after the final dose. The estimated half-life of 1, N^6-εdA was 21 ± 3 h in hepatic DNA and 20 ± 3 h in lung DNA of both male and females. Ham et al. (32) found the half-life of 1, N^6-εdA from DNA in liver and lung of male mice to be 17 h and 28 h respectively and similar results were obtained for female mice. Both Barbin and Ham observed that even in alkyl-N-purine-DNA glycosylase (ANPG, or N-methylpurine-DNA glycosylase, MPG)/Aag knock-out mice, the amounts of 1, N^6-εdA decreased after vinyl carbamate exposure and postulated that an additional repair pathway was involved. These results were recently supported by the observations that direct reversal repair is another pathway for repairing

1, N^6-εdA (34-36). Moreover, the repair of 1, N^6-εdA was also suggested by detecting εA in the urine of adult male Fischer 344 rats following *i.v.* administration of 8.5 mg chloroethylene oxide/kg body weight (51). The excretion of εA increased greatly in the first 24 h after exposure and plateaued after that. All these studies showed that 1, N^6-εdA is rapidly repaired in liver and lung of adult rodents, with no differences between gender and species. Although several studies reported the persistence of 1, N^6-εdA or εA (31, 38, 39, 41), these studies could not differentiate between adducts from VC exposure and endogenous formation through lipid peroxidation. In the present study, it is clear that continuous endogenous formation was the key factor responsible for the apparent persistence of this rapidly repaired adduct.

The accumulation of total 1, N^6-εdA was analyzed by comparing the amounts of 1, N^6-εdA in liver DNA of adult rats after a 1-day 1100 ppm VC exposure with the data from rats sacrificed immediately after 1-week of ($^{13}C_2$)-VC exposure. It was found that in both exposure scenarios total 1, N^6-εdA doubled, as shown in figure 3.8 and 3.9. Thus, the only accumulation identified was associated with the last day of exposure. Significant accumulation was not demonstrable in the kidney or lung of adult rats. Guichard et al. (38) reported the accumulation of 1, N^6-εdA in the liver of adult *Sprague-Dawley* rats exposed to 500 ppm VC for 1, 2, 4 or 8 weeks, but not in the lung and kidney. Using a simple linear regression model to fit the data of post-exposure time and 1, N^6-εdA level, the estimated accumulated rate of 1, N^6-εdA in the liver DNA of adult rats expressed as the slope of this curve was 0.682×10^{-8}/week. However, the methods employed could not differentiate between exogenous and endogenous 1, N^6-εdA. Other possibilities that must

be considered include saturation or increased expression of one or more DNA repair pathways for 1, N^6-εdA. A study by Holt et al. (52) evaluated the effect of vinyl fluoride exposure, which forms the same DNA adducts as VC, on gene expression of the N-methylpurine-DNA glycosylase (MPG), a repair enzyme for etheno adducts. Holt et al. found that the expression of MPG was similarly induced following either 25 or 2500 ppm vinyl fluoride in the liver of adult *Sprague-Dawley* rats (52). The nontarget tissues, kidney and lung, did not exhibit induction of MPG expression after VF exposure, as similar amounts of MPG were observed in both tissues in control and exposed animals. Similar gene expression studies would be needed to determine if the differences in exogenous 1, N^6-εdA adducts among different organs or between weanling and adult animals following exposure to ($^{13}C_2$)-VC could be the result of the induction of repair enzymes, Thus, a dynamic balance between 1, N^6-εdA formation and repair pathways may have age and/or organ related differences that could affect susceptibility.

Although previous studies (7) found that the number of etheno adducts (1, N^6-εdA and 3, N^4-εdC) in the liver of adult rats, the major target organ of VC, were not higher than other tissues after VC exposure, VC-induced ($^{13}C_2$)-1, N^6-εdA did show the greatest number of adducts in the liver, compared with lung and kidney in both weanling and adult rats exposed to ($^{13}C_2$)-VC. The mean number of ($^{13}C_2$)-1, N^6-εdA across these organs was liver>lung>kidney, as shown in figure 3.3. Multiple comparisons indicated significant differences for ($^{13}C_2$)-1, N^6-εdA between liver and kidney ($p < 0.05$) in the adult rats, but not between liver and lung. Significant differences for ($^{13}C_2$)-1, N^6-εdA were also detected between liver and lung, liver and kidney, but not kidney and lung in

weanling rats. Relatively homogeneous distributions of 1, N^6-εdA were observed among tissues in adult rats in studies by Barbin (7) following exposure to vinyl chloride. As shown in figure 3.4 and 3.8, without distinguishing endogenous and exogenous 1, N^6-εdA, similar amounts of 1, N^6-εdA were observed in liver, lung and kidney from both 1-day 1100 ppm VC exposed adult and weanling rats in this study. Furthermore, similar total amounts of 1, N^6-εdA (NA- 1, N^6-εdA and ($^{13}C_2$)-1, N^6-εdA) were found in the liver, lung and kidney of weanling animals exposed to ($^{13}C_2$)-VC, as shown in figure 3.5..

Previous studies showed that the concentrations of 1, N^6-εdA and N^2, 3-εG in weanling animals were higher than in adult rats exposed to VC (37, 53). Çiroussel et al. (37) measured 1, N^6-εdA in DNA of several organs of seven-day-old (group I) and 13-week-old BD VI rats (group II) after 2-weeks exposure to 500 ppm VC. It was found that the amounts of 1, N^6-εdA in liver DNA of group II was six times lower than group I. Similar results were found for N^2, 3-εG in the studies of Morinello et al. (53). The concentrations of N^2, 3-εG in liver DNA of weanling rats were 2-3 fold greater than those of the adults after VC exposure. In the present studies, ($^{13}C_2$)-1, N^6-εdA in liver DNA of weanling rats was two times higher than those of the adults exposed to 1100 ppm ($^{13}C_2$)-VC for 1 week. In addition to liver, kidney and lung also showed higher amounts of ($^{13}C_2$)-1, N^6-εdA in the weanling rats, as shown in figure 3.3. Although the number of ($^{12}C_2$)-εdA adducts did not increase in liver, lung and kidney of adult rats after 1-week exposure to 1100 ppm ($^{13}C_2$)-VC, ($^{12}C_2$)-εdA adducts did increase in the lung and kidney DNA of weanling rats. This could be the result of increased lipid-peroxidation in these tissues. The high VC adduct concentrations in young animals, coupled with increased cell

proliferation due to a steep growth rate, are key events in their greater susceptibility to VC-induced hepatic angiosarcoma and hepatocellular carcinoma, compared to adult animals. Classical long-term experimental carcinogenicity studies have shown that the susceptibility of hepatocytes to the carcinogenic effect of VC is age-dependent (54). Maltoni's study demonstrated that newborn rats, in contrast to adult animals, responded to VC with the development of hepatocellular carcinomas, and hemangiosarcomas (54). Drew et al. (55) studied age-related susceptibility to VC in rats, golden hamsters and mice. Rats held for 6 months before exposure developed VC-related neoplasm, while rats held 12 months before the start of exposure failed to show a significantly increased incidence of these neoplasm. The highest incidence of malignant neoplasm occurred in hamsters exposed for the first 12 months, whereas beginning the exposure after 12 months of age did not cause neoplasm. In Swiss CD-1 and B6C3F1 mice, VC exposure during the first 6 months of the experiment induced a high incidence of hemangiosarcomas. All these studies clearly indicated that VC exposure starting at early stage in the life was most effective. The increase in metabolism of VC resulting in increased numbers of VC adducts, coupled with greater cell proliferation provides the mechanistic basis for this increased risk for VC-induced cancer.

Weanling rats ======

Adult rats ======

1 day   1 week   3 week   5 week   9 week

Stop exposure ------------- Post exposure --------------

====== Weanling rats exposed to 1100 ppm NA-VC by inhalation

====== Weanling rats exposed to 1100 ppm $^{13}C_2$-VC by inhalation

====== Adult rats exposed to 1100 ppm NA-VC by inhalation

====== Adult rats exposed to 1100 ppm $^{13}C_2$-VC by inhalation
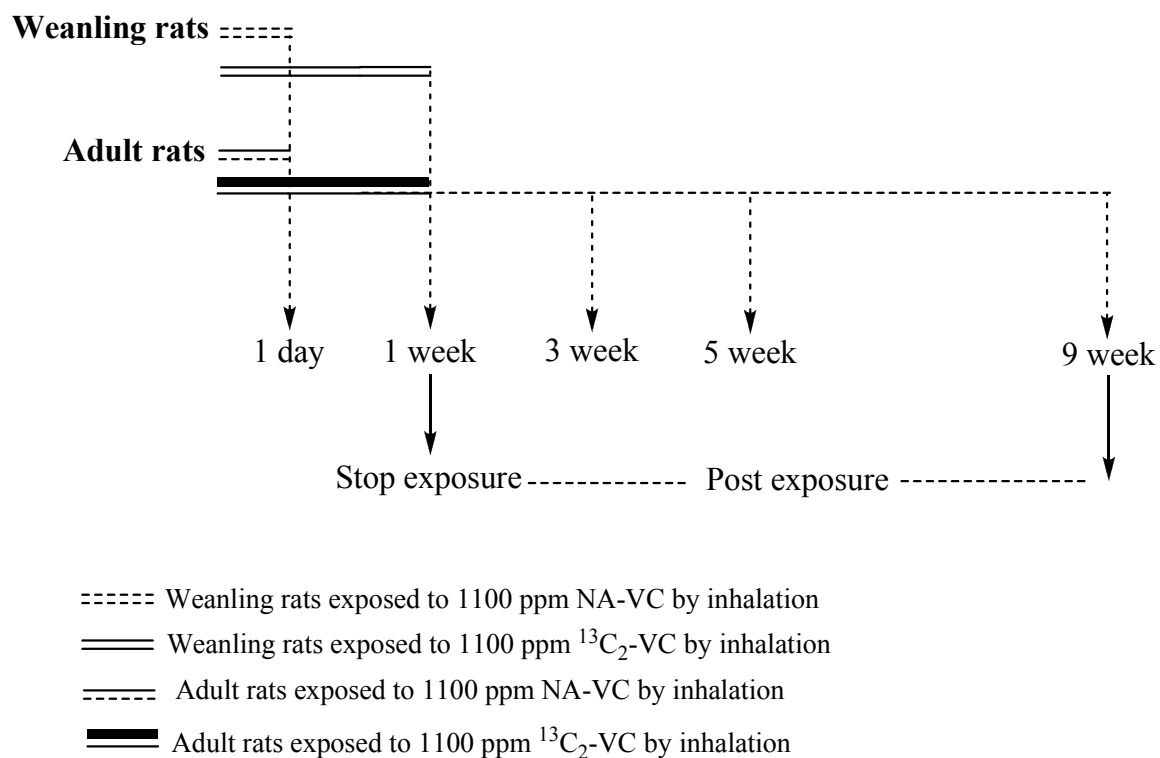
Figure 3.1 The experimental design
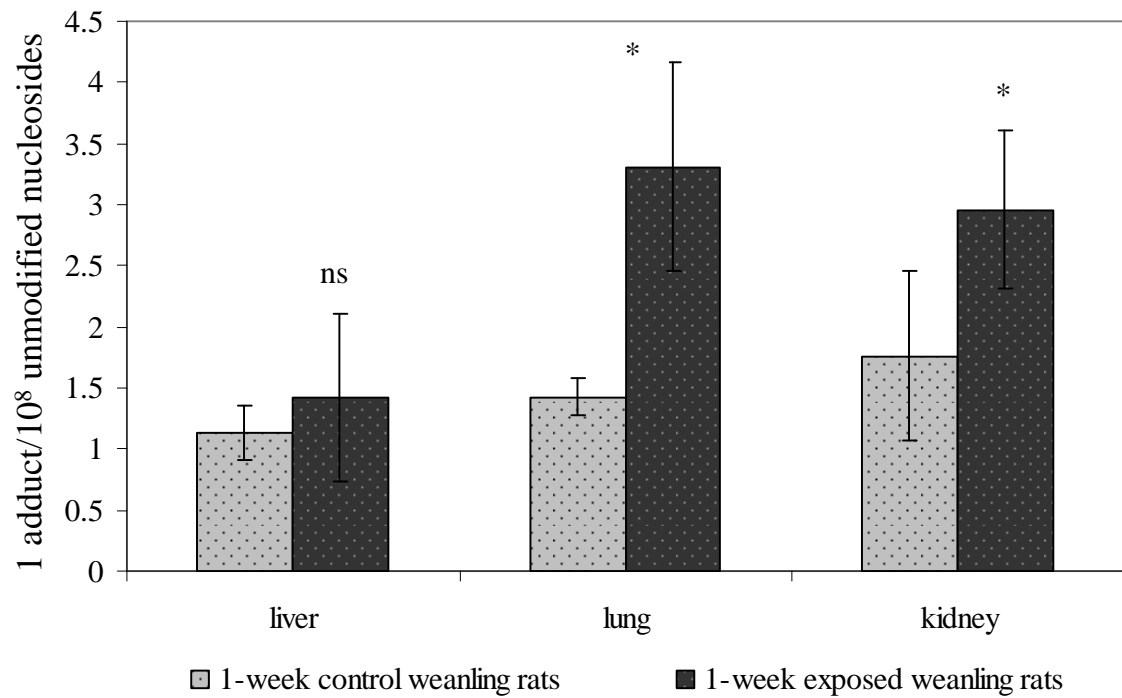
p values (two-sample t-test) for comparison between treated and control groups: *, p<0.05; ns, no significant difference, p>0.05, n=4~8. Multiple comparisons of organ were made by using one-way ANOVA

Figure 3.2 NA-1, N^6-εdA amounts in ($^{13}C_2$)-VC exposed and control weanling rats
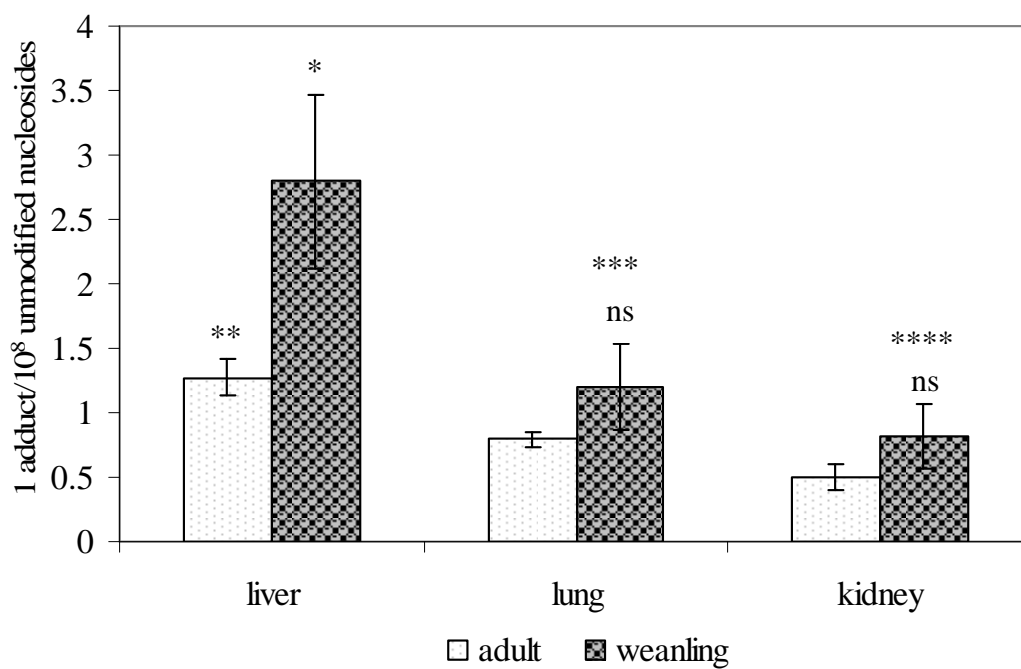
p values (two-sample t-test) for comparison between weanling and adult groups: *, p<0.05; ns, no significant difference, p>0.05. For adult, $n=4$; for weanling, $n=6\sim8$. Multiple comparisons of organ were made by using one-way ANOVA. **, p<0.05 between adult liver and kidney; ***, p<0.05 between weanling liver and lung; ****, p<0.05 between weanling liver and kidney.

Figure 3.3 ($^{13}C_2$)-1, N^6-εdA amounts in the ($^{13}C_2$)-VC exposed weanling and adult liver DNA
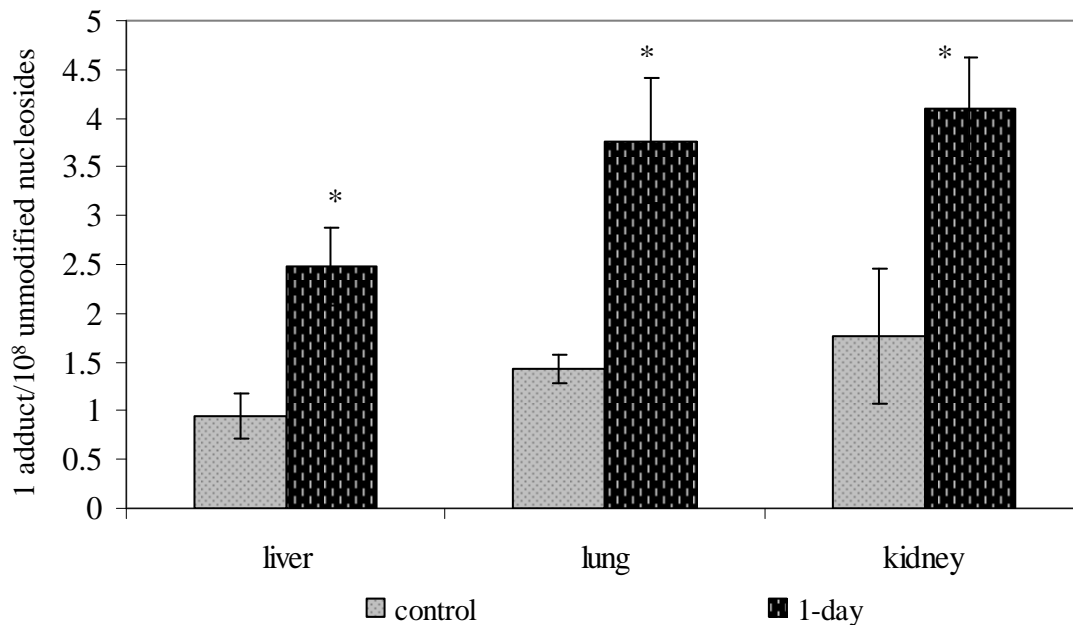
Figure 3.4 NA-1, N^6-εdA amounts in the NA-VC exposed and control weanling rats
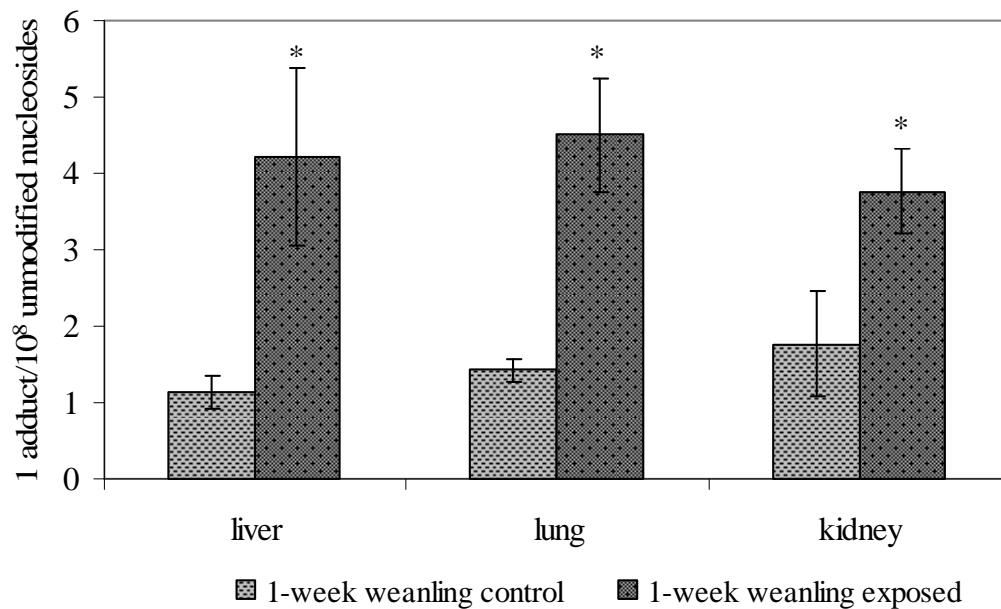
p values (two-sample t-test) for comparison between VC-exposed and control weanling animals: *, p<0.05; ns, no significant difference, p>0.05, , n=4~8. Multiple comparisons of organ were made by using one-way ANOVA

