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Review

A Review of Stable Isotope Labeling and Mass Spectrometry Methods to Distinguish Exogenous from Endogenous DNA Adducts and Improve Dose–Response Assessments

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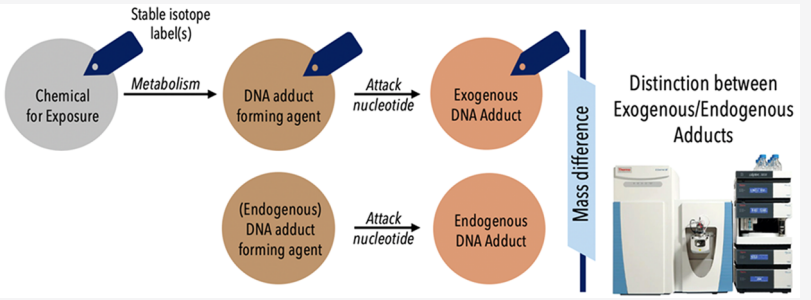

ABSTRACT: Cancer remains the second most frequent cause of death in human populations worldwide, which has been reflected in the emphasis placed on management of risk from environmental chemicals considered to be potential human carcinogens. The formation of DNA adducts has been considered as one of the key events of cancer, and persistence and/or failure of repair of these adducts may lead to mutation, thus initiating cancer. Some chemical carcinogens can produce DNA adducts, and DNA adducts have been used as biomarkers of exposure. However, DNA adducts of various types are also produced endogenously in the course of normal metabolism. Since both endogenous physiological processes and exogenous exposure to xenobiotics can cause DNA adducts, the differentiation of the sources of DNA adducts can be highly informative for cancer risk assessment. This review summarizes a highly applicable methodology, termed stable isotope labeling and mass spectrometry (SILMS), that is superior to previous methods, as it not only provides absolute quantitation of DNA adducts but also differentiates the exogenous and endogenous origins of DNA adducts. SILMS uses stable isotope-labeled substances for exposure, followed by DNA adduct measurement with highly sensitive mass spectrometry. Herein, the utilities and advantage of SILMS have been demonstrated by the rich data sets generated over the last two decades in improving the risk assessment of chemicals with DNA adducts being induced by both endogenous and exogenous sources, such as formaldehyde, vinyl acetate, vinyl chloride, and ethylene oxide.

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1. INTRODUCTION

Cancer remains the second most frequent cause of death in human populations worldwide. This has been reflected in the emphasis placed on management of risk from environmental chemicals considered to be potential human carcinogens. Multiple authoritative bodies engage in the science of risk assessment to evaluate the likelihood that an agent causes cancer and with what severity and under which exposure scenarios. As knowledge of the carcinogenic process has increased, the procedures by which cancer risk is assessed have evolved. For example, risk assessors are moving to low-dose modeling procedures for chemical carcinogens that more truly reflect our understanding of cancer as a complex multifactorial process. This is in contrast to earlier low-dose extrapolation based on an assumption that chemical carcinogens act by producing a single irreversible event that leads to a tumor.

Various carcinogens, including chemicals and biological agents, have been identified, and many of these agents produce cancer by inducing DNA damage, which can be a key event in carcinogenesis.[1−7] DNA damage includes base deamination, abasic sites, single-/double-strand breaks, DNA adducts, intra/interstrand DNA-DNA cross-links, and DNA-protein cross-links (DPC).[8] DNA damage, if not properly repaired, can lead to mutations and, thus, possibly trigger cancer development.[6,9−14] DNA adducts are defined as DNA nucleotides covalently bound to substances that add a functional group to

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Table 1. Selected DNA Adducts Derived from Normal Physiological Processes[a]

physiological process	adduct forming molecules	DNA adduct	mutation	ref
oxidative stress	ROS	8-oxo-dG	GC→TA transversion	11, 39, 43
		8-OH-dG	AT→GC transition	11, 43, 44
		5-OH-dC	C→T transition	15, 46, 47
aberrant DNA methylation	methylating agent	N^2-CH_3-dG	G→A transition	15, 48−50
		N^6-CH_3-dA	AT→GC transition	15, 48
		O^6-Me-dG	G→A transition	15, 51, 52
ethylation	ethylating agent	N^2-Et-dG	G→C transversion	15, 48, 53
		N^6-Et-dA	(no info. for mutagenicity)[b]	
demethylation	FA	N^2-$HOCH_3$-dG	(no info. for mutagenicity)[c]	15, 49, 54−59
one carbon metabolism		N^6-$HOCH_3$-dA	(no info. for mutagenicity)[c]	15, 49, 54−59
alcohol metabolism	AA	N^2-EtD-dG	(no info. for mutagenicity)[c]	15, 55, 60, 61
		N^6-EtD-dA	(no info. for mutagenicity)[b]	15
lipid peroxidation	4-HNE	1,N^6-ε-dA	AT→GC transition	15
			AT→TA transversion	
			AT→CG transversion	
		3,N^4-ε-dC	CG→AT transversion	15, 62
		1,N^2-ε-dG	GC→TA transversion	15, 63
			GC→CG transversion	
	acrolein	1,N^2-PdG	G→T transversion	15, 64
		α−OH-PdG	G→T transversion	65−67
	MDA	M^1-dA	A→G transition	15, 68, 69
		M^1-dG	G→T transversion	15, 69
		M^1-dC	C→T transition	15, 69
	glyceraldehyde	N^2-CE-dG	T→C transition	15, 70
immuno-response	HOCl	5-Cl-dC	C→T transition	71−73

[a]See Abbreviations for the full name of the adducts given for examples. [b]No information for mutagenicity: No literature was found to demonstrate the mutagenicity of N^6-Et-dA and N^6-EtD-dA. [c]No information for mutagenicity: Mutagenic properties of aldehyde-induced DNA adducts are mostly analyzed after $NaCNBH_3$ treatment to stabilize the primary DNA adduct structures. Therefore, the stabilized structures (N^2-CH_3-dG for N^2-$HOCH_3$-dG, N^6-Me-dA for N^6-$HOCH_3$-dA, and N^2-Et-dG for N^2-EtD-dG) were tested for mutagenic abilities rather than the original structure. Due to the instability in nucleoside condition, mutagenic properties of N^2-HOCH3-dG, N^6-$HOCH_3$-dA, and N^2-EtD-dG are largely unknown.

the DNA primary structure and can result from both endogenous substances and exogenous exposures (see Tables 1 and 2). The structures of DNA adducts may reflect the sources.[5−7,15] The pathway from DNA adduct formation to cancer is illustrated in Figure 1. Some compounds, such as formaldehyde (FA), can directly react with DNA to form DNA adducts. Other compounds require biotransformation which generates reactive intermediates that then can interact with DNA nucleotides. For instance, 4-aminobiphenyl (4-ABP) requires bioactivation into *N*-OH-ABP, and benzo[*a*]pyrene (B*a*P) requires bioactivation to metabolites such as B*a*P diol epoxide (BPDE) to create subsequent DNA adducts.[16−25] Additionally, a carcinogen may activate other biochemical pathways and induce the formation of endogenous DNA adducts. For instance, while arsenic does not itself bind covalently with DNA, it can stimulate oxidative stress that results in increases in related DNA adducts, such as 8-oxo-dG.[26,27]

The appearance of DNA adducts can trigger the DNA damage response (DDR) system that includes a set of DNA repair mechanisms, damage tolerance processes, and cell-cycle checkpoint pathways.[28,29] The repair of DNA adducts can be facilitated by a battery of polymerases (Pol) using various mechanisms such as base excision repair (BER) and nucleotide excision repair (NER).[8,30] The repair process, depending on the polymerase and mechanism, can have different error rates.[31−33] For instance, bulky DNA adducts of aryl hydrocarbons such as 3-nitro-benzathrone (NBA) and B*a*P can be either repaired or bypassed depending on which Pol is confronted with the adduct: error-prone Pol η or error-free Pol ζ.[34] Error-prone DNA repairs are more likely to generate mutations.[31,33] When a mutation occurs in genes critical for cell-cycle regulation (e.g., *ras*, *p53*), cell replication rates may increase which ultimately leads to neoplasia. The DNA adducts, if not repaired or if repaired with mutagenic errors, may result in the initiation of carcinogenesis.