Figure 3.5 Total amount of 1, N^6-εdA in weaning rats after 1-week exposure of ($^{13}C_2$)-VC by inhalation
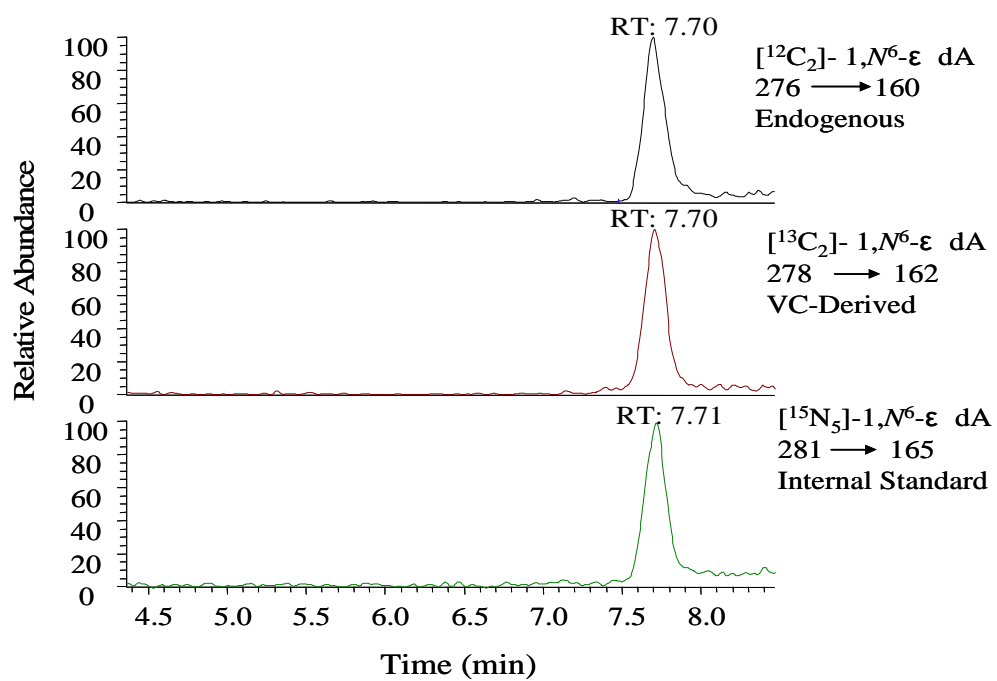
Figure 3.6 Typical chromatogram for 1, N^6-εdA detection in the liver of $(^{13}C_2)$-VC treated adult rats.
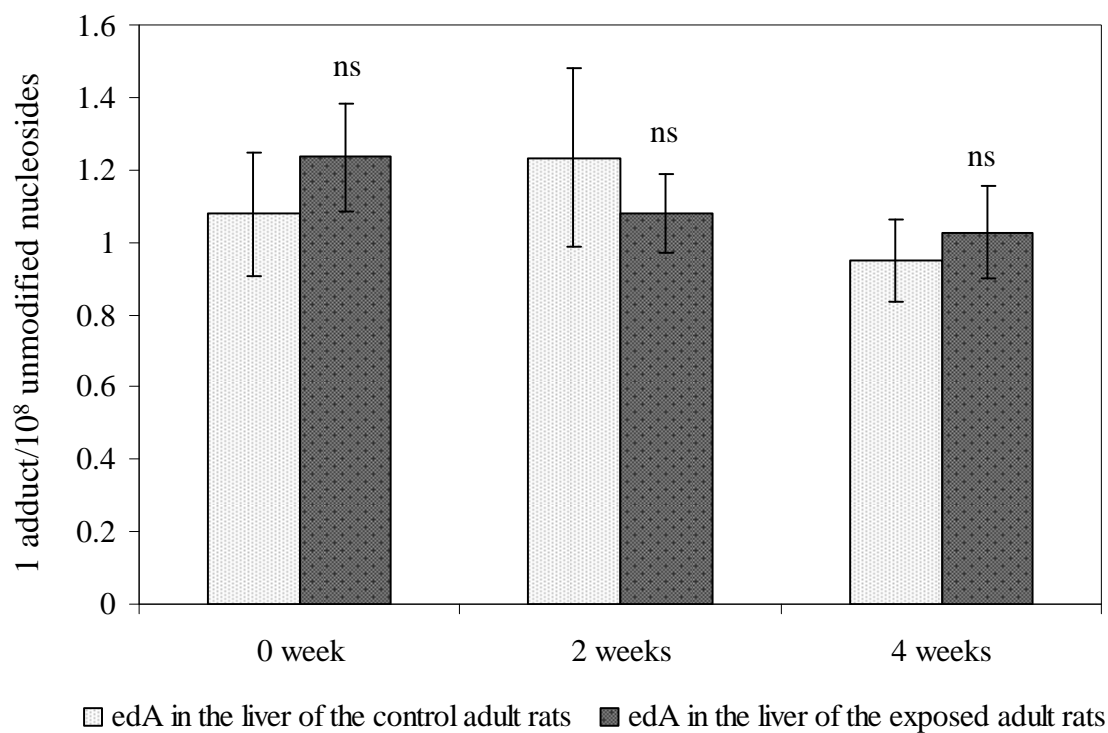
p values (two-sample t-test) for comparison between VC-exposed and control groups: ns, no significant difference, p>0.05, n=3~4. Multiple comparisons of organ were made by using one-way ANOVA

Figure 3.7 NA-1, N^6-εdA amounts in the control and (^{13}C$_2$)-VC exposed adult liver DNA with different recovery time
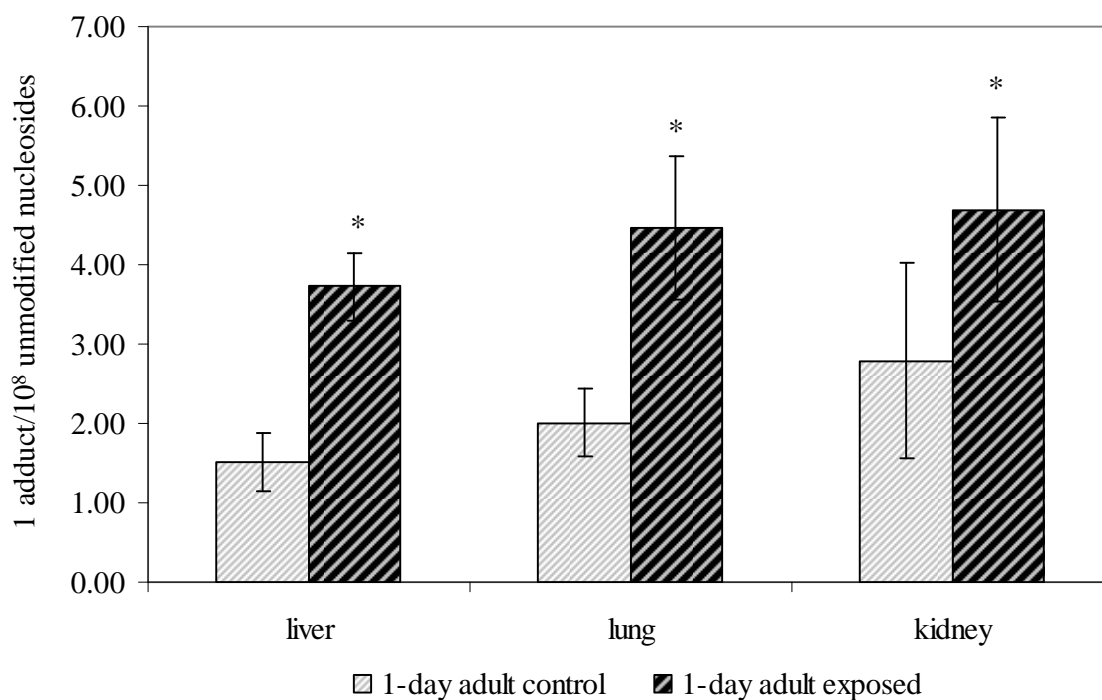
Figure 3.8 NA-1, N^6-εdA amounts in the NA-VC exposed and control adult DNA

p values (two-sample t-test) for comparison between treated and control groups: *, p<0.05; ns, no significant difference, p>0.05, n=4. Multiple comparisons of organ were made by using one-way ANOVA
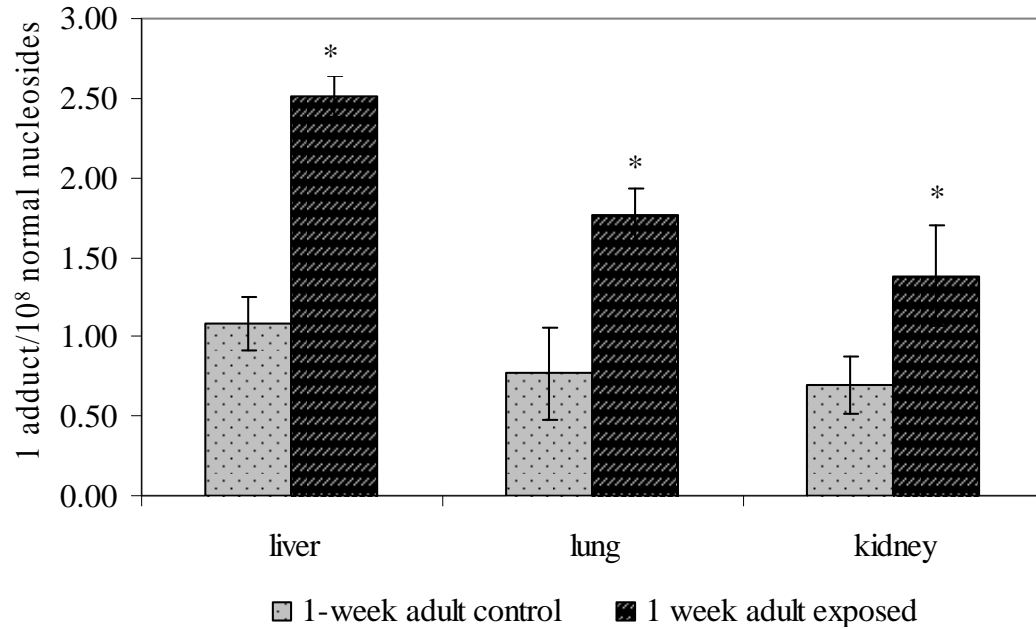
p values (two-sample t-test) for comparison between VC-exposed and control adult animals: *, $p<0.05$; ns, no significant difference, $p>0.05$, $n=3\sim4$. Multiple comparisons of organ were made by using one-way ANOVA

Figure 3.9 Total amount of 1, N^6-εdA in adult rats after 1-week exposure of ($^{13}C_2$)-VC by inhalation
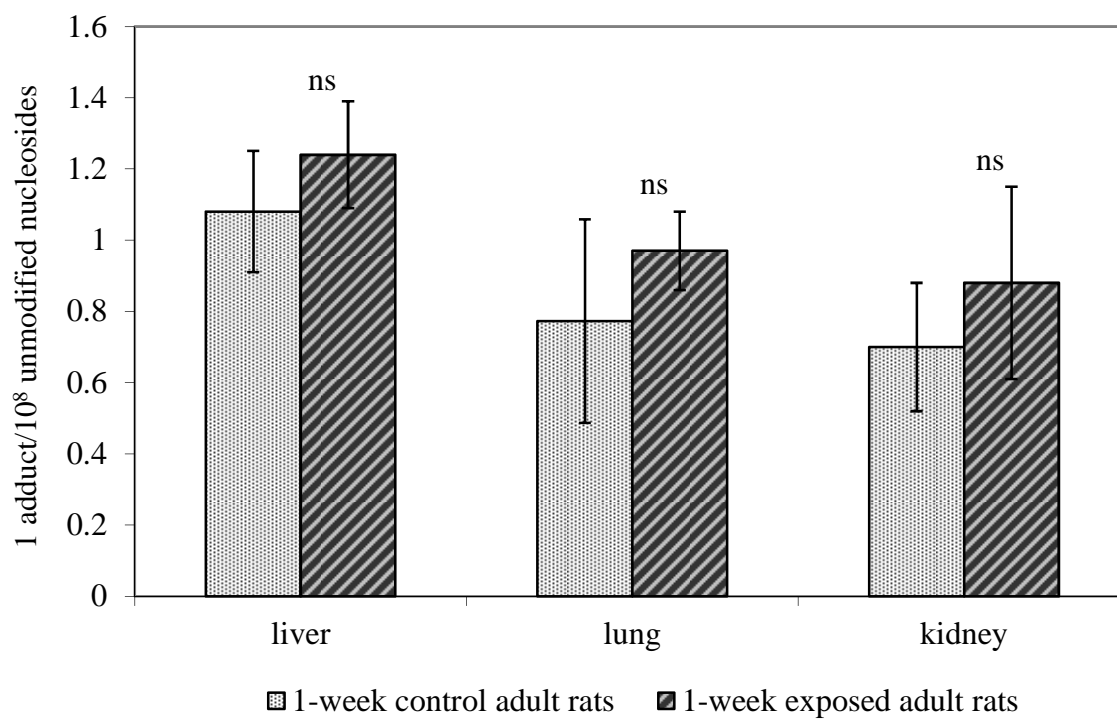
Figure 3.10 NA-1, N^6-εdA amounts in the ($^{13}C_2$)-VC exposed and control adult rats

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CHAPTER IV

SPECTRUM OF DNA LESIONS INDUCED BY REACTIVE OXYGEN SPECIES IN
SPRAGUE-DAWLEY RATS CHRONICALLY EXPOSED TO 2, 3, 7, 8-
TETRACHLORODIBENZO-p-DIOXIN AFTER DIETHYLNITROSAMINE
INITIATION

A. INTRODUCTION

2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD), commonly known as dioxin, is one

of the most toxic environmentally persistent organic pollutants (1). Although without

commercial use, it can be formed from various sources, such as the manufacture or

degradation of other chlorophenols and their derivatives, municipal incinerators, sewage

sludge and discharge from paper mills (1). Because of its lipophilicity, stability, and

resistance to biodegradation, TCDD can accumulate in human adipose tissues and cause

chronic lifetime exposure which may evoke long-term toxicity. Although with limited

data on epidimeology studies, TCDD was still classified as a group 1 carcinogen by the

International Agency for Research on Cancer (IARC) based on adequate evidence of its

multisite carcinogenicity in rodents (2-3). The key event that is thought to drive these

biological effects is TCDD binding to a receptor protein, aryl hydrocarbon receptor

(AhR). The ligand-receptor complex subsequently binds to the AhR nuclear translocator

(ARNT) in the nucleus, which binds to DNA, triggering the expression of a variety of

genes, especially CYP450 (4). The up-regulation of these enzymes may greatly speed up

the oxidative transformation of endogenous and exogenous compounds in the body and induce reactive oxygen species (ROS). Although considered a non-genotoxic compound, TCDD may cause continuous indirect oxidative DNA damage by inducing the accumulation and persistence of ROS through up-regulation of CYP450.

ROS can react with DNA directly and form various DNA lesions. One of the most studied one is 7, 8-dihydro-8-oxo-2′-deoxyguanosine (8-OHdG). It is formed in relatively high amounts *in vivo* with background amounts around $1/10^6$ guanine (5). ROS can also abstract a hydrogen atom from polyunsaturated fatty acids to initiate lipid peroxidation and produce various unsaturated aldehydes, such as malondialdehyde (MDA), 4-HNE, crotonaldehyde, and acrolein. These compounds can react with DNA and form exocyclic adducts: etheno adducts and propano adducts (6). The dominant etheno adducts are 1, N^6-etheno-2'-deoxyadenosine (1, N^6-εdA), 3, N^4-etheno-2'-deoxycytidine (3, N^4-εdC), 1, N^2-etheno-2'-deoxyguanosine (1, N^2-εdG), and N^2, 3-ethenoguanine (N^2, 3-εG). The primary propano-derived DNA adducts are 1, N^2-propano-2'-deoxyguanosines generated from acrolein (AcrdG), crotonaldehyde (CrdG) and 4-HNE (4-HNEdG). The major MDA-induced DNA adduct is 3-(2-deoxy-β-D-erythropentofuranosyl) pyrimido (1,2-α)purin-10(3*H*)-one (M$_1$dG). The accumulation of ROS-induced DNA adducts is thought to be a potential hazard *in vivo* because of their connection with mutations (7-11). Site-directed mutagenicity studies found that most ROS-induced adducts can cause specific transition and/or transversion point mutations in bacteria and/or mammalian cells. In addition, the persistent increase of 1, N^6-εdA, 3, N^4-εdC and 8-OHdG has been found in various premalignant target organs affected by disease, such as acute leukemia, colorectal cancer

and hepatic cancer (12-15). These studies provide strong support for the hypothesis that oxidative DNA damage plays an important role in carcinogenesis.

Oxidative stress has been used as a marker for the toxicity of TCDD and its congeners since the beginning of 1980s (16). Both *in vitro* and *in vivo* studies indicated increased production of ROS after TCDD exposure, which was regarded as the result of TCDD-mediated activation of AhR and a shift in the cellular redox balance (17). Therefore, it was widely hypothesized that the toxicity induced by TCDD in rodents was at least partly explained by the contribution of oxidative stress, an observation reported in many studies (18-24). Although most of these studies were acute or subchronic studies, several relevant chronic studies were also reported. All of them focused on the oxidative stress in the liver of female *Sprague-Dawley* rats, which is a sensitive strain with high hepatic tumor incidence after TCDD exposure. Tritscher et al. (18) first reported increased 8-OHdG in livers of TCDD treated intact, but not ovariectomized female rats after diethylnitrosamine (DEN) initiation. Wyde et al. (19) further found the induction of hepatic 8-OHdG by TCDD in female rats after DEN initiation and demonstrated that it was female-specific, estrogen-dependant and was a chronic effect. Recently, Hassoun et al. (20) observed increased production of superoxide anion, lipid peroxidaiton and DNA single-strand breaks in hepatic tissues of rats after 30 weeks exposure to TCDD. All of these studies supported the importance of oxidative DNA damage in carcinogenesis of TCDD.

The aim of the current study was to further study the oxidative DNA damage spectrum induced in the liver of *Sprague-Dawley* rats chronically exposed to TCDD after DEN initiation. Previous two-stage liver tumor promotion studies on TCDD observed significant increased 8-OHdG in TCDD exposed hepatic tissues of female rats (18, 19). But considering the distinct formation, repair pathways and promutagenicity of different ROS-induced DNA adducts, it's valuable to measure other adducts. Besides, the reliability of 8-OHdG as a good biomarker of ROS has been offset by artifactual formation during sample processing (5, 25), which has impeded maximal application of this adduct in understanding and assessing the risk of carcinogenesis. In order to expand our knowledge on these issues, a series of ROS-induced DNA adducts were measured in the samples provided by Wyde et al. (19). The comprehensive DNA adduct profile will include 8-OHdG, 1, N^6-εdA, CrdG, and AcrdG. These data will help us better understand the role of ROS-induced DNA damage in hepatic carcinogenesis of TCDD in this two-stage carcinogenesis model.

B. MATERIALS AND METHODS

Materials

Nucleic acid purification grade lysis buffer, protein precipitation solution and Proteinase K were purchased from Gentra Systems (Minneapolis, MN). Other chemical reagents including 1, N^6-εdA and 8-OHdG standards were from Sigma-Aldrich Chemical Company (St Louis, MO). HPLC grade water, methanol, other similar chemical solutions and 2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO) were purchased from ThermoFisher

Scientific Company (Raleigh, NC). $^{15}N_5$-1, N^6-εdA standard was synthesized and characterized as described by Ham et al. (26). $^{15}N_5$-8-OHdG and $^{15}N_5$-2′-deoxyguanosine were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The standards for AcrdG and CrdG and their $^{15}N_5$ labeled internal standards were synthesized according to previous studies (27, 28). The materials in animal exposure studies were described in detail in Wyde's reports (19).

Animal exposure

The animal study was performed in 1998 and described previously in detail by Wyde et al. (29, 30). All animals were housed in cages with control temperature (70 ± 0.5°F), humidity (50 ± 5%) and lightling (12-h light/12-h dark) and received NIH-31 diet (Zeigler Brothers Inc., Gardner, PA) and water *ad libitum*. The whole animal design is shown in figure 4.1. Four different exposure scenarios (A, B, C, and D) were applied for both male and female *Sprague-Dawley* rats provided by Charles River Labs (Raleigh, NC). Female rats were ovariectomized or sham operated (intact) at the age of 8 weeks before any treatment. All animals were initiated by single dose of 175 mg DEN/kg of body weight (BW) at 10-weeks-of-age. Intact female rats were implanted with placebo pellets and treated with olive oil (scenario A), or exposed to 700 ng/kg TCDD weekly for 30 weeks (scenario C). OVX female rats were either implanted with 0.18 mg, 1.7 mg E_2, or placebo. At 12 weeks, some OVX rats were treated with a weekly dose of corn oil (scenario A), or 700 ng/kg/week TCDD for 30 weeks without E_2 supplement (scenario C). Some OVX rats were treated with a weekly dose of corn oil (scenario B), or 700 ng/kg/week TCDD for 30 weeks with 0.18 mg or 1.7 mg E_2 supplement (scenario D).