Since the structure of DNA adducts can reflect the source of the DNA damage and the level of DNA adducts may reflect tissue-specific or individual-specific risks of cancer, DNA adducts have been explored both as markers of exposure and their formation as likely key events in modes of action (MOA) for carcinogenic chemicals.[35−37] In addition, quantifying the numbers of DNA adducts formed provides a quantitative measure of the amount of a chemical reaching the DNA, providing a dose at the molecular target which accounts effectively for both toxicokinetics and toxicodynamics. It is also known that DNA adducts of various types are produced endogenously in the course of normal metabolism. In this review, the means of detecting and quantifying DNA adducts that can result from both exogenous and endogenous exposure are described. The applications of such methodologies are demonstrated in case studies. Collectively, we suggest that these DNA adduct data can be used in improved and more realistic risk assessment.

2. DNA ADDUCT FORMATION AND CLASSIFICATION

2.1. Sources of DNA Adducts. The DNA structure is vulnerable to damage caused by chemical reactions, such as

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Table 2. Selected DNA Adducts Derived from Environmental Exposure[a]

exposure compound	adduct forming molecules	DNA adduct	mutation	ref
B*a*P	BPDE	N^2-BPDE-dG	G→T transversion	20−24
		N^6-BPDE-dA	A→G transition	20−25
A*α*C	A*α*C-*HN*^{2}-O-Gluc	dG-C8-A*α*C	GC→TA transversion	16, 19, 74
4-ABP	*N*-OH-ABP	dG-C8-4-ABP	G→T transversion G→C transversion G→A transition	16−19
MelQx	*N*-OH-MelQx	dG-C8-MelQx		16, 19, 75
IQ	*N*-OH-IQ	dG-C8-IQ dG-N^2-IQ	GC→TA transversion	76−78
FA	FA	N^2-$HOCH_3$-dG	(no info. for mutagenicity)	15
		N^6-$HOCH_3$-dA	(no info. for mutagenicity)	15
VC	CEO	7-OE-dG	not promutagenic	79, 80
		1,N^2-*ε*-dG	GC→TA transition	63, 79
VAM	AA	N^2-EtD-dG	(no info. for mutagenicity)	81−83
		N^6-EtD-dA	(no info. for mutagenicity)	81−83

[a]See Abbreviations for the full name of the adducts given for examples.

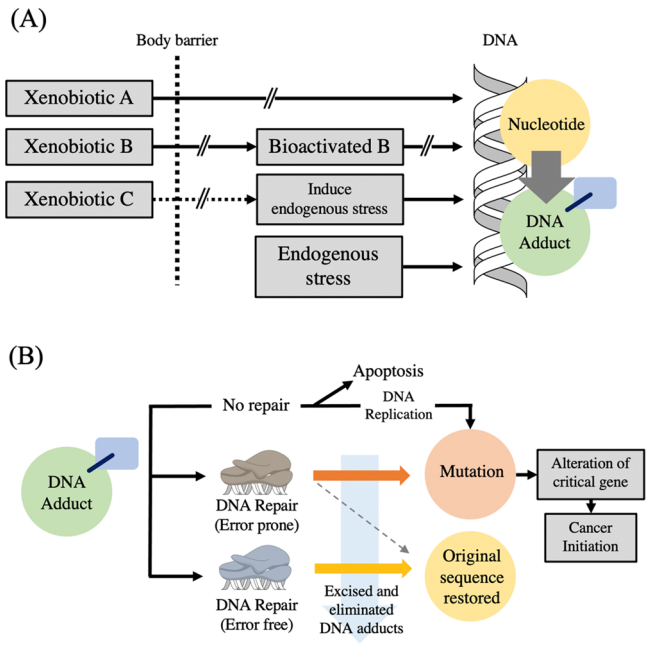

Figure 1. Pathway of chemical-derived DNA Adduct formation and carcinogenesis. (A) Xenobiotics entering the body can directly form or indirectly induce DNA adducts. (B) DNA adducts may trigger damage responses in cells that repair the DNA correctly or incorrectly (form mutations).

alkylation, oxidation, arylamination, and other damaging mechanisms. For example, *N*1, N^2, *N*7, O^6 in dG and *N*3, *N*6 in dA are common vulnerable sites for adduction.[38,39]

While environmental toxicants, such as B*a*P, can cause adduct formation, physiological endogenous processes can form DNA adducts as well. DNA is far from pristine due to ubiquitous endogenous biological processes that generate reactive molecules. For example, oxidative stress is an imbalance in the systemic oxidizing and antioxidizing agents caused by excess production of reactive species with powerful oxidizing ability capable of damaging DNA and other biomolecules.[11,39−42] Hydroxyl radicals, one of the most well-known species contributing to oxidative stress, can interact with nucleotides and generate DNA adducts such as 8-oxo-dG.[11,39,43−45] A few selected endogenous and exogenous DNA adducts, which have been used frequently as biomarkers of physiological processes or exposure, are listed in Tables 1 and 2. Both the identity and the quantity of DNA adducts help to improve understanding of the potential dose−response for mutagenicity and carcinogenic effects of a DNA damaging agent.

2.2. Endogenous and Exogenous DNA Adducts. The formation of DNA adducts leading to mutation can be a key event in carcinogenesis, and DNA adducts can also serve as biomarkers of exposure. Since both endogenous physiological processes and exogenous exposure to xenobiotics can cause DNA adducts, the differentiation of the sources of DNA adducts can be highly informative for cancer risk assessment. Endogenous DNA adducts are generally formed by metabolism, inflammation, oxidation, and other physiologically related biochemical reactions.[84] In contrast, exogenous DNA adducts are induced by exposure to xenobiotics. Some chemicals with the potential to form DNA adducts are not formed endogenously and come only from exogenous (xenobiotic) exposure; thus, the total DNA adduct loading directly reflects the exogenous exposure. For instance, the total BPDE-dG adduct loading solely reflects exogenous exposure to B*a*P.[14,85−90] In contrast, some substances are present or generated in both exogenous environments and normal physiological pathways. For instance, FA is a DNA-reactive chemical that can arise from environmental exposures, and FA can also be formed endogenously through several physiological enzymatic reactions such as oxidative demethylation/deamination and one carbon metabolism.[49,57,59,91,92] When a compound has both an exogenous and endogenous source, assessment of environmental risk is complicated in the absence of being able to accurately assess the contribution of exogenous exposure in relation to the always present endogenous background. More specifically, the questions to be answered to appropriately inform risk assessments for such substances include these:

- What is the partition/contribution between exogenous and endogenous exposure to the DNA adducts formed?
- Which tissues/organs would exogenous substances reach?
- Are there interactions between exogenous and endogenous sources/adducts (e.g., combined sources that result in metabolic saturation)?
- When does the additional tissue or cellular burden from an exogenous source become statistically and/or biologically relevant?

3. DETECTION OF DNA ADDUCTS

The detection of DNA adducts has always been challenging due to their relatively low abundance and the complexity of

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their structures. Several methodologies have been developed over the years to improve sensitivity and specificity for DNA adduct detection and quantitation. Radiolabeled compounds were the first to be used in assessing DNA damage. ^{32}P postlabeling and immunoassays were subsequently developed to overcome the difficulties associated with the use of radioactive ^{3}H and ^{14}C: These compounds present a radiohazard, it was difficult to unambiguously demonstrate covalent adduct formation, and there was a lack of adduct structure information. In the past two decades, DNA adduct characterization and quantitation have been primarily performed by mass spectrometry (MS), which provides both the high sensitivity and specificity needed for DNA adduct measurement. We herein briefly describe these methods of DNA adduct analysis and how these methods have evolved over the decades.

3.1. Radiolabeled Compound. Early studies detected DNA adducts by administrating radiolabeled compounds to animals, then measuring radioactivity of DNA isolated from the test animals. Standard protocols using radiolabeled compound include the following steps: (1) administration of the radioactive compound, (2) waiting for absorption and metabolism, (3) isolation of DNA from the desired sample, and (4) determination of the amount of radioactivity bound to the DNA.[93,94] For the label to successfully reach a nucleotide, the label needs to be retained during the metabolism, bioactivation, and adduct formation processes.[95] The measurement of radioactivity is usually obtained by liquid scintillation counting or accelerator MS.[93,95] The results of detecting the DNA adducts caused by the radiolabeled compound can be presented as a "covalent binding index" (CBI), as described below:[93,94,96]

$$\mathrm{CBI\,(kg\,BW^{-1})} = \frac{\text{damage to DNA}}{\text{administrated dose}} = \frac{\text{chemical bound per mole of DNA (g)}}{\text{chemical administered per body weight (g/kg BW)}}$$

A CBI can be used to assess the effectiveness of an administered substance in affecting DNA and can provide comparison among different substances of their relative strength of potential genotoxicity. However, it has been challenging to demonstrate that all the measured isotope signals actually come from DNA covalent binding. ^{3}H and ^{14}C are labels typically used to synthesize radiolabeled compounds for administration.[95,97] ^{3}H-labeled compounds are less expensive but are easier to lose by reactions like hydrolysis. In contrast, ^{14}C-labeled compounds, although they are more costly and generate more hazardous waste disposal problems, are less likely to lose the isotope labels throughout toxicokinetic processes.[95,97] Radio-hazards are the main drawbacks for using radiolabeled compounds in DNA adduct studies. Furthermore, the decay of the radiolabels limits their use in long-term exposure studies. Endogenous or pre-exposure DNA adducts are undetectable in radiolabeling methods; moreover, the chemical structures of the adducts formed are left unknown. Nevertheless, radiolabeled compounds provided a pioneering approach in quantifying DNA adducts attributed to exogenous exposures in early studies.

3.2. ^{32}P Postlabeling. ^{32}P postlabeling was developed to support the labeling of DNA adducts after DNA is extracted, allowing detection of the total DNA adducts (including both exogenous and endogenous DNA adducts but without distinction). The basis of the ^{32}P postlabeling method is that the radioactive label is introduced after the formation of the adducts.[98] A typical ^{32}P postlabeling is composed of four main steps: (1) DNA enzymatic digestion to nucleoside 3′-monophosphates, (2) DNA adduct enrichment, (3) 5′-OH labeling of the adducts by T4 kinase-catalyzed transference of ^{32}P-*ortho*-phosphate from [γ-^{32}P]ATP, and (4) chromatographic/electrophoretic separation of labeled adducts and detection and quantification by means of their radioactive decay.[98−101] The postlabeling allows for long-term exposure studies and retrospective analysis for DNA adducts. In addition, the label is independent of the toxicokinetics of the substance, thus avoiding losses of label and increases in sensitivity. ^{32}P postlabeling has high sensitivity, such that one adduct in 10^{9}−10^{10} nucleotides can be detected when <10 μg DNA is used.[98,102] However, detection by ^{32}P postlabeling suffers from the lack of chemical structure information. In addition, the postlabeling efficiency can vary substantially across different species of DNA adducts.[103] For instance, B*a*P-related and O^6-CH_3-dG adducts have high postlabeling efficiency, which can usually exceed 95%; in contrast, adducts resulting from 4-ABP exposure have postlabeling efficiency under 30%.[103−105] The difference in postlabeling efficiency causes uncertainty in the true adduct levels. When multiple complex DNA adducts exist in a DNA sample and ^{32}P postlabeling is applied, the variation of postlabeling efficiency among different adducts can obfuscate the results. ^{32}P postlabeling, still in common use, requires small quantities of DNA for detecting the adducts due to its high sensitivity. However, it is limited for understanding and distinguishing the chemical structures of the different adducts that may be colabeled.

3.3. Immunoassay and Immunoaffinity Methods. The demonstration that antibodies against the normal nucleosides can be generated suggested that antibodies against modified nucleosides were possible.[106,107] Intact modified DNA electrostatically complexed to methylated carrier protein, and monoadducts coupled to carrier proteins are the two types of antigens being used for the development of antibodies.[106] DNA adduct quantification is often achieved by using antibodies developed from monoadduct antigens. The early successes in monoadduct antibodies development enabled the characterization of alkylated adducts including O^6-CH_3-dG, O^6-Et-dG, 7-CH_3-dG and bulky adducts such as BPDE-dG.[106,108−112] Several thorough reviews and articles have outlined principles and protocols for developing antibodies.[107,113−116] After the generation and characterization of sensitivity of recognizing the original antigen, the cross-reactivity to other structurally similar adducts should be tested. A significant or wide cross-reactivity can result in errors in quantifying a specific antigen in samples containing multiple adducts.[106] For instance, during early development of polyclonal and monoclonal antibodies that recognize BPDE-dG, the antibodies were found to cross-react with adducts of other PAH-derived diol epoxides.[116,117] Since humans are often co-exposed to B*a*P and other PAHs, the cross-reactivity of BPDE-dG antibodies can confound the results.

The competitive mode in immunoassay, rather than the direct mode, is commonly used on microtiter plates. Inhibition of the antibody, commonly designated as "% inhibition", of the antibody binding due to the presence of a competitor represents the level of the DNA adduct in a sample. Compared to ^{32}P postlabeling, immunoassays generally present high sensitivity as well as good specificity in recognizing particular

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adducts, and outperforming ^{32}P postlabeling in many cases.[95] The main limitations for immunoassays in DNA adduct studies include the need for immunized animals and the difficulty in absolute quantification of the adducts recognized.[96] Quantification of adducts recognized is possible only when a standard is used and little cross-reactivity perturbs the antibody's specificity.[95,118,119] Lastly, immunoassay and immunoaffinity methods are incapable of distinguishing exogenous from endogenous exposures.