The dose of TCDD (700 ng/kg/week) was equivalent to a daily 100 ng/kg/day, which induced liver tumors in female rats in chronic studies (31).

All intact male rats were initiated by a single dose of 175 mg DEN/kg of body weight at 10-weeks-of-age. One week after initiation, one group was implanted with 90-day release pellets containing placebo and treated with olive oil for 30 weeks (scenario A); one group was implanted with pellets containing 0.18 mg of E_2 and treated by olive oil for 30 weeks (scenario B); one group was exposed weekly to 700 ng/kg TCDD for 30 weeks with the placebo supplement (scenario C); the last group was exposed to the same amount of TCDD for 30 weeks with 0.18 mg E_2 as supplement (scenario D). New pellets containing placebo or E_2 were implanted after 90 days in these animals. All male and female animals were sacrificed by asphyxiation with CO_2 at the end of exposure. Liver tissues were removed, frozen in liquid nitrogen and store at -80 ˚C before DNA isolation.

DNA isolation

About 500 mg of tissues were homogenized with a Tehran homogenizer (Wheaton Instruments, Millville, NJ) in PBS solution and centrifuged at 1000 g for 10 min. The formed pellet was suspended in lysis buffer. Protein was removed from the sample by adding Proteinase K (400 U/ml) in the sample, rotating overnight in 4 ˚C cold room and precipitated by Protein precipitation solution. On the second day, the sample was precipitated at room temperature by isopropanol, rinsed by 70% ice cold ethanol aqueous solution and reconditioned in cold PBS solution. Then RNAase mixture, containing RNAase T1 and RNAase A, was added to the sample and incubated for 30 min at 37 ˚C.

Finally, the sample was rinsed with 100% ethanol and its 70% aqueous solution, resuspended in HPLC grade water and stored at -80 ˚C. The concentration and purity of DNA was measured by Biomate 5 (Thermo Spectronic, NY, USA). TEMPO (20 mM) was present in the samples during the whole DNA isolation process to inhibit formation of free radical species.

Enzymatic hydrolysis and HPLC purification

The whole process has been described in detail in chapter 2. Briefly, slightly different enzymatic hydrolysis was applied for different adducts depending on their physiochemical properties. The digestion method for 1, N^6-εdA was modified based on the studies of Pang et al. *(25)* and the optimized method C was applied with 2 h incubation at 37 ˚C. For 8-OHdG detection, the same enzymatic digestion process as 1, N^6-εdA was applied to digest 50 μg DNA. TEMPO (20mM final concentration in the sample) was present through the whole sample processing. Since some studies mentioned that DNase I was necessary for bulky DNA adduct digestion (32), 50 U DNase I was added with nuclease P1 during the enzymatic digestion for AcrdG and CrdG detection. After enzymatic hydrolysis, proteins were removed by micron-10 filter followed by HPLC purification of the sample. Three different LC methods were applied for 1, N^6-εdA, 8-OHdG and AcrdG or CrdG respectively, as described in chapter 2. The column temperature was kept as 30 ˚C for all sample purification. An Atlantis T3 (5 μm, 4.5×150 mm) was utilized.

LC-MS/MS detection

Two different LC-MS/MS systems were used to analyze these adducts. 8-OHdG was analyzed by a UPLC-MS/MS system consisting of Aquity UPLC system (Waters, Milford, MA) and TSQ-Quantum ultra triple quad mass analyzer (ThermoFinnigan, San Jose, CA). Sample separation was performed on T3 HSS column (1.7 µ, 2.1×100 mm) at a flow rate of 200 µl/min. Mobile phases consisted of 0.1% acetic acid in water as A and 0.1% acetic acid in methanol as B. The quantitative analysis of 1, N^6-εdA, AcrdG and CrdG was performed on 10 kpsi nanoacquity Ultra Performance LC system (Waters, Milford, MA) coupled to a TSQ-Quantum ultra triple quad mass analyzer (ThermoFinnigan, San Jose, CA). A nanoacquity symmetry trap column (180 µm × 20 mm, 5 µm) was connected with a nanoUPLC BEH C18 analytical column (1.0 mm × 100 mm, 1.7 µm) through a control valve. Mobile phase A was 5 mM ammonium formate for 1, N^6-εdA detection, and 0.1% formic acid in water for AcrdG and CrdG detection respectively. Phase B was acetonitrile with 0.1% formic acid for the detection of allthree adducts. The detailed LC gradient program and MS parameters were as described in chapter 2.

Statistical analysis

Statistical analyses were performed using R (version 2.11). Since the variances in DNA adduct data were not homogeneous and samples sizes were limited for certain groups, nonparametric Wilcox Rank Sum test was used to assess the difference between control and exposed groups. Multiple pair-wise comparisons were corrected calculating permutation p-values using 100,000 permutations. *p*-value was considered significant if they were less than 0.05. Data from the present study were reported as mean ± SD to

allow a direct comparison with other previously published relevant studies in which mean values were reported.

C. RESULTS

Following 30 weeks exposure to TCDD, significant increases of 8-OHdG were observed in the liver of intact female rats after DEN initiation compared with the control group (19.5 ± 10.7 adduct/10^6dG vs 6.4 ± 2.13 adduct/10^6 dG, $p = 0.016$), as shown in figure 4.2. With E_2 supplement, 8-OHdG level was enhanced in the liver of OVX female rats after 30-week exposure of TCDD with DEN as initiator. However, a significant increase was only observed in the exposed group with 1.7 mg E_2 supplement ($p = 0.016$), not with 0.18 mg E_2 ($p = 0.29$), by comparing with their respective control groups. Similar amounts of 8-OHdG were detected in the TCDD treated OVX group with 1.7 mg E_2 (22.3 ± 13.4 adduct/10^6 dG) to the intact TCDD treated female rats. Without the E_2 supplement, no significant formation of 8-OHdG was observed in the OVX rats after 30 weeks exposure of TCDD ($p = 0.095$). The number of 8-OHdG adducts in the control and exposed groups was 2.9 ± 0.24 adduct/10^6 dG and 3.2 ± 0.5 adduct/10^6 dG respectively. Ovariectomy and E_2 supplement showed a slight effect on background numbers of 8-OHdG in control animals. Lower mean levels of 8-OHdG were found in the control OVX group than the intact one, but the difference was not significant ($p = 0.095$). Slightly higher background 8-OHdG was detected in the OVX rats with E_2 than the nonsupplemented OVX rats, as shown in figure 4.2.

1, N^6-εdA, AcrdG and CrdG were also measured in the same samples and the results are shown in figure 4.3A and B. Following 30 weeks of TCDD treatment, 1, N^6-εdA was significantly increased in intact female rats and OVX rats with 1.7 mg E_2 supplement. The number of 1, N^6-εdA in intact TCDD treated and control group was 20.33 ± 8.9 and 5.76 ± 2.4/10^8 dA, respectively (p = 0.0095). The number of 1, N^6-εdA in OVX TCDD treated and control groups with 1.7 mg E_2 supplement was 9.1 ± 2.1 and 3.9 ± 1.7/10^8 dA, respectively (p = 0.001). Although an increase of AcrdG and CrdG was found in intact female rats and OVX rats with 1.7 mg E_2 supplement after TCDD treatment compared with their corresponding control group, neither of them was statistically significant compared with the control, as shown in figure 4.3B. The number of AcrdG adducts in intact TCDD treated and control female groups was 21.3 ± 6.2 and 6.8 ± 2.2/10^7 dG, respectively; and the difference between them was marginally significant (p = 0.056). The number of AcrdG in OVX TCDD treated and control female group with 1.7mg E_2 supplement were 9.1 ± 3.9 and 4.5 ± 1.0/10^7 dG, respectively (p = 0.1). Higher amounts of AcrdG and CrdG were found in TCDD treated intact female rats than the OVX female rats with E_2 supplement, as shown in figure 4.3B. In addition, slightly lower background numbers of 1, N^6-εdA, AcrdG and CrdG were found in OVX control rats than the intact female control rats, although no significant difference between them was present. With E_2 as supplement, the background amounts of 1, N^6-εdA, AcrdG and CrdG in OVX control female rats were similar to the control without E_2.

8-OHdG and 1, N^6-εdA were measured in TCDD treated male rats after DEN initiation with and without 0.18 mg E_2 supplement, as shown in figure 4.4. Following 30

weeks of treatment, increases of 8-OHdG were observed in male rats, with or without the E_2 supplement. Without E_2 supplement, the amount of 8-OHdG was 4.72 ± 0.6 and $4.2 \pm 0.4/10^6$ dG in the TCDD treated and control male group, respectively. With the E_2 supplement, higher amounts of 8-OHdG were detected in the control and TCDD-treated male group. The background of 8-OHdG in hepatic DNA of male rats was slightly lower than in the intact female, as shown in figure 4.2 and 4.4. Although TCDD induced an increase of 8-OHdG in male rats, it was not statistically significantly. Besides, no significant difference of 1, N^6-εdA was found in the livers of these animals. The amounts of 1, N^6-εdA in TCDD treated and control male groups without E_2 were 3.26 ± 0.96 and 3.01 ± 1.12 /10^8 dA, respectively. In the 0.18 mg E_2 supplemented group, the values of 1,N^6-εdA were 3.11 ± 0.45 and 3.21 ± 0.63 adduct /10^8 dA for the control and treated group, respectively.

D. DISCUSSION

Oxidative DNA damage has been identified as a potential important contributor in a myriad of diseases, especially cancer (12-15). Various oxidative DNA lesions have been investigated for their role in carcinogenesis (12, 13). Among them, 8-OHdG and 1, N^6-εdA were the most widely studied oxidative DNA adducts. Site-directed mutagenicity studies found that they can induce specific point mutations in bacteria and/or mammalian cells. The presence of 8-OHdG during DNA replication can cause G to T transversions (13). The presence of 1, N^6-εdA can cause A to G transitions, and A to T, A to C transversions (6-8). Although reports concerning the biological significance of AcrdG

and CrdG are limited, both have been found to be capable of inducing G to T transversions in COS-7 system as the dominant mutation (11). Because of the controversy over the significance of 8-OHdG as a good biomarker for oxidative DNA damage, 1,N^6-εdA, AcrdG and CrdG were also measured in this study to explore the breadth of oxidative DNA damage in the hepatic toxicity of female *Sprague-Dawley* rats induced by TCDD in the two stage carcinogenesis study.

Using LC-ECD and direct injection after enzyme digestion, Wyde et al, (30) measured 8-OHdG in the liver of intact and OVX female rats exposed to TCDD for 30 weeks after DEN initiation. In his study, 18-fold higher 8-OHdG was found in the TCDD treated intact female group than their control. The mean levels of 8-OHdG in these groups were 47.10 adduct/10^6 dG and 2.6 adduct/10^6 dG, respectively. In 0.18 mg E_2 supplemented OVX female rats, TCDD significantly induced the formation of 8-OHdG, and similar mean amounts of 8-OHdG were found as those in TCDD-treated intact female rats. However, no significant increase of 8-OHdG was detected in TCDD-treated OVX female rats without E_2 supplement ($p > 0.05$). By comparing his results and ours, it was found that similar consistent increased trends were detected in both studies, which indicated that induction of hepatic 8-OHdG by TCDD in OVX female *Sprague-Dawley* rats was estrogen-dependent. This conclusion was further confirmed by the observation that 1.7 mg E_2 supplement induced higher 8-OHdG in TCDD-treated OVX female rats than 0.18 mg E_2 in the present study. Meanwhile, it was found that no significant increase of 8-OHdG was observed in TCDD treated male rats in his study and a much smaller increase of 8-OHdG was observed in male than in female rats in the present study, which

implies that the induction of 8-OHdG by TCDD was sex-dependent. Although larger increases of 8-OHdG were observed in TCDD treated intact and OVX rats with E_2 supplements in Wyde's study than this study, such differences can be explained by the use of different assays. The special advantage for this study is the application of UPLC column. With smaller particle size than the traditional LC column, UPLC column provides more efficient separation. In addition, sample purification was always regarded as an important step for the detection of DNA adducts (33-41). Moreover, mass spectrometry provides more chemical structural information and improves the specificity for the detection of DNA adducts compared with the electrochemical detectors (33-35). However, comprehensive evaluation of these two assays for 8-OHdG detection still needs further study.

Different DNA adducts have different metabolic pathways. 1, N^6-εdA, AcrdG and CrdG are all formed by indirect oxidative stress, but 8-OHdG was formed by direct reaction between ROS and DNA. The dominant repair pathways for these four DNA adducts are also different. At present, two repair pathways are known to be responsible for the repair of 1,N^6-εdA, base excision repair (BER) by alkyl-N-purine DNA glycosylase (ANPG) and direct reversal repair by oxidative demethylase of N1-methyladenine (AlkB) (42). Multiple repair pathways are also implied in the repair of 8-OHdG, BER mainly by OGG1, nucleotide excision repair and mismatch repair (43, 44). As for AcrdG and CrdG, NER is thought to be the primary repair pathway (11). Although different metabolic pathways exist for different DNA adducts, 8-OHdG, 1, N^6-εdA, AcrdG and CrdG exhibit similar patterns in liver DNA of female rats following 30 weeks

exposure to TCDD, as shown in figures 4.2, 4.3A and 4.3B. All of them support the induction of oxidative DNA damage being sex- and estrogen-dependent in the liver of *Sprague-Dawley* rats following 30 weeks exposure to TCDD in this study. The only difference between 8-OHdG and the other DNA adducts is that higher mean levels of $1,N^6$-εdA, AcrdG and CrdG were found in intact female rats than the OVX female rats with E_2 supplement after 30-week TCDD treatment, but not 8-OHdG.

It was previously reported that the volume fraction of γ-glutamyltranspeptidase (GGT)–positive preneoplastic enzyme-altered hepatocellular foci (AHF), a common bioindicator for determining the extent and efficacy of tumor promotion, was induced 14-fold over controls in intact female rats with $p < 0.01$, following 30-week exposure of TCDD (700 ng/kg/week) after DEN initiation (45). With 0.18 mg E_2 supplement, the volume fraction of GGT-positive AHF was also significantly induced in TCDD treated OVX rats compared with their OVX control group, with $p < 0.05$. However, the median value of the volume fraction of GGT-postive AHF was much higher in TCDD treated intact female rats than the OVX rats with 0.18 mg E_2 supplement, although large within-group variance was found in the intact treated group (45). Without E_2, no significant induction of AHF was observed in the TCDD treated OVX group. In addition, following 30 weeks of TCDD exposure, the mean BrdU labeling index in intact TCDD-treated female rats was higher (12 ± 14.5) than in intact control fenale rats (1.6 ± 0.4), although no significant difference between them was found. Significantly increased BrdU labeling indices were observed in E_2 (0.18 mg) supplemented OVX rats compared with their control group (45). Without E_2, no significant difference of BrdU labeling indices was

observed between TCDD-treated OVX and the control OVX rats. By combining the preneoplastic AHF and cell proliferation data with our DNA adduct results, it was found that all of them consistently supported that the hepatoxicity associated with TCDD in female rats was estrogen-dependent. Oxidative DNA damage and the volume fraction of GGT-positive AHF consistently indicated that greater toxic effects were found in TCDD-treated intact female rats than the OVX rats with E_2 supplementation. Moreover, the volume fraction of GGT-positive AHF per cm^3 was significantly higher in OVX control rats than in intact or OVX control with E_2 supplementation (45), oxidative DNA adducts measured in this study were less definitive, which may be the result of sensitivity and variability issues of these biomarkers for hepatoxicity.

The volume fraction of GGT-positive AHF and BrdU labeling index were also determined in TCDD treated male rats by Wyde et al. (29). Although significant induction of the volume fraction of GGT-positive AHF was observed in TCDD-treated male rats without E_2 supplement, only 2-fold increases were observed in these animals, compared to the 14-fold increase found in female rats. BrdU labeling index was significantly enhanced in TCDD and E_2 co-treated male rats compared with its control group receiving the E_2 supplement. Combining these results with our DNA adduct data, it was found that 8-OHdG was a potential biomarker capable of indicating the hepatoxicity of TCDD in male rats. In contrast, 1, N^6-εdA was not predictive, which may be associated with the milder toxic effect of TCDD on the liver tissue of male rats than female rats. These gender differences in estrogen metabolism, especially the relative differences in

formation and clearance of specific active catechol estrogen metabolites are associated with greater oxidative stress in the females (29).

Oxidative stress induced by quinone redox cycling of estrogen metabolites has been regarded as one of the important potential mechanisms of estrogen carcinogenesis (46). The accumulation of ROS formed by the redox cycling between the *o*-quinones and their semiquinone radicals is capable of inducing oxidation of the purine/pyrimidine residues of DNA (47). If this mechanism is the dominant contributor for oxidative DNA damage in TCDD exposed hepatic tissues of female rats, equivalent of higher amounts of oxidative DNA adducts should be induced in TCDD treated OVX rats with E_2 supplement than the intact rats, since serum estradiol concentrations in the former were continuous at levels at or above peak physiological levels (30) and similar CYP450 was induced in the intact and OVX female rats exposed to TCDD (48). However, higher lipid peroxidation adducts were detected in TCDD treated intact rats than the OVX rats with E_2 supplementation and similar amounts of 8-OHdG were found in these two groups, as shown in figure 4.2, 4.3A and 4.3B. These results imply that the effect of estrogen on oxidative DNA damage cannot completely explain the differences in oxidative DNA damage induced in these two groups. Other unknown contributors may exist, which also induced oxidative DNA damage in intact female rats with TCDD promotion.

Oxidative DNA damage was induced in rodents exposed to TCDD probably by enhancing oxidative stress and up-regulation of CYP450 (17-19). The dose-responses of CYP1B1, CYP1A1 and CYP1A2 were characterized in the liver of female *Sprague-*

Dawley rats following 30-week exposure to TCDD with DEN initiation (48). Immunoblot

analysis showed that the protein levels for CYP1B1, CYP1A1 and CYP1A2 exhibited a

dose-dependent induction by TCDD with more induction of CYP1A1 and 1A2, than 1B1.

CYP1A1 catalyzes the metabolic activation of many chemicals, which may contribute to

the formation of active carcinogens, such as PAHs (49). CYP1A1 and 1B1 induction

occurs in virtually every tissue of the body after TCDD exposure (50). CYP1A2 was

found to be linked to TCDD-inducible TCDD binding in the liver. Mice with the intact

CYP1A2 genes had 10 times more TCDD in total liver and concentration than was

present in knockout mice (51). The induction of CYP1A1 by TCDD was associated with

oxidative DNA damage in the Hepa1c1c7 cell line (52). No significant difference was

found for lipid peroxidation, indicated by thiobarbituric acid-reactive substances and

reduction of cytochrome C, in CYP1A2 knockout mice and their parental lineage strains

following a single oral dose of TCDD (25 μg/kg) (53). To date, no connection between

induction of CYP1B1 and oxidative stress in rodents following TCDD exposure has been

determined. Therefore, it is still unclear how these enzymes contribute to oxidative

stress, including oxidative DNA damage and hepatic carcinogenesis of TCDD in female

rats.