3.4. Mass Spectrometry. Detecting DNA adducts with radiolabeled compounds or ^{32}P postlabeling leaves the structure of the adduct undetermined. Although detecting DNA adducts with immunoassays provides verified structural information, the specificity of the antibody developed determines reliability since the antibodies may be perturbed by the cross-reactivity of other similar adducts. Therefore, using MS in characterizing and quantifying DNA adducts has the greatest potential for achieving both high sensitivity and high specificity, due to its ability to provide specific structural information along with high quantitative sensitivity.[95,120]

The elucidation of adduct chemical structures can inform mechanisms of formation (e.g., adduction or covalent binding) of structure−activity relationships and metabolic polymorphisms, leading to differences in reactive metabolite generation. The high chemical specificity of technologically advanced MS methods currently allows unequivocal characterization of the modified DNA. Moreover, quantifying adducts with their fragment ions by tandem mass spectrometers assures satisfying signal-to-noise (S/N) ratio and thus sensitivity can be substantially enhanced. DNA adduct studies with MS were first developed by coupling the MS(s) to GC, which suffered from the complex and laborious work of derivatizing the adducts into analytes and the low sensitivity of GC-MS assays. The rapid development and application of LC-MS to DNA adduct studies has eased DNA sample pretreatment and lowered the detection limits, thus enhancing the versatility and power of MS-based DNA adduct analyses.[95,102] The ionization efficiency increases when the flow rate of a coupling LC decreases, substantially improving the sensitivity of LC-MS assays.[121,122] The emerging of UHPLC or nanoLC, with corresponding ionizing techniques such as nano ESI, further increased the sensitivity of LC-MS methods in detecting DNA adducts by orders of magnitude. When multiple mass analyzers are simultaneously used, the different modes of tandem mass analysis can facilitate holistic DNA adduct studies. For example, a top-down DNA adduct scan, newly coined as "DNA adductomics", can be performed by monitoring fragmentations that detach 2′-deoxyribose (dR) (116 or 116.04735 Da) or nucleobases.[123−126] Thorough reviews on adductomics have been described elsewhere.[123,126]

Besides identifying novel DNA adducts, analyzing DNA adducts by MS is known for its reliable quantification. The MS method typically monitors the stable isotope-labeled internal standards (ISs) and their corresponding DNA adduct analytes simultaneously. This results in highly powerful DNA adduct quantification due to its high specificity, sensitivity, and accuracy.[54,57,79,95,120,127−129] In summary, DNA adduct analysis by MS provides structural confirmation, and the advances in MS technology (e.g., HRMS) continue to improve the reliability and accuracy of such methods. Furthermore, it has enabled technological leaps in identifying and quantitating DNA adducts. With current state-of-the-science nanoLC and/or UHPLC, nano ESI, and tandem MS, DNA adducts can be accurately identified and quantitated at remarkably low concentrations. For example, N^2-HOCH$_2$-dG adducts in rat nasal epithelium related to FA inhalation exposure can be unequivocally identified and quantified with high precision at levels approaching 1 adducted dG in 10^{10} unadducted dG nucleosides. Lastly, as detailed in the following sections, using isotopic labeling, advanced MS techniques are unique in being able to distinguish exogenous from endogenous DNA adducts.

4. DISTINCTION OF ENDOGENOUS AND EXOGENOUS DNA ADDUCTS IN MASS SPECTROMETRY-BASED METHODOLOGY

4.1. Mass Spectrometry-Based Quantitation. MS is currently considered the most accurate and reliable method for DNA adduct quantitation and has been extensively reviewed elsewhere.[54,57,79,95,120,127−129] MS-based analysis typically involves the use of stable isotope-labeled analogues of the analytes (e.g., ^{2}H, ^{15}N, ^{13}C) as the internal standards (ISs). The use of ISs increases reproducibility and accuracy, especially when the ISs are introduced to a sample in early sample preparation. The ISs are structurally identical to the corresponding analytes (except for the heavy isotopes in the structure) and can account for the substantial variability in recovery from sample to sample.[130−132] ISs and their corresponding analytes also have the same ion suppression property on MS, thus the matrix effect can also be compensated for by the addition of ISs.

A calibration curve between the IS and its corresponding analyte is required for DNA adduct quantitation. Since the amount of spiked IS is known, the amount of the analyte can then be calculated using the calibration curve. The synthesis and the purity of the IS are essential for MS-based DNA adduct measurement. ^{2}H, ^{15}N, and ^{13}C are often used as the stable isotope labels in synthesizing the ISs. Multiple labels should ideally be used in synthesizing ISs to avoid the confounding of naturally occurring isotopes of the analyte to the IS.[133] MS-based methods take into account recovery, matrix effects, ion suppression, and other uncertainties during sample preparation, and they have become the technological gold standard for advanced DNA adduct studies.

4.2. Stable Isotope Labeling and Mass Spectrometry. Endogenous DNA adducts are ubiquitously formed by numerous cellular physiological processes, and exogenous DNA adducts are formed after cells are exposed to certain xenobiotics. However, exposure to some exogenous and endogenous chemicals lead to the formation of DNA adducts with identical chemical structures even though the originating/parent chemicals are quite different. For instance, 7-OE-dG can be formed from endogenous processes such as lipid peroxidation and also by environmental or occupational exposure to vinyl chloride.[79,134−137] Not only can the DNA adduct formed be identical from different endogenous and exogenous sources, but some chemicals with both endogenous and exogenous sources can form identical adducts. FA, a highly reactive chemical species that induces DNA adducts such as N^2-HOCH$_2$-dG, can enter the body via inhalation. Meanwhile, FA is also an essential metabolic intermediate in endogenous processes.[49,54,57,59,79,92] Therefore, FA-induced DNA adducts have both endogenous and exogenous sources. In a typical DNA adduct measurement, differentiation of identical endogenous and exogenous adducts cannot be achieved. Thus, it is not possible to assess the contribution of exogenous exposure separately from the substantial endogenous back-

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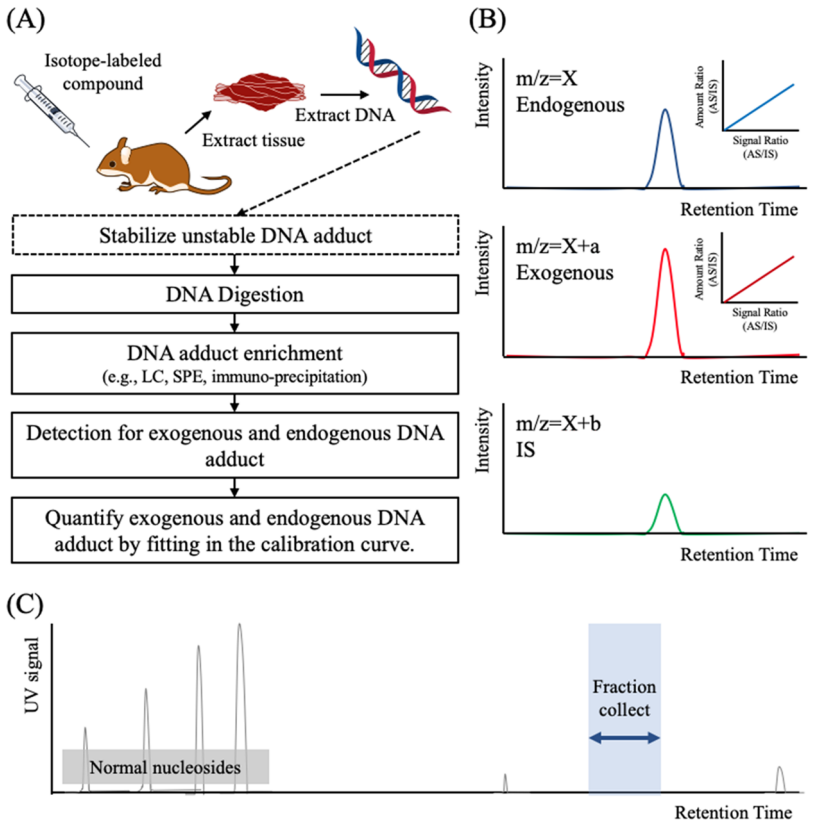

Figure 2. Experiment workflow of SILMS for the detection of DNA adducts induced by exogenous exposure and endogenous sources (A). Simultaneous quantitation of both endogenous (m/z = X) and exogenous DNA adducts (m/z = X + a, typically a > 2 Da compared to the endogenous one) using the internal standards (m/z = X + b, usually several ^{15}N and/or ^{13}C are introduced in chemical synthesis to distinguish internal standards from endogenous and exogenous adducts) and calibration curve (B). Illustration of the separation and enrichment of DNA adducts from normal nucleosides by HPLC to reduce the matrix effects and improve the sensitivity of adduct detection by MS (C).

ground, which hinders science-based risk assessment of chemicals with both endogenous and exogenous sources.

These limitations are surmounted with stable isotope labeling of the exogenously administered test substance, coupled with MS for detection of biomarkers. Using stable isotopic-labeled test materials greatly improves our ability to track the metabolism, bioactivation, and DNA adduct formation in experimental models. If the stable isotopic atom(s) reach and react with DNA nucleotides, the stable isotopic labels are present in the adducts formed. The difference in mass between exogenous (heavy isotope labeled) and endogenous (unlabeled) adducts can be analyzed by MS, resulting in the successful differentiation between endogenous and exogenous adducts. With the ability to distinguish the exogenous adducts, the proportion of exogenous and endogenous adducts can be obtained to determine the exact contribution of the external exposure to the total tissue or molecular target burden. In addition, the levels of endogenous adducts may be perturbed by exogenous exposure. Therefore, comparing the level of endogenous adducts between unexposed and exposed subjects can help tease out whether exogenous exposure affects the homeostasis of endogenous adducts or the total adduct burdens, that is, the sum of the endogenous and exogenous DNA adducts.

The workflow of stable isotope labeling and mass spectrometry (SILMS) is illustrated in Figure 2A. The stable isotope-labeled substances are administrated (e.g., intravascular injection, oral gavage, inhalation) into the test animals, followed by DNA isolation after the exposure is completed. It should be noted that SILMS are applicable to both *in vitro* or *in vivo* studies. After purification, digestion of the DNA into nucleosides (in some cases, nucleotides) is essential for DNA adduct quantification. The level of DNA adducts is often expressed as the proportion of DNA adducts to normal nucleosides. Endogenous DNA adducts are usually present at one per 10^6–10^{10} nucleotides. Some DNA adducts are unstable in nucleotide forms and may need pretreatment to stabilize them before DNA digestion. For example, a DNA adduct resulting from FA exposure, labile N^2-$HOCH_2$-dG, requires reduction by $NaCNBH_3$ to convert it into stable N^2-CH_3-dG for detection and quantitation. The abundance of DNA adducts is normally low; hence, enrichment of DNA adducts is usually essential for identifying and quantifying the adducts. Methods for DNA adduct enrichment or separation include solid-phase extraction, liquid–liquid extraction, immune precipitation, and on/off-line LC prior to adduct detection.[102,129,130,138–141] After DNA adduct enrichment, the DNA adducts in the sample can be more sensitively detected by MS. The identity of the DNA adducts and absolute quantification can be obtained by MS. ISs are spiked into the sample during early sample preparation to account for matrix effects and loss of analytes. The amount of endogenous and exogenous DNA adducts can be simultaneously quantified by comparing the peak intensities or areas of these adducts with corresponding spiked ISs using the calibration curves, as illustrated in Figure 2B. Of note, prior to MS detection, off-line HPLC is commonly used to separate DNA adducts from normal nucleosides to reduce matrix effects and ensure the

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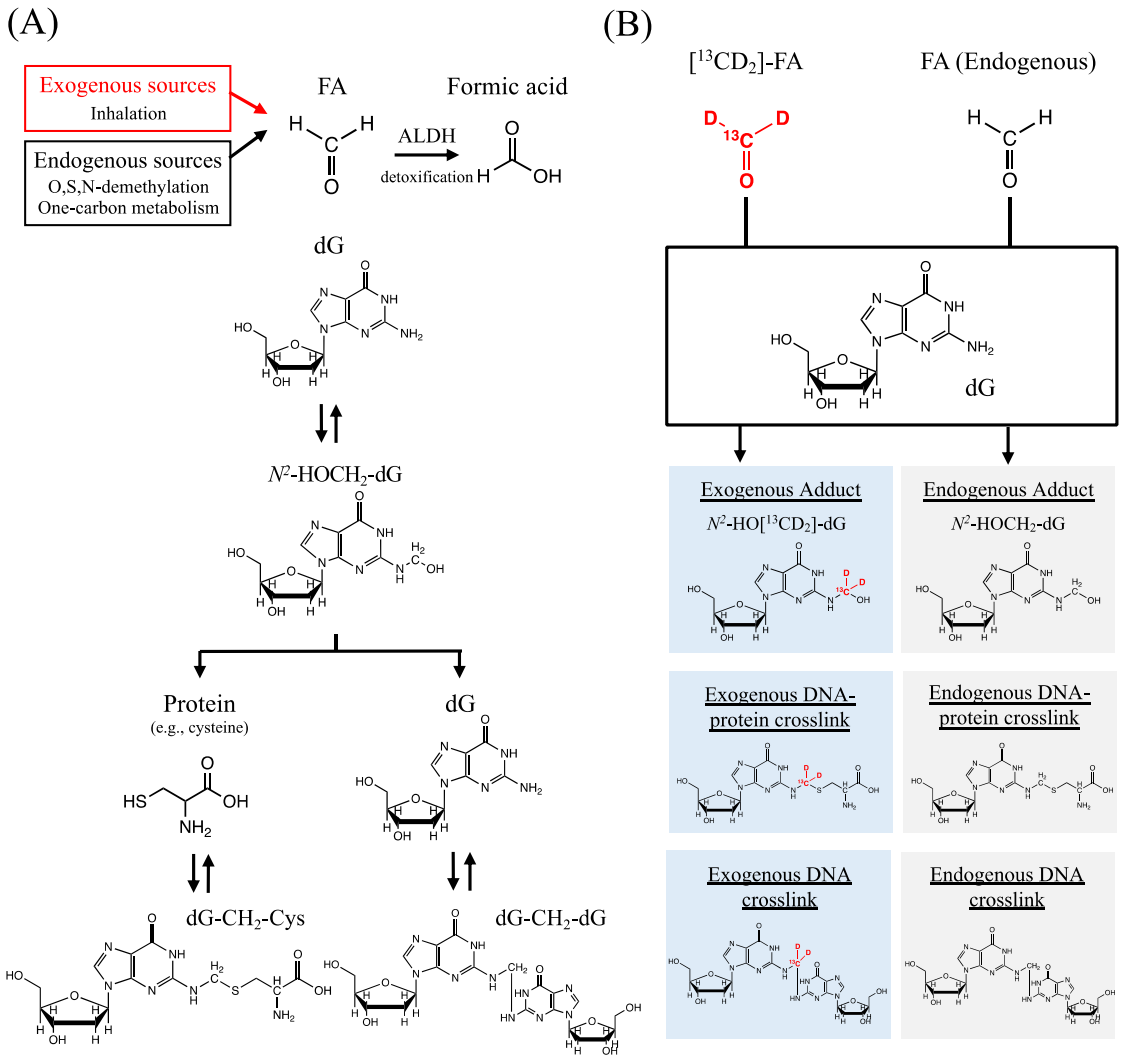

Figure 3. FA directly targets DNA to form diverse DNA lesions, including DNA monoadducts, DNA-DNA cross-links and DNA-protein cross-links (A). Endogenous and exogenous DNA damage can be distinguished due to stable isotope labeling ([$^{13}CD_2$]-FA) (B).

sensitivity of adduct detection by MS, as illustrated in Figure 2C.