The mechanisms for TCDD-estrogen interactions appear to be tissue specific in

female *Sprague-Dawley* rats, which may contribute to the extent of oxidative DNA

damage induced in hepatic DNA of these animals. Liver contains a fully functional

estrogen receptor (ER), with similar characteristics to those identified for ER in

mammary gland and uterus. Liver also showed high affinity binding for E_2 and other

potent estrogens (2). TCDD decreases rat hepatic ER in both chronic and acute exposure experiments, which was also shown in certain AhR-dependent ways (2). In addition to effects on hepatic ER, the up-regulation of CYP450 by TCDD may enhance the metabolism of hepatic estrogen, especially by CYP1A1/1A2 and CYP1B1. CYP1A1/1A2 and CYP1B1 selectively catalyze hydroxylation at the 2- and 4-postions of estrone (E_1) and E_2 respectively, which can be further oxidized into o-quinones *in vivo*, as shown in figure 4.5 (46). *o*-Quinones can undergo redox cycling with the semiquinone radical generating superoxide radicals mediated through CYP450 and enhance oxidative DNA damage (46). The connection between 4-hydroxyestradiol or E_2 and oxidative stress has been observed in several studies (54-56). But very limited studies were available for comparison of oxidative stress induced by 2- ($2\text{-}OHE_1/E_2$) and 4-hydroxylated E_1 or E_2 ($4\text{-}OHE_1/E_2$) except the *in vitro* study by Han et al. (57). However, in his study, only 8-OHdG was monitored to evaluate the oxidative DNA damage and the difference of induced 8-OHdG between $2\text{-}OHE_1/E_2$ ($2\text{-}OHE_1$, $1.00 \pm 0.53/10^5$ dG; $2\text{-}OHE_2$, $0.71 \pm 0.28/10^5$ dG) and $4\text{-}OHE_1/E_2$ ($4\text{-}OHE_1$, $1.62 \pm 0.79/10^5$ dG; $4\text{-}OHE_2$, $1.27 \pm 0.31/10^5$ dG) exposed group was not high. The much higher amounts of 8-OHdG presented in this paper suggest a prominent amount of artifact in this study. Besides oxidative DNA damage induced by quinone redox cycling, estrogen quinones can also directly damage cellular DNA and form bulky DNA adducts, as shown in figure 4.5 (57, 58). However, the number of $E_1/E_2\text{-}2, 3\text{-}Q$ adducts were considerably lower than corresponding adducts from $E_1/E_2\text{-}3, 4\text{-}Q$ (58). Several studies have confirmed the importance of $4\text{-}OHE_1/E_2$ and their bulky DNA adducts during estrogen carcinogenesis (59-62). At present, the relative promutagenic properties of these two bulky classes of DNA adduct remain unclear (63,

64). By altering the metabolism of estrogen in female rats, the relative contribution of oxidative DNA damage and bulky DNA adducts induced by estrogens in TCDD hepatic carcinogenesis is still unknown.
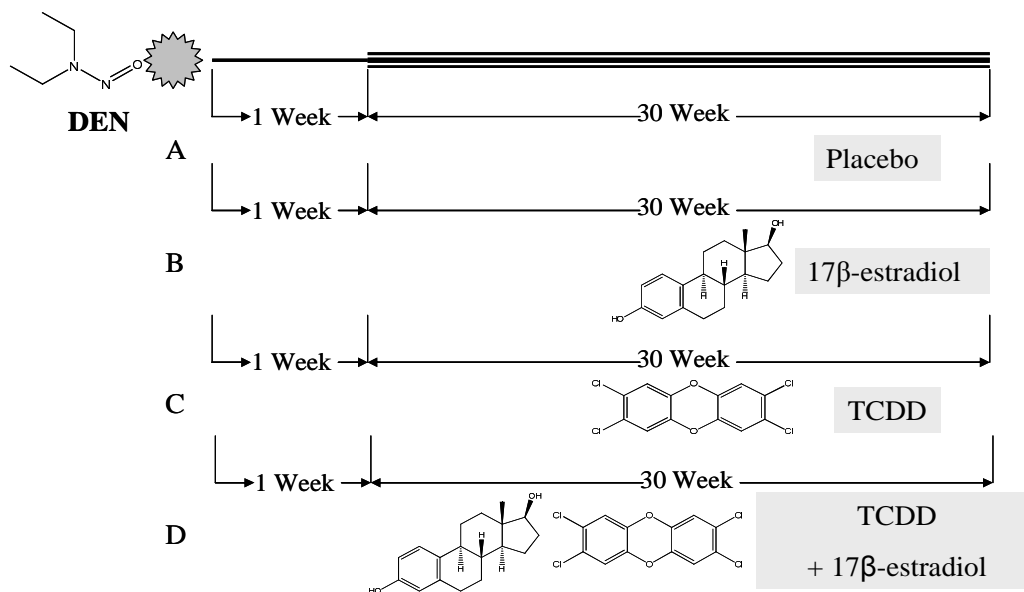
Male rats were treated by A, B, C, D four different exposure scenarios with 0.18 mg 17β-estradiol; OVX female rats were treated by A, B, C, D four scenarios with both 0.18 and 1.7 mg 17β-estradiol; intact female rats were treated by A and C exposure scenarios.

Figure 4.1 The experiment design of this study
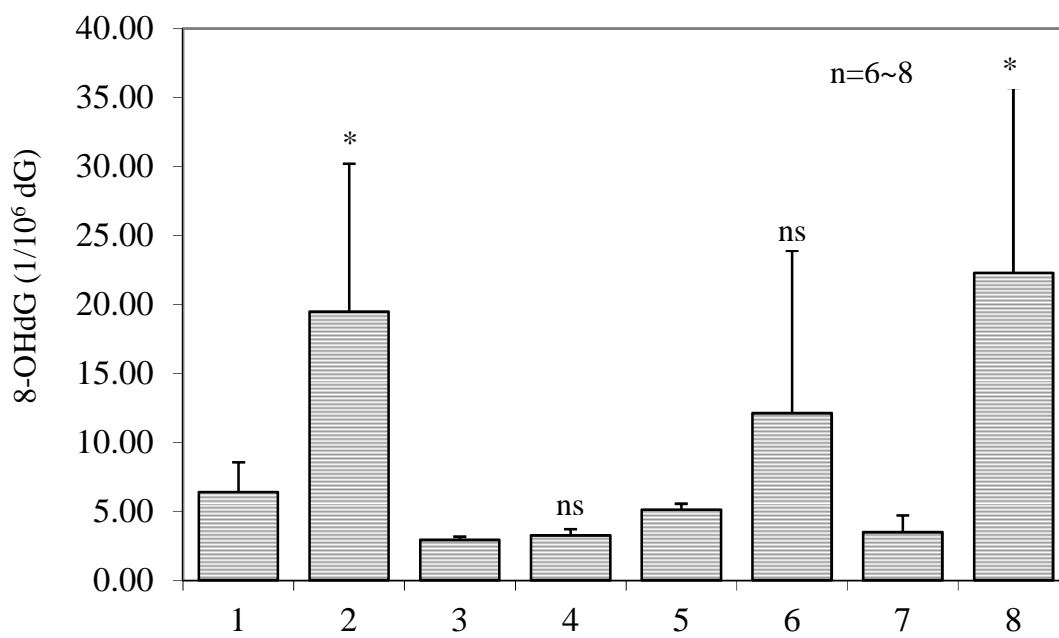
Differences between groups were determined by nonparametric Wilcox Rank Sum test, *p* values for comparison between treated and control groups: *, $p<0.05$; ns, no significant difference, $p>0.05$

Group 1, vehicle control group for intact female rats with DEN initiation; Group 2, 700 ng/kg/week TCDD treated group of intact female rats with DEN initiation; Group 3, vehicle control group for OVX female rats with DEN initiation; Group 4, 700 ng/kg/week TCDD treated group of OVX female rats with DEN initiation; Group 5, vehicle control group of OVX female rats with 0.18 mg/pellet E_2 supplement and DEN initiation; Group 6, 700 ng/kg/week TCDD treated group of OVX female rats with 0.18mg/pellet E_2 supplement and DEN initiation; Group 7, vehicle control group for OVX female rats with 1.7 mg/pellet E_2 supplement and DEN initiation; Group 8, 700 ng/kg/week TCDD treated group of OVX female rats with 1.7 mg/pellet E_2 supplement and DEN initiation.

Figure 4.2 8-OHdG in the liver of intact or OVX female *Sprague-Dawley* rats

Differences between groups were determined by nonparametric Wilcox Rank Sum test, p values for comparison between treated and control groups: *, $p<0.05$; ns, no significant difference, $p>0.05$

Group 1, vehicle control group for intact female rats with DEN initiation; Group 2, 700 ng/kg/week TCDD treated group of intact female rats with DEN initiation; Group 3, vehicle control group for OVX female rats with DEN initiation; Group 4, 700 ng/kg/week TCDD treated group of OVX female rats with DEN initiation; Group 7, vehicle control group for OVX female rats with 1.7 mg/pellet E_2 supplement and DEN initiation; Group 8, 700 ng/kg/week TCDD treated group of OVX female rats with 1.7 mg/pellet E_2 supplement and DEN initiation.

Figure 4.3A 1, N^6-εdA in the liver of intact or OVX female *Sprague-Dawley* rats

Differences between groups were determined by nonparametric Wilcox Rank Sum test, p values for comparison between treated and control groups: *, $p<0.05$; ns, no significant difference, $p>0.05$

Group 1, vehicle control group for intact female rats with DEN initiation; Group 2, 700 ng/kg/week TCDD treated group of intact female rats with DEN initiation; Group 3, vehicle control group for OVX female rats with DEN initiation; Group 4, 700 ng/kg/week TCDD treated group of OVX female rats with DEN initiation; Group 7, vehicle control group for OVX female rats with 1.7 mg/pellet E_2 supplement and DEN initiation; Group 8, 700 ng/kg/week TCDD treated group of OVX female rats with 1.7 mg/pellet E_2 supplement and DEN initiation.

Figure 4.3B AcrdG and CrdG in the liver of intact or OVX female *Sprague-Dawley* rats
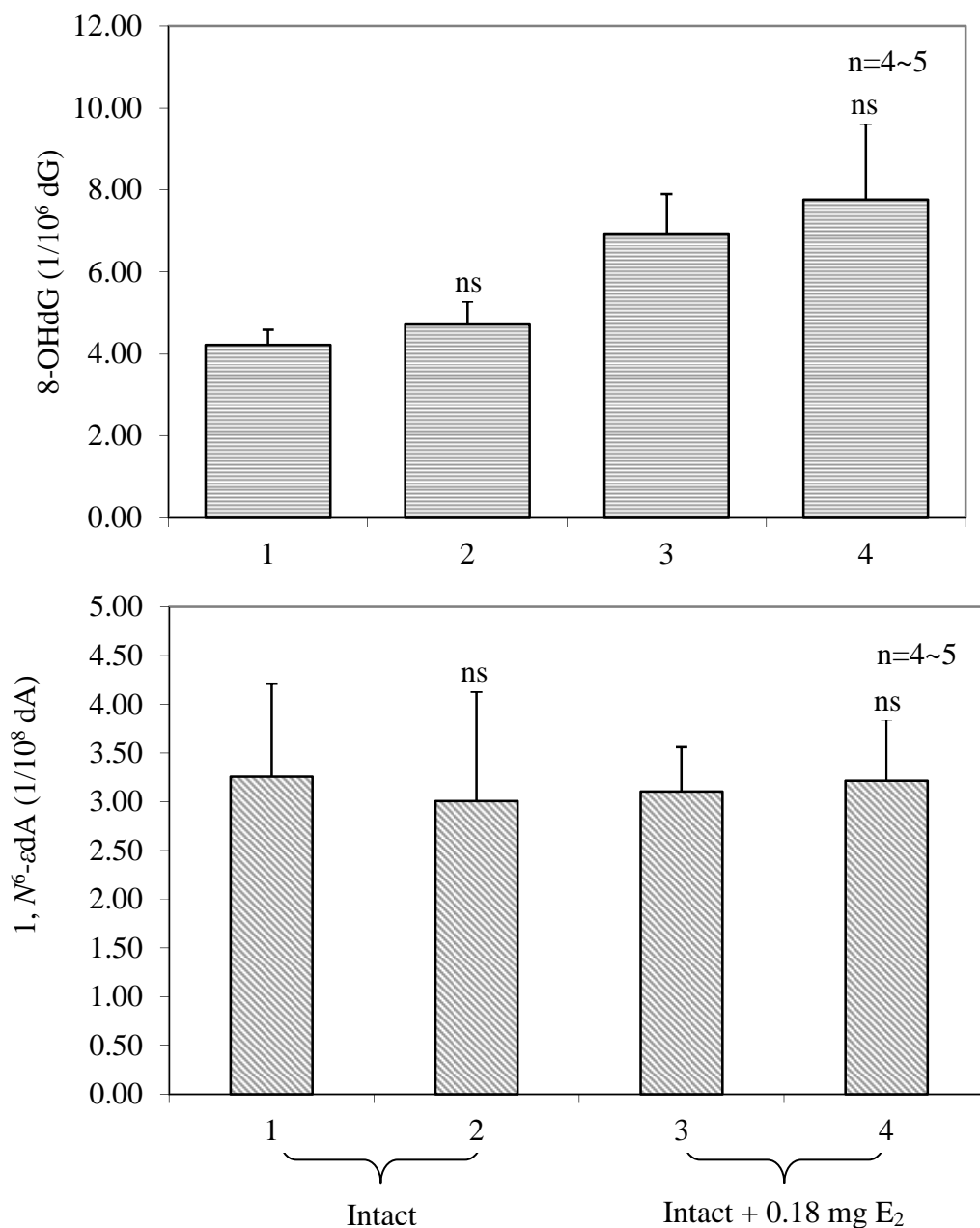
Differences between groups were determined by nonparametric Wilcox Rank Sum test, p values for comparison between treated and control groups: *, $p<0.05$; ns, no significant difference, $p>0.05$

Group 1, vehicle control group for intact male rats with DEN initiation; Group 2, 700 ng/kg/week TCDD treated group of intact male rats with DEN initiation; Group 3, vehicle control group for male rats with 0.18 mg/pellet E_2 supplement and DEN initiation; Group 4, 700 ng/kg/week TCDD treated group of male rats with 0.18 mg/pellet E_2 supplement and DEN initiation.

Figure 4.4 8-OHdG and 1, N^6-εdA in the hepatic DNA of male *Sprague-Dawley* rats
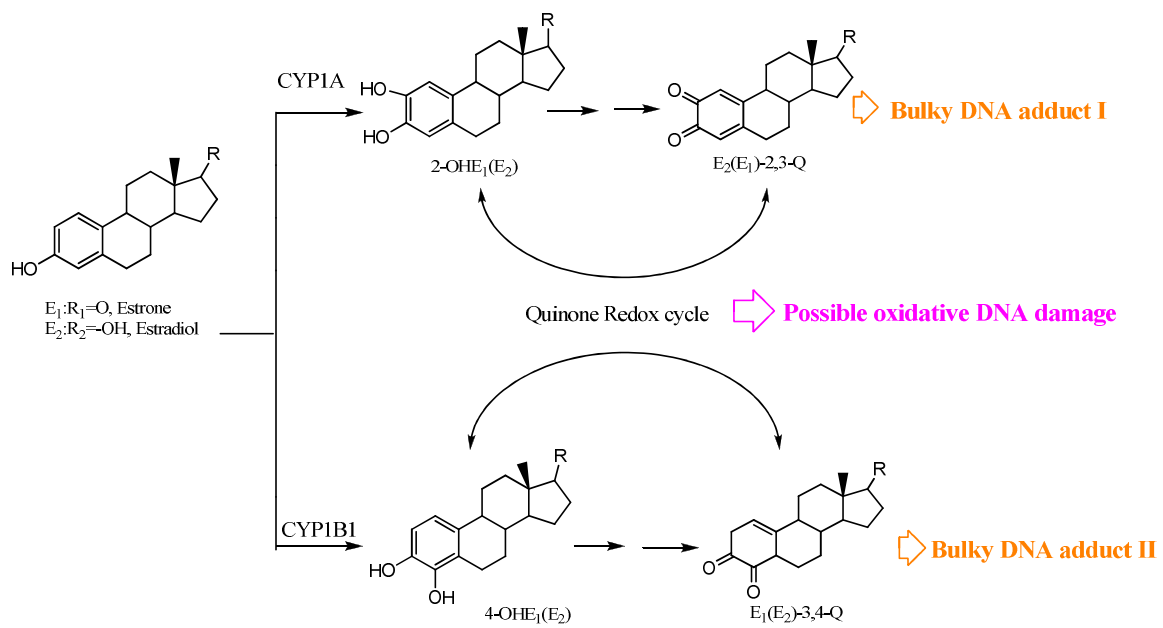
Figure 4.5 The formation of ROS and bulky DNA adducts by estrogen

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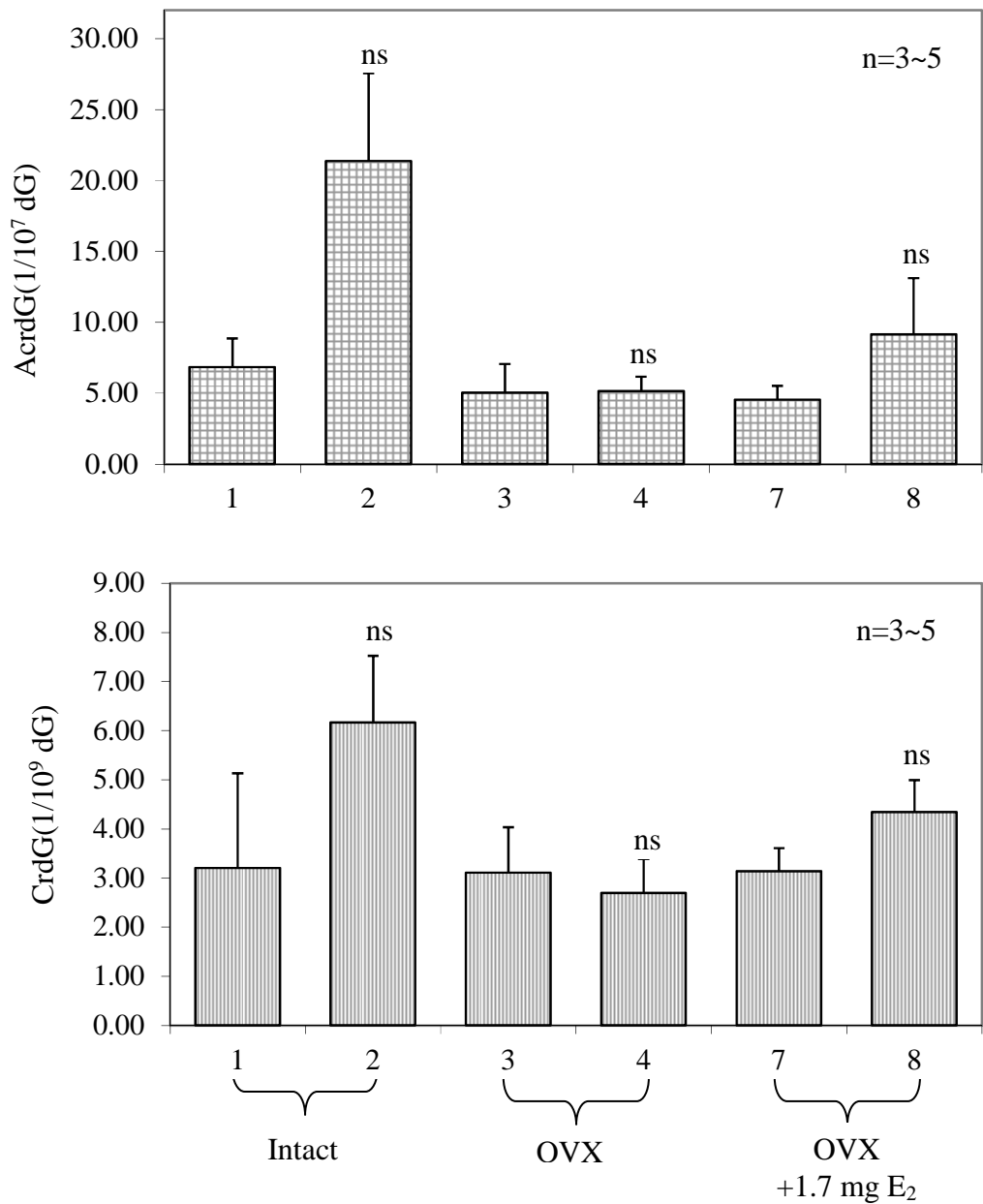
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CHAPTER V

OXIDATIVE DNA DAMAGE IN THE LIVER OF FEMALE SPARAGUE-DAWLEY
RATS CHRONICALLY EXPOSED TO POLYHALOGENATED AROMATIC
HYDROCARBONS (PHAHS)

A. INTRODUCTION

Oxidative stress is thought to play an important role in many diseases including cancer, neurodegeneration and aging (1). Many endogenous processes, as well as exogenous chemicals or their metabolites, are known to induce ROS, which can interact with cellular constituents, especially lipids and nucleic acids, and further induce various DNA adducts (1). To date, several DNA adducts are considered as key endogenous DNA adducts induced by ROS. They include 8-OHdG, 1, N^6-εdA, N^2, 3εG, 1, N^2-εdG, M_1dG, acrolein derived dG adduct (AcrdG), crotonaldehyde derived dG adduct (CrdG) and 4-HNE derived dG adduct (4-HNEdG) (2-3, 31-35). Site-directed mutagenicity studies found that these DNA adducts can induce specific transition and/or transversion point mutations in bacteria and/or mammalian cells (4-6, 39), which contributes to the formation of a hypothesis that the accumulation of ROS-induced DNA damage can induce cancer. Meanwhile, growing evidence supports that these promutagenic DNA adducts are significantly induced in patients with various chronic inflammatory diseases, including cancer (2, 6, 36-38). Although DNA adduct formation is generally regarded as

an important stage for the carcinogenesis of genotoxic environmental chemicals, it may also be an important contributor for the tumorgenesis of nongenotoxic chemicals, especially chemicals which can induce endogenous active metabolites and ROS in the promotion and progression stages of carcinogenesis, such as polyhalogenated aromatic hydrocarbons (PHAHs) (7, 47-52).