4.3. Applications of SILMS in DNA Adduct Studies. SILMS is well suited for distinguishing between endogenously and exogenously formed DNA adducts due to mass differences arising from stable isotope labeling; thus, both endogenous and exogenous DNA adducts can be detected and quantified simultaneously by comparing with spiked ISs. SILMS offers unique power to differentiate the contribution of exogenous exposure under the ubiquitously present endogenous background, and this methodology has generated important data sets to advance science-based risk assessment for chemicals that have identical endogenous and exogenous DNA adducts. For instance, Lu et al., in 2010, administrated [$^{13}CD_2$]-FA to Fischer 344 rats, followed by DNA adduct analysis. SILMS allowed the authors to distinguish DNA adducts and DNA-DNA cross-links originating from endogenous and inhalation-derived FA exposure for the first time, laying the foundation for a series of milestone studies to advance FA risk assessment in an unprecedented manner.[49,54,56] Previously, FA-induced DNA binding and DPC measurements utilized nonchemical-specific methods, which were primarily based on radiolabeled FA and physical chemistry. Until 2010, chemical-specific DNA biomarkers had not been evaluated following inhalation exposure to FA, a primary route of exposure.[49] The original rat study (10 ppm [$^{13}CD_2$]-FA for 1 day or 5 days, 6 h/day) using SILMS was the first one to examine inhalation-specific DNA adducts of FA and distinguish endogenous and exogenous FA-induced DNA adducts.

SILMS is also applicable to the examination of the potential systemic distribution and MOA of a compound using DNA adducts as a biomarker of exposure. The level of DNA adducts may indicate tissue-specific compound dosimetry, bioactivity, metabolic rate, DNA repair efficiency, tissue turnover rate, and other related factors. Thus, the detection and quantitation of exogenous DNA adducts can characterize potential systemic distribution, target tissue dosimetry, and molecular target dosimetry throughout the dose−response curve.[95,142,143] In addition to distinguishing exogenous and endogenous DNA adducts, stable isotope labeling for exposure can be used to trace potential systemic distribution and determine the target tissues where the DNA adducts are formed: Only the tissues to which the substance can be distributed following external exposure can result in the detection of labeled exogenous adducts. For example, in rats exposed to 10 ppm [$^{13}CD_2$]-FA for 5 days, exogenous N^2-$HOCH_2$-dG (N^2-HO[$^{13}CD_2$]-dG)

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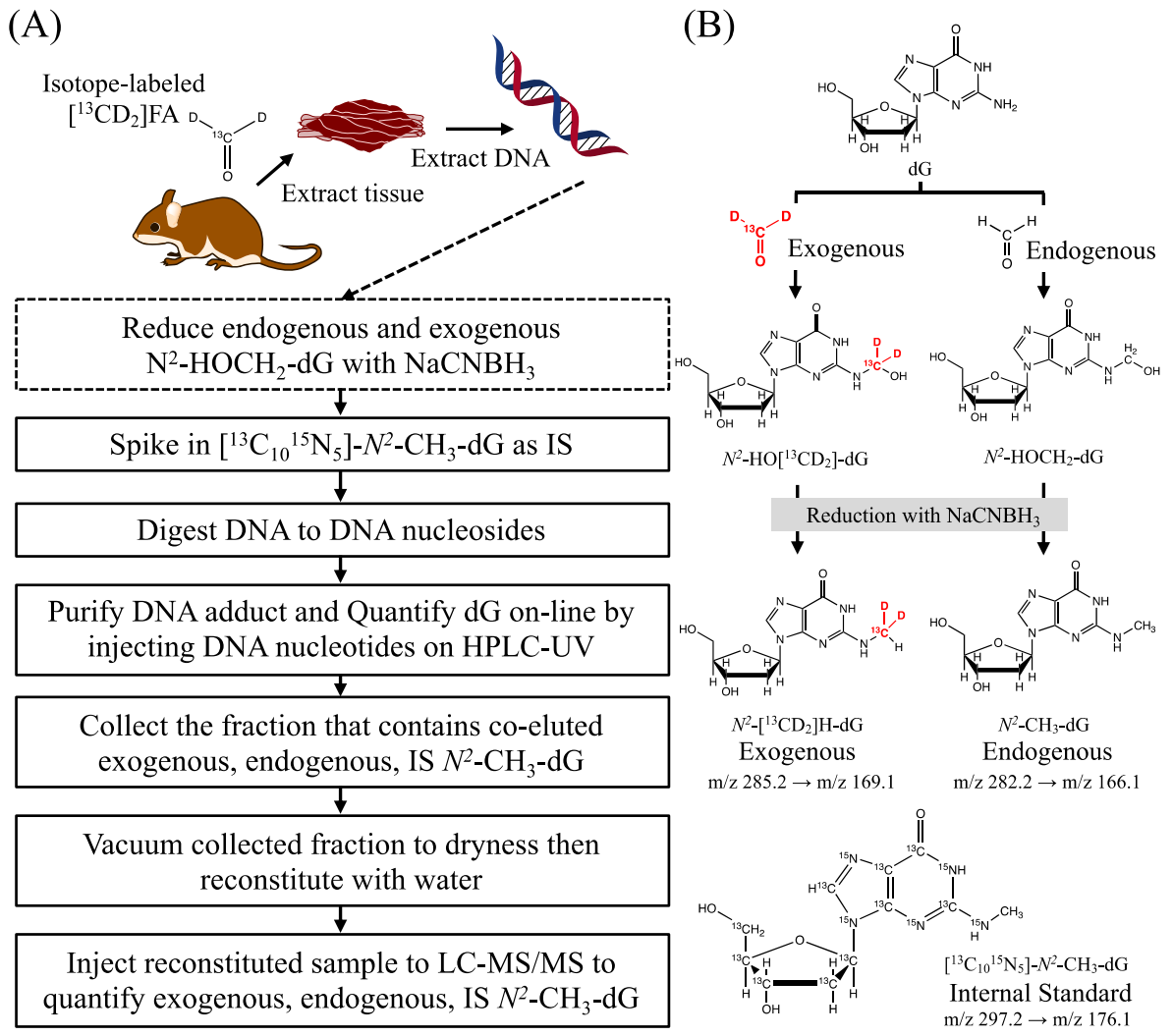

Figure 4. Experimental workflow for the quantitation of DNA adducts from both exogenous and endogenous FA (A). Chemical structures and SRM transitions of the exogenous and endogenous FA-induced DNA adducts and the internal standard spiked in samples for adduct quantitation (B).

was detected only in nasal epithelium, but not detected in bone marrow, peripheral blood mononuclear cells (PBMC), lungs, liver, or any other noncontact site tissues analyzed.[49,56] This study illustrates the powerful application of SILMS in characterizing systemic distribution and possible MOA of compounds of interest in the application of risk assessment.

SILMS can also be used to address the kinetics of formation and loss/repair of DNA adducts induced by exposure. SILMS can distinguish exogenous DNA adducts from their endogenous counterparts, making it possible to monitor the formation and repair/loss of specific DNA adducts in time course experiments. The kinetics of the formation of DNA adducts is essential to estimate or calculate the steady-state level of DNA adducts after exposure, as exogenous DNA adducts only reach the steady-state after repeated exposures. The information on how fast a DNA adduct is eliminated from the body, that is, the adduct's half-life, reflects the active repair and/or spontaneous loss of DNA adducts of interest. Therefore, a kinetic study is often needed to determine the half-life of adducts, data which are necessary to determine the number of repeated exposures required to reach steady state. For example, in a previous study, F344 rats were exposed to 2 ppm [$^{13}CD_2$]-FA for 7, 14, 21, or 28 consecutive days (6 h/day) with postexposure tissue harvesting at 6, 24, 72, and 168 h postexposure, followed by DNA adduct analysis at each time point. The results show exogenous FA-induced DNA adducts reached approximate steady-state concentrations in rat nasal epithelium over the 28 days of exposure, followed by elimination with a half-life ($t_{1/2}$) of 7.1 days. Such toxicokinetic data play a critical role in establishing novel models to improve science-based risk assessment of inhaled FA exposure.