PHAHs comprise a large class of compounds, including polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) (8). They are one of the most prevalent groups of pollutants in the environment. PCDDs and PCDFs have no industrial use, but are produced as unwanted by-products of many anthropogenic activities, such as chlorine bleaching of paper and pulp and combustion of wastes and fuels (8). 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 (9). Because these compounds are resistant to degradation and persistent in environment, they have the ability to bioaccumulate and concentrate in humans and animals, which may result in chronic lifetime exposure, possible toxicity and carcinogenicity (8-10). Depending on the site and type of the halogenation, some PHAHs induce a similar spectrum of biochemical and toxic responses in experimental animals. They are regarded as 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 (10). 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. Therefore, these structurally related compounds are commonly referred to as dioxin-like compounds (DLCs). Due to similarity in toxicity and its mechanisms, the concept of toxic equivalency factors (TEF) has been used for the assessment of risk and regulatory control for dioxin-like compounds (DLCs) (10). By using those factors, the toxicity of a PHAHs mixture 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.

The association of oxidative stress and PHAHs, especially TCDD, has been studied for several decades (11-13). Substantial evidence has accumulated to support that TCDD can induce oxidative stress *in vitro*, mammalian cells and rodents. Different biomarkers have been applied to assess the oxidative stress induced by TCDD (11), including hepatic lipid peroxidation (LPO), DNA single strand breaks, hepatic membrane fluidity, glutathione, nonprotein sulfhydryl, and NADPH. Significantly increased LPO with large strain differences was also detected in other organs of rats and mice exposed to TCDD, including kidney, thymus, heart, tests and brain (11). Similarly, PCB-induced oxidative stress was also observed in multiple studies (14-19). These studies indicated 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 ROS-induced DNA adducts (11-19). Because TEF has been widely applied for evaluation of the toxic effects of PHAHs and oxidative stress is universally induced in

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animals exposed by these compounds, it is meaningful to evaluate the application of the TEF approach in the toxicity of PHAHs using oxidative DNA lesions, especially in chronic animal toxicity and carcinogenesis studies.

In cooperation with National Toxicology Program (NTP), multiple oxidative DNA lesions were measured in liver DNA of female *Sprague-Dawley* rats following 53 weeks of exposure of PHAHs by a battery adduct assays. Several typical PHAHs were selected as model compounds in NTP's studies, including TCDD, 3,3′,4,4′,5-pentachlorobiphenyl (PCB126), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB153). PCB126 is a non-*ortho*-substituted PCB with TEF value of 0.1. As the most potent DLC in the environment, PCB126 accounts for 40% to 90% of the toxic potency of dioxin-like PCBs (26). PeCDF, with a TEF value of 0.5, represents the most potent PCDF present in human tissues. PCB153 is a di-*ortho*-substituted nonplanar PCB with the highest concentrations in human samples on a molar basis with no TEF value (27). The structures of these compounds are shown in figure 5.1. In order to evaluate the TEF approach in assessing of the carcinogenesis of PHAHs mixtures, female *Sprague-Dawley* rats were exposed to the ternary mixture of TCDD, PCB126 and PeCDF, the binary mixture of PCB153 and PCB126, as well as PCB153 alone, PCB126 alone and TCDD alone in NTP's studies (8). An important assumption for TEF methodology is that the toxicity of DLCs mixture is dose additive based on the TEF value of the individual component. This study will evaluate this assumption from the standpoint of oxidative DNA damage induced and its relationship to toxicity and carcinogenicity of PHAHs. Since different DNA adducts have different formation and repair pathways *in*

vivo, the application of this oxidative DNA adduct battery will provide more comprehensive information on oxidative DNA damage to estimate the toxicity of PHAHs. This will improve the scientific basis of human risk assessment of PHAHs in environment.

B. MATERIALS AND METHODS

Chemicals

Nucleic acid purification grade lysis buffer, protein precipitation solution and proteinase K were purchased from Gentra Systems (Minneapolis, MN). Other chemical reagents without further explanation were from Sigma-Aldrich Chemical Company (St Louis, MO). HPLC grade water, methanol and other similar chemical solutions were from Thermo Fisher Scientific Company (Raleigh, NC). $^{15}N_5$-1, N^6-εdA standard was synthesized and characterized as described by Ham et al. (20). $^{15}N_5$-8oxodG, $^{15}N_5$-dG and $^{13}C_{10}$-dG were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). 1, N^2-εdG and $^{13}C_{10}$-1, N^2-εdG were synthesized as reported by Kusmierek et al. (21). MDA modified $^{15}N_5$ and $^{14}N_5$ DNA was made by the method of Jeong's (22). AcrdG, CrdG and 4-HNEdG standards and their $^{15}N_5$ labeled internal standards were synthesized according to previous studies (23-25).

Animal exposures

Rat liver tissues were provided by NTP Battelle Laboratories (Columbus, OH), which conducted the studies according to NIEHS contract (N01-ES-75411) (8, 26, 27, 61, 65). Female *Sprague-Dawley* rats were exposed to one of the following materials by gavage 5 days per week for 53 weeks: PCB 153 alone, PCB 126 alone, TCDD alone, the binary mixture of PCB153 and PCB126 or the ternary mixture of TCDD, PCB126 and PeCDF. The TEQ doses used for TCDD and the ternary mixture were 0, 22, 46 and 100 ng/kg/day body weight. The doses used for PCBs were 0, 300 and 1000 ng/kg/day body weight for PCB126, and 0, 550 and 1000 µg/kg/day for PCB 153. In addition, oxidative DNA damage was also measured in liver DNA of rats receiving the following dose of the binary mixture of PCB153 (µg/kg/day) and PCB126 (ng/kg/day), 0+0 and 1000+1000. Liver tissues were collected from 5~8 female rats per group one day after the final exposure and stored frozen at -80 ˚C.

DNA isolation

400 mg tissue samples in 6 ml PBS solution was homogenized by a Tehran homogenizer (Wheaton Instruments, Millville NJ) and then centrifuged at 1700 g for 5 min. After decanting the supernatant, the formed nuclear pellet was suspended in cell lysis buffer. Proteinase K (400 U/ml) was added and samples were rotated overnight in 4 ˚C cold room. On the second day, protein was removed by protein precipitation solution. Then the sample was washed with isopropanol and 70% ethanol subsequently. RNase A was added into the sample before incubation for 30 min at 37 ˚C. Finally, DNA of high purity was obtained and suspended in water at -80 ˚C until adducts analysis. Besides,

TEMPO (20mM final concentration in the sample) was present during the entire DNA isolation process to inhibit the possible formation of free radical species.

Enzymatic digestion and HPLC purification

Slightly different enzyme digestion and HPLC purification approaches were applied for different DNA adducts. Considering the possibility of artifact formation of 8-OHdG during sample work-up, a quick assay was developed especially for the detection of this adduct. The enzymatic hydrolysis was slightly modified based on Pang's studies (3). 100 µg DNA was dissolved in sodium acetate buffer I (sodium acetate 30mM, pH 5.6, 0.2 mM zinc chloride) and incubated at 37 ˚C for 1 h with addition of nuclease P1 (5 µg). 20 units of alkaline phosphate (AP) and 0.0118 units of phosphodiesterase (PDE) were added into the sample with sodium acetate buffer II (sodium acetate 30mM, pH 8.1) and incubated for additional 1 h at 37 ˚C. 5 µl TEMPO (200 mM), and $^{15}N_5$-8-OHdG were added to the sample at the beginning of the digestion. A similar digestion procedure was used for 1, N^6-εdA and AcrdG, 1, N^2-εdG, M_1dG, CrdG or 4-HNEdG, except that DNase I (200 U) was added for the propano adducts. The mixture of $^{15}N_5$-AcrdG, $^{15}N_5$-CrdG, $^{15}N_5$-4-HNEdG (50 fmol for each) and malondialdehyde (MDA) modified IS DNA corresponding to 100 fmol of $^{15}N_5$-M_1dG was added to the sample at the beginning of the digestion. The proteins were removed by micron-10 filter and the hydrolysate was concentrated by speed vacuum before HPLC sample purification.

Different HPLC purification methods were developed for these DNA adducts. A short LC gradient program was applied to purify the sample for 8-OHdG and 1, N^6-εdA.

The fractions containing them were collected based on retention time of standards on the Agilent 1200 Series analytical fraction collector system. All nucleosides were monitored by UV at a wavelength 264 nm. The separation was performed on Atlantis T3 (5 μm, 4.5 × 150 mm) column at 30 °C with flow rate of 1 ml/min. Mobile phases A and B were 10 mM ammonium acetate in water and 100% methanol, respectively. The elution started with 5% B held for 5 min, increased to 10% in 5 min, 20% in 10 min and 80% in the next 10 min before returning to 5% in the final 10 min. Using the same mobile phase and column, the fractions containing CrdG and 4-HNEdG were collected by a different LC program. The initial conditions were 35% B with the flow rate of 0.5 ml/min for 10 min. From 10 to 25 min, B was increased to 70% and held for 10 min before returning to 35% in the final 15 min. For LC purification of 1, N^2-εdG, AcrdG and M_1dG, mobile phase A was 5 mM ammonium formate with 0.1% formic acid in water (pH 3.5); mobile phase B was 100% methanol. The sample was separated with the initial condition 10% B at flow rate 0.6 μl/min. From 5 to 17 min, B was increased to 30%, held for 10 min and then increased to 60% for 10 min to clean the column before returning to 10%.

LC-MS/MS detection

Two different systems were applied for detect the ROS-induced DNA adducts, UPLC-MS/MS and nanoUPLC-MS/MS. The UPLC-MS/MS system, consisting of Aquity UPLC system (Waters, Milford, Massachusetts) and TSQ-Quantum ultra triple quad mass analyzer (ThermoFinnigan, San Jose, CA), was applied for the measurement of 8-OHdG. The Sample was separated on a T3 HSS column 2.1×100mm at a flow rate of 200μl/min. A nanoUPLC-MS/MS system, comprised of 10kpsi nanoacquity Ultra

performance LC system (Waters, Milford, Massachusetts) and a TSQ-Quantum ultra triple quad mass analyzer (ThermoFinnigan, San Jose, CA), was applied to detect the remaining adducts. For 1, N^6-εdA analysis, mobile phase A was 5 mM ammonium formate in water and B was 1% formic acid in acetonitrile. For 1, N^2-εdG, M$_1$dG, AcrdG, CrdG and 4-HNEdG, mobile phase A and B were 0.1% formic acid in water and acetonitrile respectively. The sample was first loaded on the trap column by 99% A in the first 3 min with flow rate 5 μl/min. Then the trap column was connected to the analytical column through the trap valve in the following analysis. For 1, N^6-εdA, 1, N^2-εdG, M$_1$dG, and AcrdG assay, the flow rate was 1.0 μl/min with the gradient program 95% A for 1 min and a linear decrease of phase A from 95% to 70% in 10 min before reconditioning in 95% A for 15 min. For CrdG assay, the flow rate was 1.0 μl/min and the gradient program was 90% A for 1 min, decreasing to 50% A in 10 min and held for 5 min before returning to 90% A. For 4-HNEdG assay, the gradient program was 85% A for 2 min, decreasing to 30% over the next 10 min. Similar MS parameters were utilized for 1, N^6-εdA, 1, N^2-εdG, M$_1$dG, AcrdG, CrdG and 4-HNEdG monitoring the appropriate transitions in SRM mode. The capillary temperature was 285 ˚C. The spray voltage was 1500 V. Neither nebulizing nor drying gas was applied in this system. With positive ESI mode and collision energy 16 eV, two transitions were monitored for the detection of 1, N^6-εdA, 1, N^2-εdG, M$_1$dG and 4-HNEdG respectively, m/z 276.0 → 160.0 and 281.0 → 165.0 for 1, N^6-εdA, m/z 304.0 → 188.0 and 308.0 → 193.0 for M$_1$dG, m/z 292.0 → 176.0 and 302.0 → 181.0 for 1, N^2-εdG and m/z 424.0 → 308.0 and 429.0 → 313.0 for 4-HNEdG. Three and four transitions were monitored for CrdG and AcrdG detection respectively, m/z 338.0 → 222.0, 343.0 → 227.0 with collision energy 12 eV and m/z

338.0 → 178.0 with collision energy 32 eV for CrdG, m/z 324.0 → 208.0, 329.0 → 213.0 with collision energy 12 eV and 324.0 → 164.0, m/z 329.0 → 190.0 with collision energy 32eV for AcrdG. The transitions with collision energy 12 eV were used for quantification of these two adducts.

N^2, 3-εG assay

Briefly, 200 fmol of $^{13}C_4^{15}N_2$-N^2, 3-εG was spiked into 250 μg DNA and incubated at 100 ˚C for 40 min. After neutral thermal hydrolysis, the DNA backbone was removed by micron 10 filters and the hydrolysate was enriched in analyte on a Beckman Ultrasphere C18 column (5 μ, 250 x 4.6 mm). The fraction containing N^2, 3-εG was collected by Agilent 1200 HPLC system. Mobile phase A and B were 10 mM ammonium acetate in water and methanol respectively. The sample was then analyzed on a Waters Acquity UPLC coupled to a Thermo Finnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer. Separation was performed on an Aquasil 10 mm i.d. C18 column (3 μ, 150 x 1mm) at a flow rate of 0.05 ml/min with 0.1% acetic acid in water as phase A and acetonitrile as phase B. Similar MS parameters were utilized to detect N^2, 3-εG to 8-OHdG. The transitions monitored were m/z 176.0 → 176.0 for N^2, 3-εG, and m/z 182.0 → 182.0 for $^{13}C_4^{15}N_2$-N^2, 3-εG.

Statistical analysis

Statistical analyses were performed using R (version 2.11). The effect of chemical exposures on the accumulation of DNA adducts was assessed with nonparametric

isotonic (monotonic) regression model, and p-values were calculated based on 100,000 permutations (28). A *p*-value ≤ 0.05 was considered as a positive association between the level of DNA adducts and PHAHs exposure. Considering the nonhomogenous variance and the limited sample size in some groups, the nonparametric Wilcox Rank Sum test was used to assess the differences of DNA adduct concentrations between control and PHAHs treated rats. Multiple comparisons across pairwise Wilcox Rank Sum tests were adjusted by calculating permutation p-values using 100,000 permutations (29). Two-sided and one-sided *p* values were considered significant if they were ≤ 0.05.

C. RESULTS

Following 53 weeks exposure, increases of 1, N^6-εdA, N^2, 3-εG and 8-OHdG were observed in liver DNA of female rats treated with 1000 µg/kg/day PCB153, although they did not reach statistical significance, as shown in figure 5.2. About a 2-fold increase of 8-OHdG was found in the 1000 µg/kg/day PCB153 exposed group (1.2 $\pm$ 0.49 adduct/10^6 unmodified nucleosides), compared with 0.59 $\pm$ 0.07/10^6 unmodified nucleosides in the control. Slight increases of 1, N^6-εdA and N^2, 3-εG were also observed in these two groups, with 0.55 $\pm$ 0.7 adduct/10^8 unmodified nucleosides in the exposed and 0.27 $\pm$ 0.11/10^8 unmodified nucleosides in the control for 1, N^6-εdA, 0.69 $\pm$ 0.12 adduct/10^8 unmodified nucleosides in the exposed and 0.4 $\pm$ 0.1/10^8 unmodified nucleosides in the control for N^2, 3-εG. No significant increase of these three adducts was found in the 300 µg/kg/day PCB153 exposed group compared with their control group.

Among these three adducts, the amount of 8-OHdG was the highest, almost 100 times higher than those of 1, N^6-εdA and N^2, 3-εG in the control animals.

53-week exposure to PCB126 at 1000 ng/kg/day induced a significant increase in 1, N^6-εdA from 0.48 ± 0.09 adduct/10^8 unmodified nucleosides in the control group to 1.09 ± 0.33/10^8 unmodified nucleosides in the treated group ($p = 0.02$). Induction of N^2, 3-εG ($p = 0.07$) and 8-OHdG ($p = 0.08$) were also observed in the liver DNA of these female rats, as depicted in figure 5.2. 8-OHdG was increased to 1.7 ± 0.76 adduct/10^6 unmodified nucleosides relative to 1.1 ± 0.38 adduct/10^6 unmodified nucleosides in the control group. N^2, 3-εG increased to 0.69 ± 0.15 adduct/10^8 unmodified nucleosides in the exposed group relative to 0.45 ± 0.08 adduct/10^8 unmodified nucleosides in the control group. In addition, a significant increase of 1, N^6-εdA was also observed in the group exposed to PCB 126 at 550 ng/kg/day, with the mean value 1.04 ± 0.26 adduct/10^8 unmodified nucleosides. No significant difference for either of these three adducts was found between the two PCB126 exposure groups at 1000 ng/kg/day and 550 ng/kg/day.

Following 53-week exposure to the binary mixture of PCB153 (1000 µg/kg/day) and PCB126 (1000 ng/kg/day), 8-OHdG, 1, N^6-εdA and N^2, 3-εG were all significantly increased in liver DNA of female rats, compared to the respective control groups, as shown in figure 5.3. More than a 2-fold increase of 8-OHdG was present in the exposed group (3.33 ± 0.59 adduct/10^6 unmodified nucleosides), compared with the control (1.24 ± 0.16 adduct/10^6 unmodified nucleosides). Likewise, significant increases of 1, N^6-εdA (1.09 ± 0.22 adduct/10^8 unmodified nucleosides in the exposed, 0.52 ± 0.17 adduct/10^8

unmodified nucleosides in the control) and N^2, 3-εG (0.70 ± 0.21 adduct/10^8 unmodified

nucleosides in the exposed, 0.32 ± 0.07 adduct/10^8 unmodified nucleosides in the control)

were also observed in the exposed samples compared with their controls. The mean

background level of 8-OHdG was the highest of the three adducts in the control animals,

more than 100 times higher than the other two.

Following 53-week exposures of TCDD by gavage, slight increases of 8-OHdG

were observed in all TCDD exposed groups as shown in figure 5.4, although no

significant increase was observed in all exposed groups compared with the control. The

highest 8-OHdG was detected in 1000 ng/kg/day TCDD exposed group, $1.93 \pm 0.33/10^6$

unmodified nucleosides. 1, N^6-εdA was significantly induced in the liver of all female

rats after TCDD exposure, compared with the control group. Unexpectedly, the highest

mean level of 1, N^6-εdA was observed in the exposed group with the dose of 22 ($1.58 \pm$

0.39 adduct/10^8 unmodified nucleosides) and 46 ng/kg/day (1.52 ± 0.57 adduct/10^8

unmodified nucleosides) instead of the highest dose 100 ng/kg/day (0.53 ± 0.13

adduct/10^8 unmodified nucleosides), as shown in figure 5.4. In addition, a significant

positive association was found between the dose of TCDD and the number of 1, N^6-εdA

adducts in the exposed animals ($p = 0.0006$), which was not observed for 8-OHdG.

Following 53-week exposure of the ternary mixture of TCDD, PCB126 and PeCDF,

a significant increase in 8-OHdG was only observed in the highest TEQ dose group, 100

ng/kg/day. The mean value of 8-OHdG in 100 ng/kg/day ternary mixture exposed groups

was 2.4 ± 0.4 adduct/10^6 unmodified nucleosides, with 1.4 ± 0.1 adduct/10^6 unmodified

nucleosides in the control group. In contrast, 1, N^6-εdA showed a significant increase even at 22 ng/kg/day (0.98 ± 0.29 adduct/10^8 unmodified nucleosides) compared with the control group (0.23 ± 0.14 adduct/10^8 unmodified nucleosides), besides the doses of 46 (1.24 ± 0.41 adduct/10^8 unmodified nucleosides) and 100 ng/kg/day (1.03 ± 0.22 adduct/10^8 unmodified nucleosides), as shown in figure 5.4. The significant positive association was found for both 8-OHdG and 1, N^6-εdA with the TEQ dose of all the ternary mixture exposed animals ($p < 0.0001$).

Several other DNA adducts induced by lipid peroxidation products were also measured in the liver DNA of control and 100 ng/kg/day TCDD or the ternary mixture exposed female rats, which included 1, N^2-εdG, M_1dG, AcrdG, CrdG and 4-HNEdG. The results are shown in figure 5.5. Following 53-week exposure, none of these adducts showed significant increases in the TCDD treated female rats. On the contrary, significant increases were observed for CrdG and 1, N^2-εdG in the ternary mixture exposed animals. Induction of AcrdG ($p = 0.07$) and M_1dG ($p = 0.08$) were also observed in these groups. More than 3-fold increases of M_1dG and AcrdG were found in the 100 ng/kg/day ternary mixture exposed animals followed by approximately 2-fold increases of 1, N^2-εdG, CrdG and 4-HNEdG compared with the control group.