5. CASE STUDIES OF QUANTIFYING DNA ADDUCTS WITH SILMS

SILMS has been used in a number of laboratories to distinguish endogenous and exogenous DNA adducts in order to improve chemical risk assessments. These have included FA, acetaldehyde (AA), vinyl acetate monomer (VAM), vinyl chloride (VC), and ethylene oxide (EO). The use of SILMS now allows assessment of the relative contribution of external exposure to the ubiquitously present background of endogenous DNA adducts. Furthermore, the ultrahigh sensitivity of SILMS enables the quantitation of DNA adducts at very low abundance. SILMS makes it possible to evaluate the systemic distribution of specific compounds

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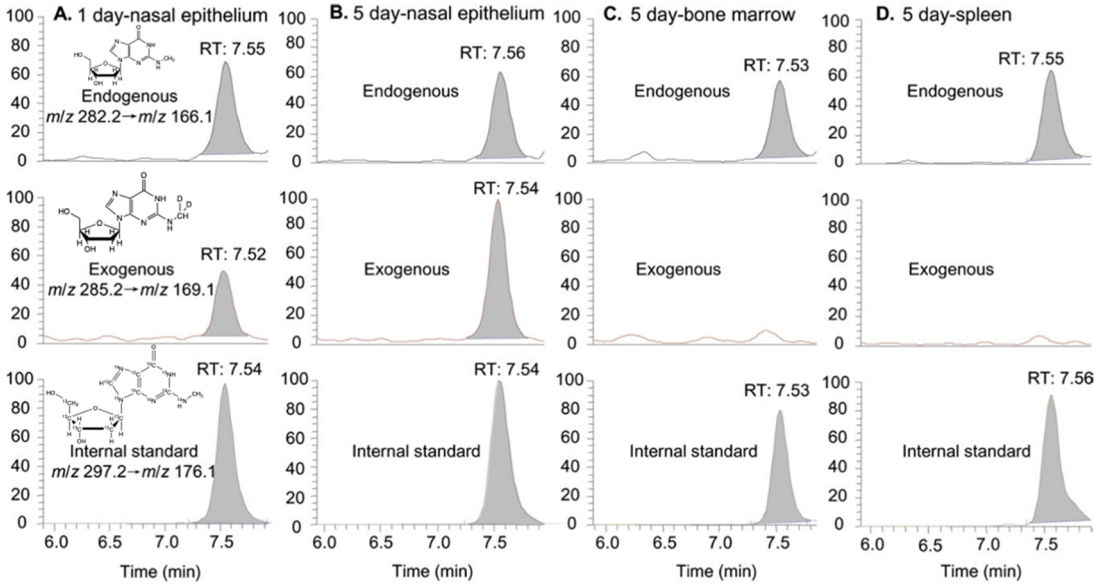

Figure 5. Representative UPLC-MS/MS SRM chromatograms of endogenous and exogenous DNA adducts (N^2-CH_3-dG) caused by FA in rat nasal epithelium after the 1 day exposure (A) and 5 day exposure (B) and in bone marrow (C) and spleen (D) after 5 day exposure. The adducts are measured by LC-ESI-MS/MS-SRM. The SRM transitions for the endogenous adduct N^2-CH_3-dG, the exogenous adduct N^2-[$^{13}CD_2$]H-dG, and the internal standard [$^{13}C_{10}{}^{15}N_5$]-N^2-CH_3-dG are m/z 282.2 → 166.1, m/z 285.2 → 169.1, and m/z 297.2 → 176.1, respectively.

following exposure and to obtain kinetics on the formation and repair/loss of DNA adducts.

Herein, FA and VAM are selected as examples to demonstrate the utilities of SILMS in studying DNA adducts induced by chemicals with different reactivity, metabolism, and MOA. FA targets DNA directly and, as one of the most extensively studied chemicals, has the richest DNA adduct data set achieved over the past decade using advanced nanoLC-MS/MS technologies. In contrast, VAM needs to first be metabolized to AA, which reacts with DNA to form DNA adducts. The entire dose−response curve for VAM-related DNA adducts in rat nasal epithelium and systemic tissues at steady-state has not yet been completely characterized, but there is an ongoing research program to further develop this data set.

5.1. Formaldehyde. FA, one of the top 20 highest volume production industrial chemicals, induces nasal tumors in rats at high doses (6 ppm and above) and has been classified as a Group 1 Human Carcinogen by International Agency for Research on Cancer (IARC), whereas European Chemical Agency (ECHA) classifies FA as a Group 1b carcinogen, solely based on its potential to cause nasal tumors.[56,58,144−147] It remains highly debatable whether FA causes nasopharyngeal cancer and leukemia in humans.[148,149] FA is a highly reactive chemical, and its DNA-damaging ability is well documented.[49,55,57,58,79,92,145,146] FA can directly target DNA to form diverse DNA lesions, including DNA monoadducts, DNA−DNA cross-links and DNA−protein cross-links (DPC), as illustrated in Figure 3.

In addition to exogenous exposure, FA is produced from normal cellular metabolism and is present in all human tissues.[79,150,151] In a human study, the pre-exposure FA concentration in blood was quantitated at an average concentration of 2.61 ± 0.14 μg/g of whole blood.[152] Similar blood concentrations (2.24 ± 0.07 μg/g) were observed in rats prior to inhalation exposure. Following exposure to inhaled FA at concentrations of 14.4 ppm for 2 h or 1.9 ppm for 40 min in rats and humans, respectively, Heck et al. found no increases in blood FA concentrations.[152] These results have been confirmed in the biomarker studies using multiple species, demonstrating that FA measured systemically after inhalation exposures up to 15 ppm remains limited to endogenous sources.[49,56,57,59,79]

A key question that has been raised is whether inhaled FA can enter into the systemic circulation and cause cancer at tissues beyond the portal of entry such as the nasal epithelium. FA is also an important intermediate/metabolite of cellular endogenous processes, such as enzymatic demethylation and one carbon metabolism; thus, DNA adducts originating from endogenous FA can confound the characterization of adducts that arise from inhalation exposure.[57,66,79,91,92] To address these challenges to understand the contribution of exogenous exposure of FA to adduct formation and to reduce uncertainty in FA assessments, stable dual isotope-labeled FA ([$^{13}CD_2$]-FA) exposure (Figure 3B), coupled with highly sensitive MS, has been employed to distinguish and quantify both endogenous and exogenous FA-induced DNA adducts.