D. DISCUSSION

Mutation studies have suggested that oxidative stress, especially from chronic inflammation, has been associated with carcinogenesis (1). Since the induction of cancer

is a multistage process characterized by accumulation of genetic changes, much effort has been focused on understanding the effects of DNA damage and induction of mutations. At present, oxidative DNA damage is generally regarded as a major source of mutations in living organisms, which includes base modifications (DNA adducts), 2´-deoxyribose modification, DNA strand breakage and DNA cross-linking (30). Among them, DNA adducts are the most widely applied biomarkers in carcinogenesis studies (2, 3, 6, 31-33, 35-39). 8-OHdG is presently the most extensively studied and most abundant oxidative DNA adduct *in vivo*. It has been shown to induce G to T transversions in both bacterial and mammalian cells (37). Many assays have been developed to detect this DNA adduct in animal or human tissues, such as LC-ECD (electrochemical detector), immunoassay and LC-MS/MS (6, 36, 37, 40), but the artifactual formation during sample working-up has hampered its application as a reliable biomarker (40). 1, N^6-εdA is another popular biomarker, widely applied to evaluate the oxidative DNA damage in animal or human tissues (2). As one of the DNA adducts induced during lipid peroxidation (LPO), its background level is much lower than 8-OHdG *in vivo*. Similar to 8-OHdG, 1, N^6-εdA can induce multiple single base changes, with A to G transitions as the dominant mutation. Compared with 1, N^6-εdA, other etheno adducts are less widely studied in carcinogenesis, but all of them have promutagenic properties. Different from etheno adducts, the mutation study on propano adducts is still under development and the promutagenic properties for some of them have been demonstrated (39). Besides differences in promutagenic properties, different DNA adducts have different formation pathways *in vivo*. Considering these differences between oxidative DNA adducts,

multiple adducts were measured in this study to provide a more comprehensive profile of the oxidative DNA damage in the liver of female rats exposed to PHAHs.

PHAHs are predominant nongenotoxic pollutants in environment, and thus their mechanism of carcinogenesis has been studied for decades (41-46). Although several studies indicated no direct genotoxic effects induced by PHAHs, some studies found the induction of oxidative stress by these compounds, which may cause oxidative DNA damage and thereby contribute to their toxicity indirectly (7, 11-19, 47-53). Among them, TCDD is the most studied. The oxidative DNA damage induced by TCDD has been measured *in vitro*, in cell culture and in animal studies (7, 47-49, 53, 54). However, most of the studies were high dose acute studies. Similar to some other toxic effects of TCDD, oxidative DNA damage and oxidative stress was also thought to be associated with AhR and activation of CYP450, especially CYP1A1 (13, 54, 55). The up-regulation of these enzymes triggered changes in metabolism of endogenous compounds *in vivo*, which resulted in oxidative DNA damage. The TEF methodology was developed based on the AhR affinity of PHAHs. Therefore, we hypothesized that oxidative DNA adducts would be highly correlated with the predictions based TEFs for PHAHs.

Using a battery of assays for oxidative DNA adducts, the oxidative DNA damage induced by PCB153 was measured in the liver of *Sprague-Dawley* rats after 53-weeks of exposure. Although PCB153 does not have a TEF value, it slightly increased oxidative DNA damage at the highest dose evaluated, 1000 μg/kg/day, as indicated by 8-OHdG, N^2, 3-εG and 1, N^6-εdA in figure 5.2. Using the same animal tissues, no increase of M_1G was

observed in a previous study (51). As a non-dioxin like compound, the mechanism of oxidative stress induced by PCB153 was regarded as regulated by AhR-independent pathway. PCB153 is a phenobarbital-like inducer of hepatic CYP450, which may significantly induce CYP450 other than CYP1A1 and 1A2 (27) and increase endogenous metabolites that result in reactive oxygen species. With a single i.p. dose of PCB153 (150 µg/kg) in male *Sprague-Dawley* rats, increased hepatic lipid peroxidation was found by Fadhel et al. (56). More than 2 fold increases of thiobarbituric acid reactive substances (TBARS) were detected in the liver of male rats after 150 µmol/kg PCB153 exposure for 6-days. Significant increases of glutathione-S-transferase activity were also observed in acute animal studies by Lamartiniere et al. (57). Subchronic PCB 153 exposure (13 weeks) significantly induced treatment-related depletion of hepatic vitamin A in both male and female *Sprague-Dawley* rats exposed to 50 ppm PCB 153 in the diet (58). However, a high dose chronic exposure to PCB153 seemed necessary for induction of oxidative DNA damage in our study. These results indicate that an AhR-independent pathway is capable of inducing oxidative DNA damage in the liver of PCB 153 exposed rats, since ROS can be enhanced by multiple pathways in biological systems (1).

Compared with PCB153, a 53-weeks exposure to PCB126 induced higher amounts of oxidative DNA damage, as indicated by 8-OHdG and 1, N^6-εdA in figure 5.2. Hassoun et al. studied the abilities of 13-week and 32-week exposure of PCB126 to induce oxidative stress in hepatic tissues of female rats (19, 49, 59). In their studies, approximately 2-fold increases in the production of superoxide anion and lipid peroxidation were observed in both 13-week and 32-week PCB126 (1000ng/kg/day)

exposed female rat livers. DNA single-strand breaks were also significantly induced in the PCB126 exposed samples, with slightly more than 3-fold increases, compared with the control groups. Previously, less than 2-fold increases of M_1G were found in the liver DNA of female rats after 53-week PCB126 (1000ng/kg/day) exposures (51). Moreover, dose-related increases in active acute and subchronic to chronic inflammation was observed in the livers of high dose PCB126 treated animals after 31, 53 and 2-year exposure in the NTP studies (26). Collectively, these results provide strong support for the involvement of oxidative stress and oxidative DNA damage in the toxicity of PCB126.

As shown in figure 5.6, after 53-weeks exposure to PCB153, no dose-dependent increases in hepatocellular adenoma or cholangiocarcinoma were observed in PCB153 exposed animals. Different from PCB153 exposed animals, increased incidences of both cholangiocarcinoma and hepatocellular adenoma were observed in all groups of PCB126 exposed animals after 53-week exposures, with the highest incidence in the group exposed to the highest dose ($p<0.05$). Therefore, our oxidative DNA adduct data consistently agreed with the relative carcinogenicity of PCB153 and PCB126 as evaluated by tumor incidence. As shown in figure 5.6, significant induction of CYP1A1, evaluated by 7-ethoxyresorufin-O-deethylase (EROD) activity, was observed in all PCB126 exposed animals with similar median values in the two exposed groups, 550 and 1000 ng/kg/day ($p < 0.05$). By comparing the number of 8-OHdG, 1, N^6-εdA and N^2, 3-εG between the two PCB126 exposed groups respectively, it was found that none of them showed statistically significant between-group differences, which can not completely explain the difference of the tumor incidence in these two groups. Although significant

induction of EROD was observed in PCB153 exposed animals, the extent of induction by PCB 153 was less than two fold over vehicle controls, which implies the possiblity that oxidative DNA damage could be regulated by other pathways.

Different from oxidative DNA damage induced in PCB153 or PCB126 exposed animals, 8-OHdG, 1, N^6-εdA and N^2, 3-εG were all significantly induced in liver DNA of female rats after 53-weeks of exposure of the binary mixture of PCB153 (1000 μg/kg/day) and PCB126 (100 ng/kg/day), as shown in figure 5.3. Almost 2-fold higher 8-OHdG was found in the binary mixture exposed animals than in PCB153 or PCB126 alone, with a smaller increase in 1, N^6-εdA and N^2, 3-εG. Comparing the oxidative DNA damage data with the histopathology results of the 2-year cancer bioassay from NTP, it was found that higher cholangiocarcinoma incidence (75.5%) and hepatocellualr adenoma (67.7%) was accompanied with higher oxidative DNA damage in the binary mixture exposed animals compared with the animals exposed to PCB153 or 126 alone. Neither oxidative DNA damage, nor tumor incidence can simply be estimated by the TEF approach. Furthermore, more induction of CYP1A1 (3,219.5 ± 271.93 (pmol/minute per mg microsomal protein) was observed in the binary mixture exposed animals than the in the PCB126 treated animals, which may explain the higher oxidative DNA damage in these samples.

Although oxidative stress and oxidative DNA damage are regarded as one of the important mechanisms for hepatic toxicity of TCDD in rats, this hypothesis was proposed mainly based on previous acute high dose studies. Since cancer is a multistage chronic disease, an explanation of the relationship of these studies to the carcinogenic mechanism

of TCDD is likely to require additional key events, such as increased cell proliferation due to toxicity. To date, there are only three chronic studies focusing on oxidative stress or DNA damage in TCDD treated animals. Among them, two studies applied TCDD as a promoter in two-stage carcinogenesis models. Significant increases of 8-OHdG was observed in the female rat liver DNA after 30-week promotion by TCDD and diethylnitrosamine (DEN) initiation (48, 53). Without TCDD, no 8-OHdG was induced after DEN initiation. The increase of 8-OHdG in the liver of rats was also found to be gender and estrogen dependent (48). Recently, Hassoun et al. (19) measured several biomarkers of oxidative stress in the livers of female rats following 32-weeks of TCDD exposure, including increased superoxide anion, lipid peroxidation and DNA single-strand breaks. The highest increase of the mean values of superoxide anion, lipid peroxidation and DNA single-strand breaks was observed in the highest dose group of TCDD (100 ng/kg/day). However, in our studies, no significant increase of 8-OHdG was found in 100 ng/kg/day TCDD-treated female rat liver DNA after 53 weeks exposure. The disparity between all above studies may be due to differences between a two-stage model and one-stage model, poor understanding of metabolic pathways, deficiencies of 8-OHdG as a biomarker for ROS stress, different periods of exposure, or various combinations of these reasons. In contrast to 8-OHdG, significant increases of 1, N^6-εdA were observed in TCDD-treated rat liver DNA. Even the low dose, 22ng/kg/day, induced significant increases of this adduct compared to the 46ng/kg/day group, as shown in figure 5.4. Similar to 8-OHdG, no significant increase of 1, N^2-εdG, M$_1$dG, AcrdG, 4-HNEdG and CrdG was observed in TCDD-treated rat liver DNA with the dose of 100 ng/kg/day after 53 weeks exposure, which further indicated that TCDD did not induce

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extensive amounts of oxidative DNA damage at high doses. Thus oxidative DNA damage may not be a significant contributor to TCDDs hepatic toxicity at high doses in chronic female rat studies.

Compared with the oxidative DNA damage induced by TCDD, higher mean levels of oxidative DNA damage were found in the liver DNA of female rats treated by the ternary mixture of TCDD, PCB126 and PeCDF with the same TEQ dose 100ng/kg/day, as shown in figure 5.5. Therefore, it can be concluded that oxidative DNA damage by DLCs cannot be simply estimated by TEF approach. Besides, Vezina found a limited subset of genes activated by PeCDF and PCB126, but not TCDD in 13-week exposed female rat livers, which included CAT, cytochrome b5 (CYB5), and COX oxidative stress stress response genes (60). This result suggests that PeCDF and PCB126 exposures may be more capable of inducing oxidative stress than TCDD. Both PCB126 and PeCDF were regarded as DLCs, and their dominant toxic effect was considered to be mediated by AhR-dependent pathways. Compared with the up-regulation of CYP450, it was found that the mean value of CYP1A1 was almost two fold higher in the livers of rats treated with the ternary mixture than in rats exposed to TCDD at a 100ng/kg/day TEQ dose (8, 61), as shown in figure 5.7. The mean value of CYP1A2 in the ternary treated liver was almost half that of the same TEQ dose of the TCDD treated group (8, 61). Park et al. (54) found that the induction of CYP1A1 by TCDD was associated with oxidative DNA damage in the hepatoma Hepa1c1c7 cell line. Slezak et al. reported that CYP1A2 did not play a critical role in the oxidative stress response following TCDD exposure in animals (55). However, the connection between oxidative DNA damage and specific CYP450 up-

regulation in TCDD treated animals still needs further study. The 2-year hepatocellular adenoma incidence data supported the application of the TEF approach in the risk assessment of DLCs, since similar dose-response curves were obtained in the TCDD and the ternary mixture exposed female rats (60). However, different cholangiocarcinoma incidence was observed in these groups, as shown in figure 5.7. These inconsistencies further hhighlight the complex interactions of oxidative DNA damage and carcinogenesis for DLCs in the liver of female rats.

Although oxidative stress and chronic inflammation are thought to play multifaceted roles in carcinogenesis, the relative contribution of oxidative DNA damage compared to other possible factors for carcinogenesis is still difficult to evaluate. At present, many PHAHs have been shown to induce oxidative stress in multiple biological systems, supporting a role for oxidative DNA damage, and genomic instability in PHAHs carcinogenicity. In addition, possible indirect genotoxicity, cytotoxicity, stimulation of cell proliferation, inhibition of apoptosis and induction of enzymes are all suggested to be involved in the toxicity of these compounds (41-45). Recent advances in scientific understanding of cancer biology also support the view that environmental chemicals can act through multiple toxicity pathways, modes and/or mechanisms of action to induce cancer (63, 64). However evaluating the relative weight of each possible important contributor to cancer induction is far more complicated than identifying them. Just like the saying, "there are at least two sides to a story and the truth is usually somewhere in between." The role of oxidative DNA damage in the hepatic toxicity effect of PHAHs in female rats may be a case in point.
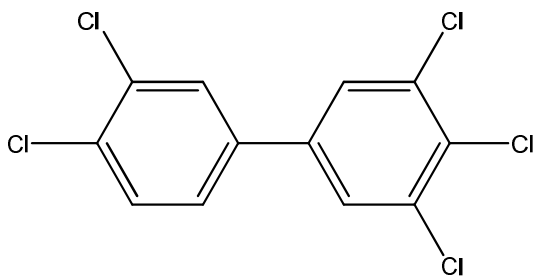
3,3´,4,4´,5-Pentachlorobiphenyl
PCB 126
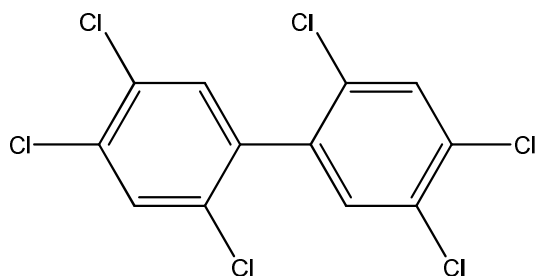
2,2´,4,4´,5,5´-Pentachlorobiphenyl
PCB 153
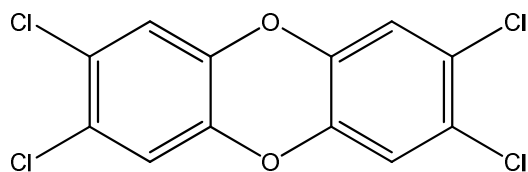
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TCDD
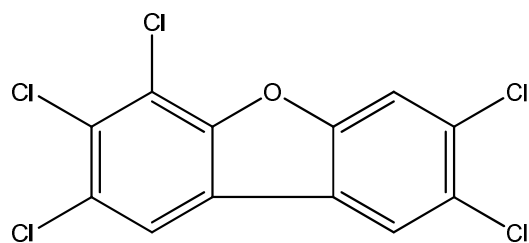
2,3,4,7,8-Pentachlorodibenzofuran
TCDD

Figure 5.1 The structures of typical PHAHs in this study
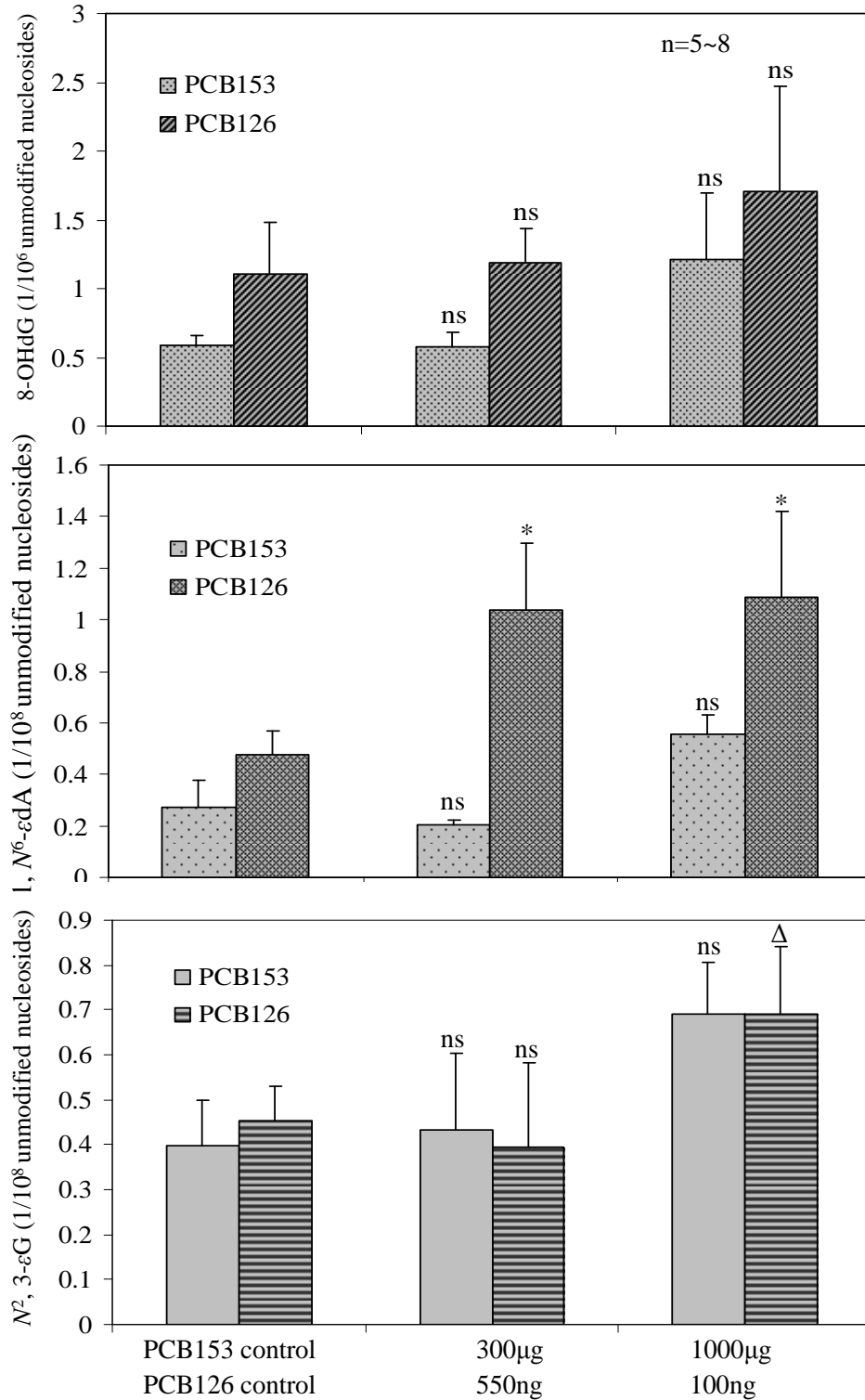
*, p<0.05, ns, no significant difference, Δ, marginally significant, 0.05<p<0.10, comparison between control and exposed groups

Figure 5.2 N^2, 3-εG, 1, N^6-εdA and 8-OHdG in the liver of female Sprague-Dawley rats exposed to PCB153 alone or PCB126 alone for 53 weeks
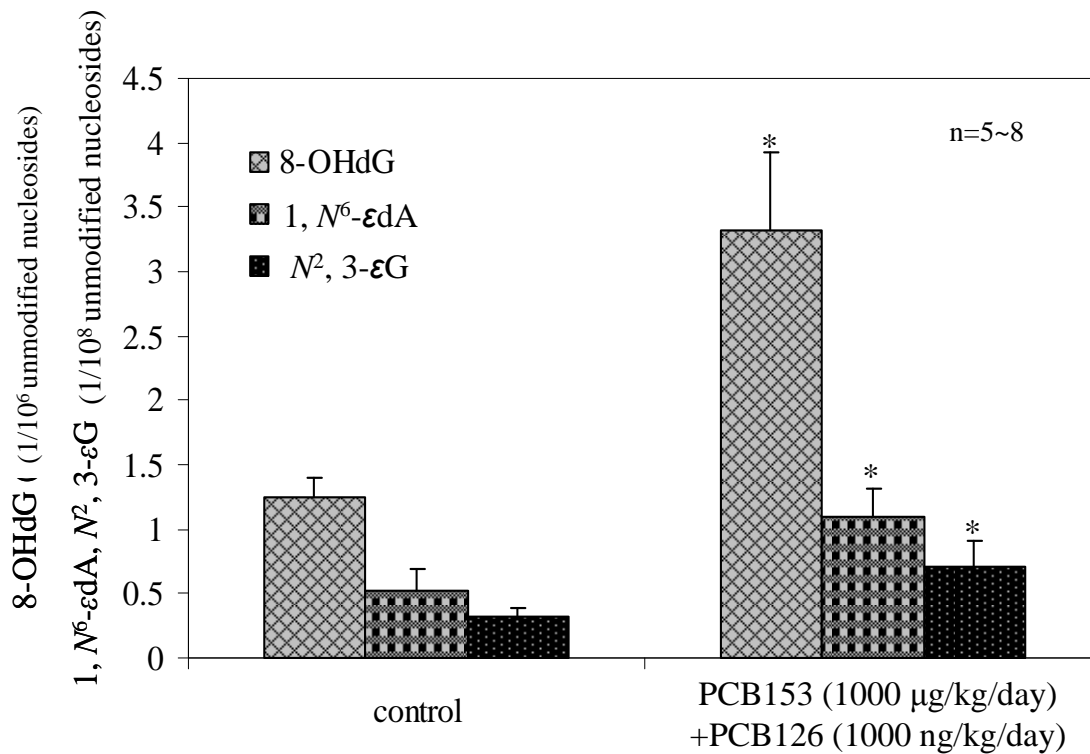
Y-axis label: 8-OHdG ($1/10^6$ unmodified nucleosides)
1, N^6-εdA, N^2, 3-εG ($1/10^8$ unmodified nucleosides)

Legend:
- 8-OHdG
- 1, N^6-εdA
- N^2, 3-εG

n=5~8

control

PCB153 (1000 μg/kg/day)
+PCB126 (1000 ng/kg/day)

*, p<0.05, ns, no significant difference, Δ, marginally significant, 0.05<p<0.10, comparison between control and exposed groups

Figure 5.3 N^2, 3-εG, 1, N^6-εdA and 8-OHdG in the liver of female Sprague-Dawley rats exposed to the binary mixture of PCB153 and PCB126 for 53 weeks
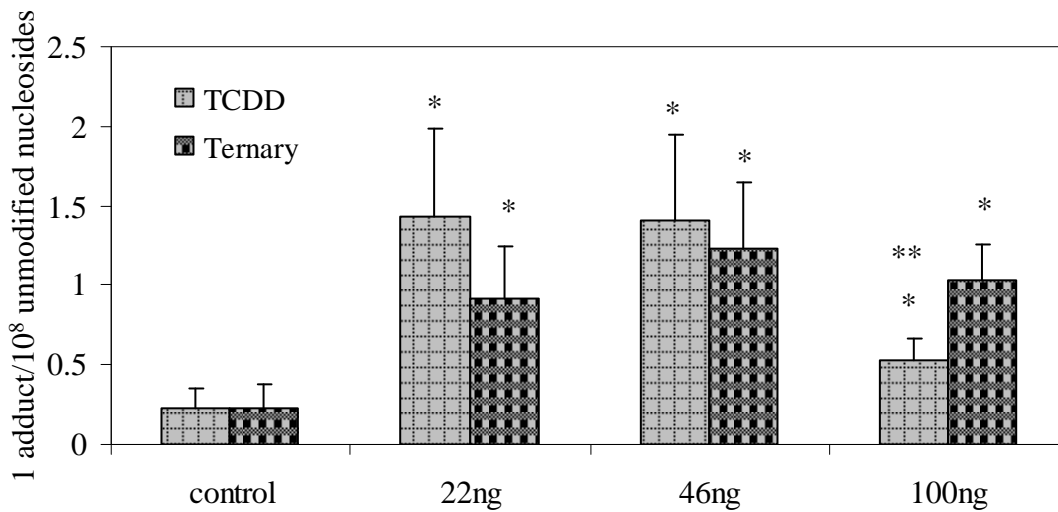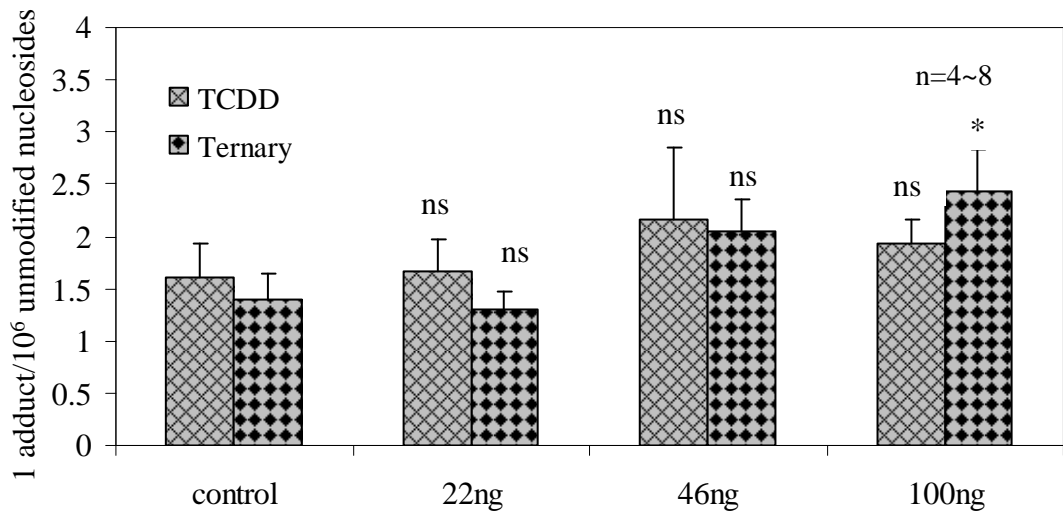
*, p<0.05, ns, no significant difference, comparison between control and exposed groups, **, p<0.05, comparison between ternary mixture and TCDD with the dose of 100 ng/kg/day; n=4~8

Figure 5.4 1, N^6-εdA and 8-OHdG in the liver of female Sprague-Dawley rats exposed to TCDD or the ternary mixture of TCDD, PCB126 and PeCDF for 53 weeks
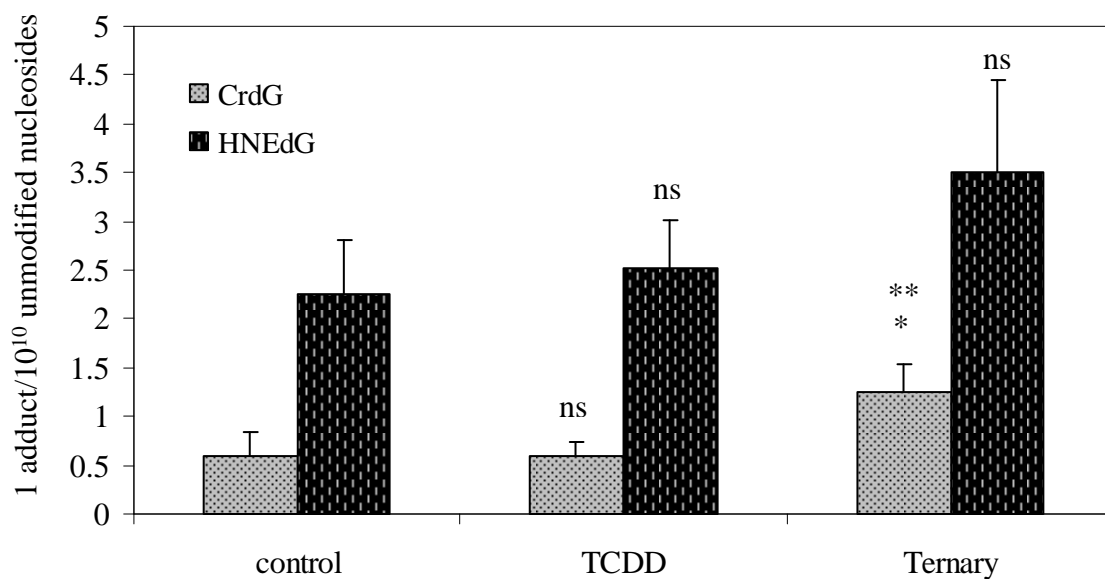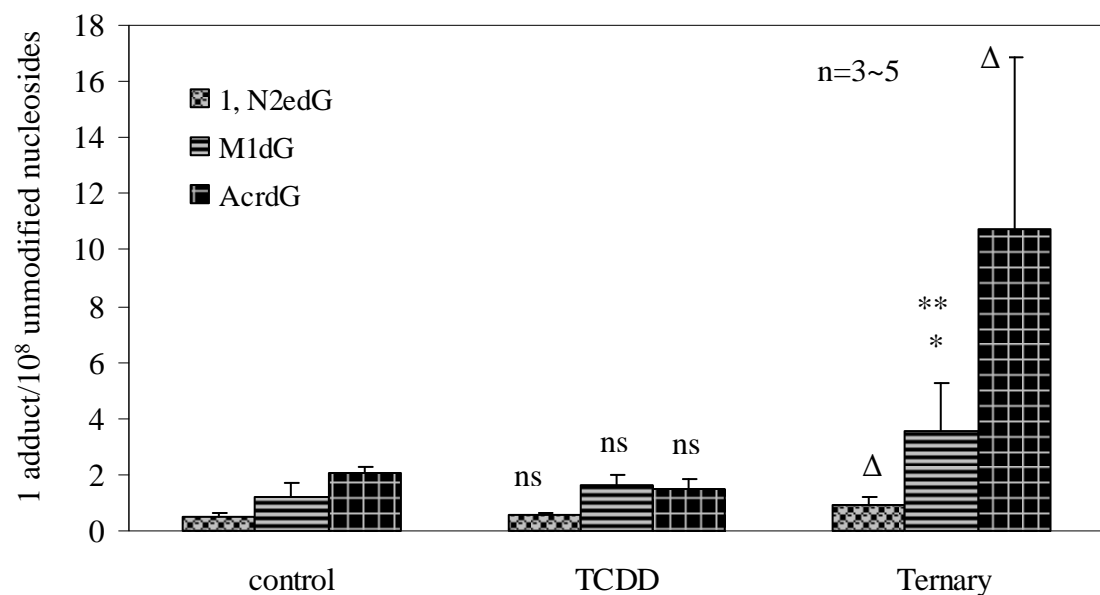
*, p<0.05, ns, no significant difference, Δ, marginally significant, 0.05<p<0.10, comparison between control and exposed groups, **, p<0.05, TCDD vs Ternary

Figure 5.5 1, N^2-εdG, M_1dG, AcrdG, CrdG and 4-HNEdG in the liver of female Sprague-Dawley rats exposed to TCDD or the ternary mixture of TCDD, PCB126 and PeCDF for 53 weeks
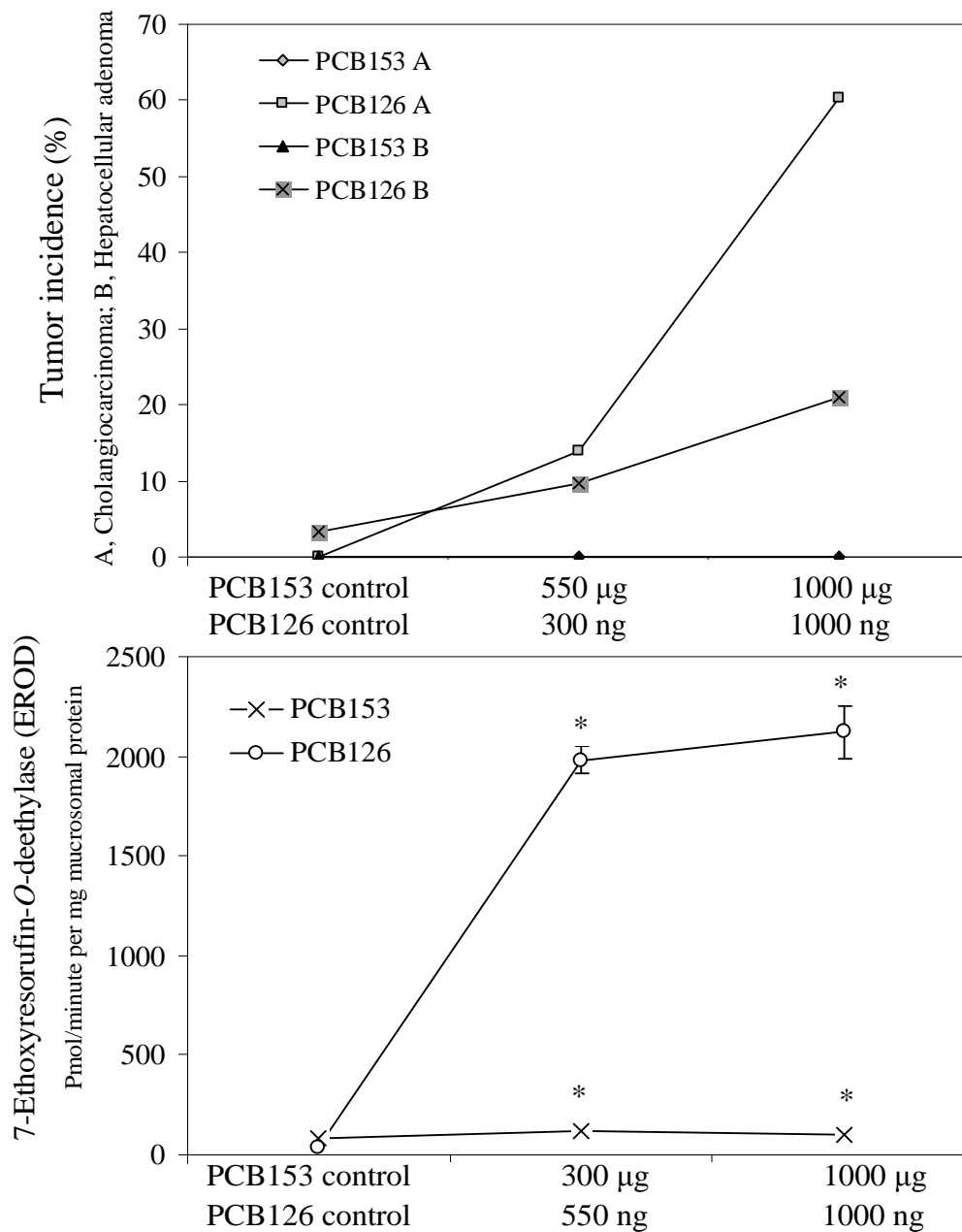
Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test, n=10

Figure 5.6 Tumor incidence (2 years) and enzyme induction in the liver of female Sprague-Dawley rats exposed to PCB153 or PCB126 for 53 weeks

Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test, n=10

Figure 5.7 Tumor incidence (2 years) and enzyme induction in the liver of female Sprague-Dawley rats exposed to TCDD or the mixture of TCDD, PCB126 and PeCDF for 53 weeks

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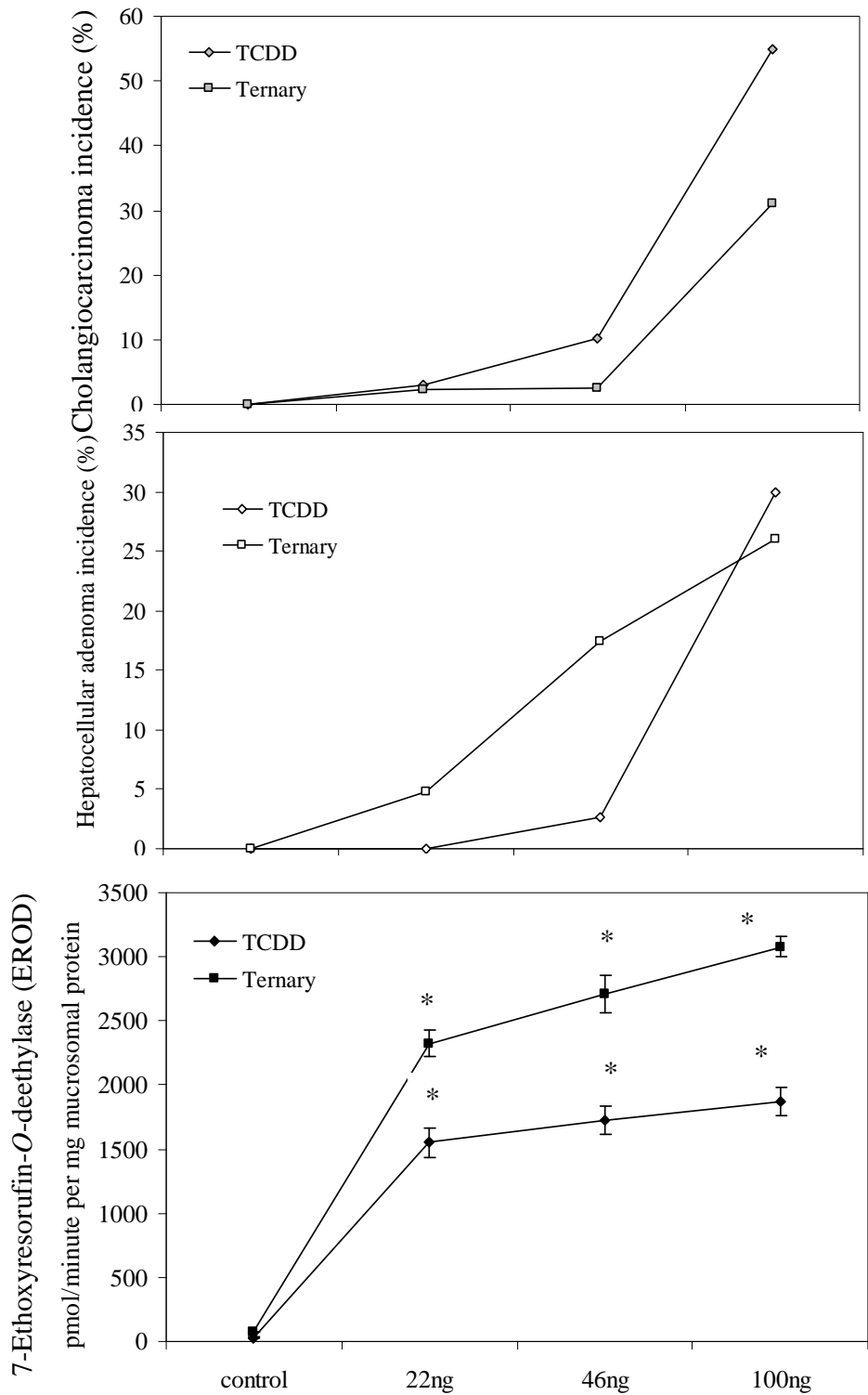(1) Lyrenas L., Zotova E., Ekström L., Morgenster R (2006) Oxidative Stress, Genetic Variation, and Disease. In *Oxidative stress, disease and cancer* (Keshav K Singh, Ed.); pp 371-460, Imperial College Press, Roswell Park Cancer Institute, Buffalo, New York.

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CHAPTER VI

GENERAL DISCUSSION

A. SUMMARY OF FINDINGS

Reactive oxygen species (ROS) are highly reactive molecules formed by the incomplete one-electron reduction of oxygen or electronic excitation of O_2 and produced as normal products of cellular metabolism. Under oxidative stress, considerable amounts of ROS are produced *in vivo*, which contributes to oxidative damage in nucleic acids, proteins and lipids. Among them, oxidative DNA damage has received the most study and is regarded as a possible key event in the mode of action of certain chemical carcinogens, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (4, 5). Prior to this study, the possible association of oxidative DNA damage with hepatocarcinogenesis has been suggested, including monitoring of multiple forms of oxidative DNA damage.

- Each ROS-induced DNA adduct has different mutagenic potential, as well as distinct formation and repair pathways (1, 2).

- The induction of 1, N^6-εdA in vinyl chloride (VC) exposed rodents was observed, which may represent contributions of both endogenous sources (lipid peroxidation) and exogenous (chemical exposure to vinyl chloride) sources (3).

- Significant induction of 8-OHdG was observed in the liver of intact female *Sprague-Dawley* rats exposed to TCDD for 30 weeks after diethylnitrosamine (DEN) initiation (4, 5).

- The induction of hepatic lipid peroxidation (LPO), DNA single strand breaks and some oxidative DNA damage were observed in the livers of animals treated with acute doses of polyhalogenated aromatic hydrocarbons (PHAHs) (6-11).

The aims of this study were to develop a battery of assays for the measurement of ROS-induced DNA damage and to determine whether the oxidative DNA damage was increased in rats after various chemical exposures, including VC, TCDD, polychlorinated biphenyls (PCB) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF). The findings from this study were:

1. Short-term (1-week) high dose (1100 ppm) VC exposure didn't significantly induce endogenous 1, N^6-εdA of weanling or adult rat.

2. Several ROS-induced DNA adducts (8-OHdG, 1, N^6-εdA, AcrdG and CrdG) showed a sex- and estrogen-dependent increase in the two-stage hepatocarcinogenesis studies, which agreed with the induction of 8-OHdG reported previously (4, 5). Higher amounts of LPO-induced DNA adducts were observed in the liver of intact female rats without E_2 supplement than ovariectomized (OVX) female rats with 17β-estradiol (E_2) supplement after TCDD promotion and DEN initiation.

3. The induction of ROS-induced DNA adducts was observed in animals chronically treated with both dioxin-like compounds (DLCs) and nondioxin-like compounds

(NDLCs), indicating that oxidative stress was formed by both AhR-dependent and independent pathways. Higher concentrations of LPO-induced DNA adducts were detected in the ternary mixture TCDD, PeCDF and PCB126 than pure TCDD exposed samples with the same toxic equivalency factor (TEF) values, indicating TEF methodology can not accurately estimate ROS-induced DNA adducts. Much lower increase of oxidative DNA adducts were detected in pure TCDD-treated animals (chapter 5) than the animals exposed to TCDD as a promotor after DEN intiation (chapter 4) (5).

The detection of ROS-induced DNA adducts

Different LC-MS/MS systems were evaluated in this study, including conventional UPLC-MS/MS, capillary LC-MS/MS and nano UPLC-MS/MS. Using 1, N^6-εdA, the sensitivity of nano UPLC-MS/MS was almost 4 times higher than the capillary system, and 20 times higher than the conventional UPLC-MS/MS. The robustness of this system was assessed by measuring 1, N^6-εdA in the DNA of ($^{13}C_2$)-VC exposed *Sprague-Dawley* rats. Using nanoLC-MS/MS with sensitive nanospray interface, sub-fematomole limits of detection (LOD) were obtained for most of ROS-induced DNA adducts in this study. The LOD of 1, N^6-εdA and 1, N^2-εdG was several hundred atmole; the LOD of AcrdG, CrdG and 4-HNEdG was 50 atmole on column. Enzymatic digestion and sample purification by LC were the other two steps optimized for the detection of ROS-induced DNA adducts. System B (NP1+AP+PDE) (12) was selected as the final digestion method for these ROS-induced DNA adducts. Because 4-HNEdG and CrdG were bulky DNA adducts, DNase I was added into the system to ensure the complete digestion. TEMPO (5μl,

1.5mM) was added to inhibit artifact formation for 8-OHdG detection. A short LC purification method was developed for 8-OHdG and 1, N^6-εdA respectively in order to remove possible interference and artifact. Complete separation of AcrdG and dA was realized when 0.1% formic acid was added into 5mM ammonium formate in water as mobile phase A. CrdG and 4-HNEdG were processed together using high composition of organic solvent in mobile pahse. M_1dG was detected directly by nanoUPLC-MS/MS after LC purification..

The endogenous and exogenous 1, N^6-εdA in weanling and adult *Sprague-Dawley* rats exposed to VC by inhalation

Both adult and weanling *Sprague-Dawley* rats were exposed to 1100 ppm ($^{13}C_2$)-VC for 1 week (6 h/day, 5 days/week). Weanling animals were sacrificed immediately. Adult animals were sacrificed immediately, 2 weeks, 4 weeks, and 8 weeks post exposure. Using the sensitive nanoUPLC-MS/MS assay developed in this study, both endogenous NA- and exogenous ($^{13}C_2$)-1, N^6-εdA was measured in the liver, lung and kidney of these animals. The distribution pattern of ($^{13}C_2$)-1, N^6-εdA in both adult and weanling animals clearly indicated that liver was the primary metabolic organ of VC in rodents (p<0.05), followed by lung and kidney respectively. The NA-1, N^6-εdA level did not show a significant difference in the liver of adult and weanling rats after VC exposure. ($^{13}C_2$)-1, N^6-εdA was detected only in the liver, lung and kidney in the exposed adult animals without recovery, but was not detected in post exposure animals. This implied that 1, N^6-εdA was repaired rapidly in adult rats after VC exposure. Short-term high dose VC

exposure (1 week, 1100ppm) did not induce significant ROS related 1, N^6-εdA in the liver of these animals, which implied that 1, N^6-εdA from endogenous sources did not contribute to the genotoxicity of VC in the liver of *Sprague-Dawley* rats after short-term high dose exposure.

ROS-induced DNA adducts in the liver of *Sprague-Dawley* rats after DEN initiation and TCDD promotion

In this study, several major oxidative DNA adducts were detected in the liver DNA from the same animal study reported by Wyde (5), including 8-OHdG, 1, N^6-εdA, 1, N^2-propano-2'-deoxyguanosines generated from acrolein (AcrdG), chrotonaldehyde (CrdG). Significant induction of 8-OHdG and 1, N^6-εdA was found in the liver of TCDD-treated both intact female rats and E_2 supplemented OVX female rats. The mean values of these DNA adducts in the intact TCDD exposed and control female hepatic DNA were $19.5 \pm 10.7/10^6$ dG and $6.4 \pm 2.2/10^6$ dG for 8-OHdG, $20.3 \pm 8.9/10^8$ dA and $5.8 \pm 2.4/10^8$ dA for 1, N^6-εdA, $21.4 \pm 6.2/10^7$ dG and $6.8 \pm 2.0/10^7$ dG for AcrdG, and $6.2 \pm 1.4/10^9$ dG and $3.2 \pm 1.9/10^9$ dG for CrdG respectively. Following 30 weeks TCDD promotion, higher levels of 1, N^6-εdA, AcrdG and CrdG were detected in intact female rats than E_2 supplemented OVX female rats (1, N^6-εdA, $9.1 \pm 2.2/10^8$ dA; AcrdG, $9.1 \pm 4.0/10^7$ dG; CrdG, $4.3 \pm 0.8/10^9$ dG). 1.7 mg E_2 supplement induced higher 8-OHdG than 0.18 mg E_2 supplement in the liver of OVX TCDD-treated female rats, with the mean levels of $22.3 \pm 13.4/10^6$ dG for 1.7 mg E_2 and $12.1 \pm 11.8/10^6$ dG for 0.18 mg E_2 supplemented group respectively. Without E_2, none of these DNA adducts were significantly induced in the

liver of OVX female rats treated by TCDD. No significant increase of 1, N^6-εdA or 8-OHdG was observed in male rats with or without E_2 supplement following 30 weeks TCDD promotion. These results further confirmed that the induction of ROS relevant DNA adducts occurs via a sex-specific and estrogen-dependent mechanism. A more complicated interaction among TCDD, estrogen and oxidative DNA damage was implied in this study, considering that higher amounts of DNA adducts induced by LPO were found in intact female rats, than E_2 supplemented OVX rats after TCDD promotion.

ROS-induced DNA adducts in the liver of *Sprague-Dawley* rats after chronic exposure of PHAHs and their mixtures

ROS-induced DNA adducts were measured in liver DNA of female *Sprague-Dawley* rats following 53-week exposures to PHAHs, which included PCB153 (nondioxin-like compound), PCB126 (dioxin-like compound), TCDD, the binary mixture of PCB153 and PCB126, and the ternary mixture of TCDD, PCB126 and PeCDF. Eight DNA adducts were measured to provide the comprehensive profile of oxidative DNA lesions, including 8-OHdG, 1, N^6-εdA, N^2, 3-εG, 1, N^2-εdG, M_1dG, AcrdG, CrdG, and 4-HNEdG. Following 53-week exposures, significant increases of 1, N^6-εdA (1.09 ± 0.33/10^8 vs 0.48 ± 0.09/10^8 unmodified nucleosides, p = 0.029) and marginally significant increase of N^2, 3-εG (0.69 ± 0.08/10^8 vs 0.45 ± 0.15/10^8 unmodified nucleosides, p = 0.07) were observed in the highest dose of PCB126 treated animals compared with the control group. 8-OHdG was slightly increased (1.70 ± 0.76/10^6 vs 1.10 ± 0.38/10^6 unmodified nucleosides, p = 0.083). Although statistically significant

increase of 8-OHdG, N^2, 3-εG and 1, N^6-εdA were not observed in PCB153 exposed animals, all these adducts were slightly enhanced, suggesting that both AhR-dependent and independent pathways may induce oxidative DNA damage. Following 53-week exposures to the binary mixture of PCB153 (1000µg/kg/day) and PCB126 (100ng/kg/day), higher amounts of 8-OHdG were detected in the binary group than in groups exposed to PCB126 or 153 alone, although both N^2, 3-εG ($0.70 \pm 0.21/10^8$ unmodified nucleosides) and 1, N^6-εdA ($1.01 \pm 0.22/10^8$ unmodified nucleosides) had numbers of adducts similar to the PCB126 (100ng/kg/day) exposed group. Oxidative DNA lesions provided consistent support for the conclusion that TEF methodology cannot fully account for ROS-derived DNA adducts in the binary mixture studies, which agrees with tumor incidence (2-year exposure), toxicity and CYP1A1 induction data (53-week exposure) in the same animals. Following 53-week exposures, a significant increase of 1, N^6-εdA was observed in all animals exposed to TCDD or the ternary mixture of TCDD, PeCDF and TCDD, including TEQ dose 22, 46 and 100 ng/kg/day groups. 8-OHdG was not significantly induced in most TCDD and ternary mixture treated groups except the 100 ng/kg/day ternary mixture treated group. Significant increases of 1, N^2-εdG, CrdG, AcrdG and M_1dG were detected in the ternary mixture treated animals (100ng/kg/day) but not the TCDD treated group (100ng/kg/day) relative to the respective control groups. Although no significant increase of 4-HNEdG was found in the ternary mixture treated animals ($3.49 \pm 0.96/10^9$ unmodified nucleosides), the mean levels of 4-HNEdG were still higher than the TCDD treated group ($2.52 \pm 0.51/10^9$ unmodified nucleosides). Although similar tumor incidence was observed in TCDD and the ternary mixture treated animals with the same TEQ dose, indicating that TEF methodology could

be applied to the prediction of hepatic tumor incidence of the ternary mixture (13), this approach cannot be simply applied to estimate the oxidative DNA lesions induced by this mixture in the liver of these animals.

B. FUTURE DIRECTIONS

Evaluate the sensitivity of different ROS-induced DNA adducts as biomarker of oxidative stress

At present, we monitored seven different ROS-induced DNA adducts, 8-OHdG, formed directly from ROS, and six LPO-induced DNA adducts including 1, N^6-εdA, 1, N^2-εdG, M$_1$dG, AcrdG, CrdG and 4-HNEdG. However, under conditions of oxidative stress, multiple DNA lesions can be produced by the direct reaction of ROS with DNA, as shown in figure 6.1. The formation of 8-OHdG and 8-OHdA competes with the opening of imidazole ring in purine, which is reduced to yield 2, 6-Diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4, 6-Diamino-5-formamidopyrimidine (FapyAde) (14). 2′-deoxycytidine can deaminate and/or dehydrate when reacting with ROS, inducing the formation of 5-hydroxy-2′-deoxycytidine (5-OHdC) and 5-hydroxyuracil (5-OHUra) as the major products (15). ROS can attack several sites on thymine and induce the formation of thymine glycol, 5-(hydroxymethyl)uracil and 5-formyluracil (15). However, due to the difficulty of sample purification by reverse-phase LC for most DNA adducts directly induced by the reaction of ROS and DNA, only 8-OHdG was selected as a monitoring biomarker in this study. Therefore, it is necessary to monitor other adducts, such as FapyGua, 8-OHdA, FapyAde, 5-OHdC, 5-OHUra and Thymine glycol. Because

most of these adducts have short retention time on reverse phase (RP) columns, it is difficult to separate them from unmodified nucleosides or bases. Fortunately, the development of hydrophilic interaction liquid chromatography (HILIC) columns gave us a second choice. As a version of normal phase liquid chromatography, HILIC columns are more suitable for the separation of polar compounds, which means that compounds with short retention times on RP columns tend to have long retention times on HILIC columns (17).

One of the important aims of adductomics (18) is to find the most sensitive biomarkers for risk assessment of chemical carcinogenesis. In our study, multiple DNA adducts were measured in both OVX and TEF studies. 1, N^6-εdA showed the best sensitivities among all of the studied adducts. However, comprehensive studies are still necessary in order to identify the most sensitive biomarker for identifying ROS as an important Mode of Action for toxicology, chemical carcinogenesis and risk assessment.

Examine effect of length of exposure on ROS-induced DNA adducts

In this study, we found significant induction of ROS-induced DNA adducts in the liver of female *Sprague-Dawley* rats after exposure to PHAHs for 53 weeks. Previously, Wyde et al. studied the induction of 8-OHdG in the liver of female rats after TCDD promotion for 20 and 30 weeks after DEN initiation (5), and found that higher 8-OHdG was observed in the 30-week treated animal than the 20-week treated group. Besides, he also found that sing high dose (1000 and 3000 ng/kg) of TCDD without DEN intiation did not induce 8-OHdG in the liver of female rats. Jeong examined the induction of

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M_1dG in the liver of female C57BL/6J mice treated by a single dose of diverse PHAH mixtures and found that no increases of M_1dG in these animals although significant increase of this adduct was observed in the liver of female *Sprague-Dawley* rats after exposure to PHAH for 53 weeks (20). Although these two studies consistently implied that the induction of ROS-induced DNA adducts was a chronic effect of PHAH in the liver of rodents, comprehensive systematic studies are still limited for the effect of exposure length on ROS-induced DNA adducts in female rats after PHAH exposure. Fortunately, NTP also examined the hepatoxicity of female rats exposed to PHAHs for 13 weeks and 31 weeks. Using these unique precious samples, we can evaluate the effect of length of exposure on ROS-induced DNA adducts in the liver of these animals.

Measure estrogen-induced DNA adducts, abasic sites and ROS-induced DNA adducts in the livers of female Sprague-Dawley rats exposed to TCDD.

In our studies, ROS-induced DNA adducts showed an estrogen-dependent pattern in the liver of female OVX *Sprague-Dawley* rats exposed to TCDD for 30 weeks after DEN initiation. But the association of quinone-redox cycling from estrogen and oxidative DNA damage is still unknown. The contribution of estrogen to the indirect genotoxicity of TCDD is a mystery. Therefore, monitoring both estrogen-induced bulky DNA adducts (19), the chemical-specific biomarkers of exposure, and ROS-induced DNA adducts in these animals can help us better understand the role of estrogen in the hepatoxicity of TCDD in this two-stage model. The dominant biological effects of estrogen-induced DNA adducts is to induce abasic sites (AP sites). N3 adenine adducts of estrogen are lost from DNA rapidly; the half-lives of N7 guanine adducts of estrogen are only 3 hours in

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water (21). Therefore, in order to further confirm the important effect of estrogen on the hepatoxicity of TCDD, we can measure estrogen-induced DNA adducts, AP sites and ROS-induced DNA adducts in the liver of female OVX and intact rats and explore their difference.

Estrogen-induced DNA adducts, AP sites and ROS-induced DNA adducts can also be measured in the liver of female *Sprague-Dawley* rats after 30-week or 52-week exposure of pure TCDD. The results of the one-stage carcinogenesis samples can be compared with the two-stage carcinogenesis samples to determine whether there is difference between them.

All above results will help us better understand the role of estrogen in the induction of ROS-induced DNA adducts in the liver of female rats after TCDD exposure.

Compare the dose/time-response of ROS-induced DNA adducts with oxidative stress-related gene expression in the livers of female *Sprague-Dawley* rats exposed to PHAHs.

The rapid development of genomics provides us novel techniques to examine the gene expression profile in toxicology studies. Using DNA microarray analysis, Olson et al. identified a subgroup of genes activated by PeCDF and PCB126, but not TCDD in the liver of female *Sprague-Dawley* rats exposed to PHAHs for 13 weeks. They included *CAT*, cytochrome *b*5 (*CYB5*), and *COX* oxidatative stress response genes (22). This result may indicate that the induction of oxidative stress in the liver of these animals is

mediated through a unique, AhR-independent pathway activated by PeCDF and PCB126. Using LC-MS/MS, we found significant induction of the oxidative DNA damage in the liver of female rats after exposure to PHAHs for 53 weeks. Therefore, a dose/time-response comparison between these two distinct biomarkers (ROS-induced DNA adducts vs ROS-relevant gene expression) will help us better evaluate their sensitivity for evaluating oxidative stress.

Measure ROS-induced DNA adducts in the other target organs of female rats exposed to PHAHs

Significant induction of ROS-induced DNA adducts were found in the liver of female *Sprague-Dawley* rats chronically exposed to PHAHs in our studies, which indicates that oxidative DNA damage does contribute to the hepatoxicity of PHAHs. However, whether the accumulation of ROS-induced DNA damage contributes to the toxicity of other organs in these animals after PHAHs exposure is still unknown. Since lung and kidney are also the target organs of PHAHs and chronic inflammation is associated with diverse chronic diseases, it is worthwhile to measure ROS-induced DNA adducts in these organ and compare the results with those in the liver to further determine whether ROS-induced DNA adducts are good biomarkers to indicate different toxicity across different tissues.
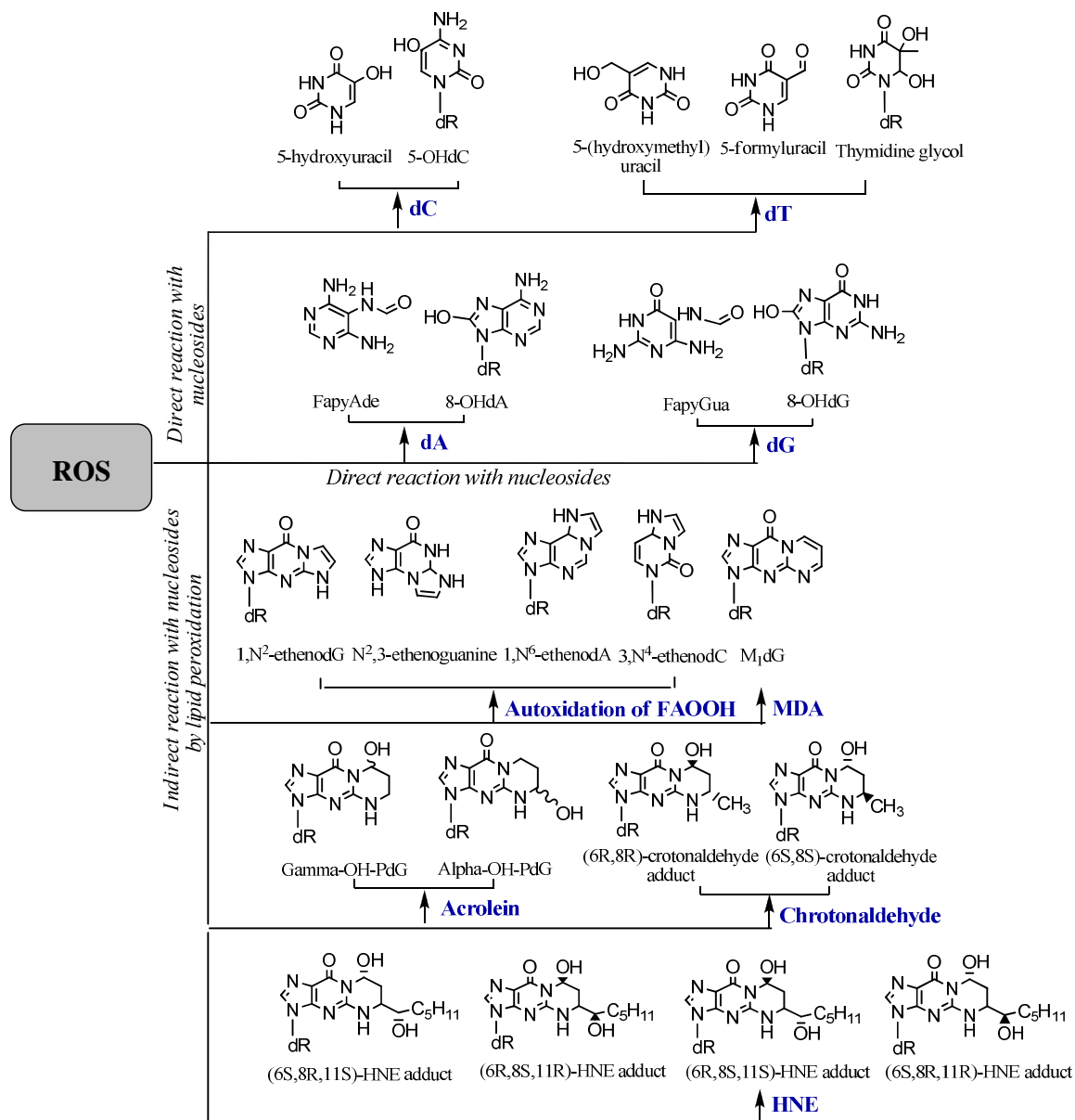
Figure 6.1 Major promutagenic DNA adducts induced by ROS

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