The workflow for FA-induced DNA adduct analysis is depicted in Figure 4A. Briefly, the extracted DNA was treated with $NaCNBH_3$ in order to convert N^2-$HOCH_2$-dG to N^2-CH_3-dG, a form that is stable as a nucleoside. After spiking [$^{13}C_{10}{}^{15}N_5$]-N^2-CH_3-dG into the samples as the IS for both the exogenous and endogenous adducts, enzymatic hydrolysis of DNA was applied subsequently. An off-line HPLC enrichment (also see Figure 2C) was next performed to collect the fractions that contain the N^2-CH_3-dG adducts to separate the analytes from the abundant normal nucleosides. The off-line HPLC was coupled with a UV detector to quantify the dG amount in each sample. Fractions with the eluted DNA adducts were dried and reconstituted with water for the characterization and quantitation of DNA adducts on MS. LC-ESI-MS/MS was performed on UHPLC and QqQ for the DNA adduct detection. The exogenous adducts, endogenous adducts, and IS were detected in the SRM mode. As shown in

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Figure 4B, the transitions of m/z 285.2 → m/z 169.1, m/z 282.2 → m/z 166.1, and m/z 297.2 → m/z 176.1 were used for the exogenous adducts (N^2-[$^{13}CD_2$]H-dG), the endogenous adducts (N^2-CH_3-dG), and the IS ([$^{13}C_{10}{}^{15}N_5$]-N^2-CH_3-dG), respectively. The same strategies were used to detect another monoadduct, N^6-CH_3-dA, DNA-DNA cross-links, and DNA-protein cross-links. Recently, we have also developed more sensitive methods using high-resolution Orbitrap MS to quantify FA-induced DNA adducts.[59] The high mass resolution and mass accuracy provided by the Orbitrap mass analyzer enabled an unbiased and accurate quantification of FA-induced DNA adducts. The high-resolution Orbitrap-based methods generate an interference-free spectrum for unambiguous quantification, resulting in good intra- and interday precisions and accuracies with <10% variation.

The first molecular dosimetry study exposed rats to 10 ppm [$^{13}CD_2$]-FA for 1 or 5 days (6 h/day) to examine systemic distribution of FA by monitoring DNA adducts in different organs.[49] Figure 5 shows the representative chromatograms of N^2-CH_3-dG in different tissues. The exogenous adduct (N^2-[$^{13}CD_2$]H-dG) was detected in the nasal epithelium of rats exposed to 10 ppm [$^{13}CD_2$]-FA for either 1 or 5 days, but not in the bone marrow, spleen, or any other distant tissues we analyzed, such as lungs, liver, thymus, and blood. The study showed that exogenous FA resulted in DNA adducts in rat respiratory nasal mucosa, but did not form [$^{13}CD_2$]-adducts in distant organs remote to the portal of entry. Therefore, the finding provides evidence to support a genotoxic and/or cytotoxic MOA for the carcinogenesis of inhaled FA in respiratory nasal epithelium, but does not support the biological plausibility of inhaled FA causing leukemia or other systemic effects. In addition, the number of exogenous N^2-$HOCH_2$-dG in 1 and 5 day nasal DNA samples from rats exposed to 10 ppm [$^{13}CD_2$]-FA were 1.28 ± 0.49 and 2.43 ± 0.78 adducts per 10^7 dG, respectively, demonstrating a sublinear (saturating) increase as exposure duration increases and suggesting an active repair/loss of exogenous FA-induced DNA adducts.

Another study to evaluate the molecular dosimetry of FA-induced DNA adducts was performed by exposing rats to 0.7, 2, 5.8, 9.1, and 15.2 ppm [$^{13}CD_2$]-FA for 1 day (6 h/day), which modeled the exposures in a previous cell proliferation and carcinogenicity study by Monticello et al.[153] In the Monticello et al. study, FA exposure induced nasal squamous cell carcinomas in a highly nonlinear fashion, with no nasal carcinomas observed following exposure to 0.7 or 2 ppm; however, nasal carcinomas were observed in 1, 22, and 47% of the animals exposed to 6, 10, and 15 ppm FA, respectively.[153] As shown in Table 3, the exogenous dG adducts show a highly nonlinear increase in the 0.7−15 ppm concentration range, suggesting saturation of metabolism and removal processes in this exposure range. In addition, the exposure did not change the numbers of endogenous FA-induced DNA adducts in any of the dosed groups. Examination of the ratio of exogenous versus endogenous FA-induced DNA adducts clearly demonstrates that endogenous DNA adducts predominate at low ppm exposure concentrations and that exposures contribute comparatively miniscule amounts of exogenous DNA adducts.

Table 3. FA-Induced N^2-$HOCH_2$-dG Adducts in the Nasal Epithelium of Rats Exposed to [$^{13}CD_2$]-FA for 6 h

tissue	[$^{13}CD_2$]-FA concentration (ppm)	exogenous adducts/10^7 dG	endogenous adducts/10^7 dG
nasal epithelium	0.7 ± 0.2	0.039 ± 0.019	3.62 ± 1.33
	2.0 ± 0.1	0.19 ± 0.08	6.09 ± 3.03
	5.8 ± 0.5	2.03 ± 0.43	5.51 ± 1.06
	9.1 ± 2.2	11.15 ± 3.01	3.41 ± 0.46
	15.2 ± 2.1	4.24 ± 0.92	4.24 ± 0.92

Improved knowledge of when exogenous inhaled FA exposures reach steady-state and alter normal endogenous or total FA tissue concentrations is critical to understanding the MOA of FA; this was noted by the US National Research Council in 2011 when reviewing the EPA's draft of the Integrated Risk Information System Toxicological Review for FA. Therefore, a 28 day study was conducted to gain new information on the kinetics of formation and loss of FA-induced DNA adducts.[57] Male F344 rats were exposed to 2 ppm [$^{13}CD_2$]-FA atmospheres for 7, 14, 21, or 28 consecutive days (6 h/day, 7 day/wk) with postexposure tissue harvesting for [$^{13}CD_2$]-FA quantification at 6, 24, 72, and 168 h using a single nose-only unit. Sensitive nanoLC-ESI-MS/MS permitted accurate determinations of endogenous and exogenous FA-induced DNA adducts. The results again showed that inhaled FA reached only the rat nose (i.e., nasal epithelium), but not tissues distant to the portal of entry (e.g., blood and brain). The amounts of exogenous adducts remained markedly lower than those of endogenous adducts in exposed nasal epithelium (1.05 ± 0.16 exogenous and 2.82 ± 0.76 endogenous adducts/10^7 dG at the 28-day time point). Exogenous adducts accumulated in rat nasal epithelium over the 28-day-exposure to reach near-steady-state concentrations, followed by elimination with a half-life ($t_{1/2}$) of ∼7.1 days.

DNA-protein cross-links (DPC) have been considered as a major form of DNA damage induced by FA and used as a biomarker of FA exposure in previous studies. However, previous studies were largely based on radiolabeled FA and physical chemistry, without any structural information being provided. In contrast, SILMS has been used to distinguish FA-specific DPC from both endogenous and exogenous sources. Based on our work on structural characterization of DPC and the stability of cross-links involving cysteine and dG, dG-CH_2-Cys was selected as a representative FA-induced DPC for detection and quantitation by MS.[154−157] The sample preparation and analytical flow have been described elsewhere.[155,156] Briefly, using a mixture of enzymes to digest DPCs into small nucleoside-amino acid cross-links (dG-CH_2-Cys) under mild conditions (pH 6.0 and room temperature), FA-induced DPCs were isolated by off-line HPLC fraction collection along with the quantification of digested dG using UV absorbance at 256 nm. The isolated endogenous and exogenous dG-CH_2-Cys are differentially quantified by MS. As listed in Table 4, exogenous DPCs were measured at 5.52 ± 0.8 cross-links/10^8 dG in the rat nasal tissues from animals exposed to [$^{13}CD_2$]-FA at a targeted concentration of 15 ppm for 1 day, while no exogenous DPC was detected in PBMC, bone marrow, or other distant tissues.[156] In contrast, endogenous FA DPC was detected in all tissues analyzed in rats. These results are consistent with the findings obtained when the N^2-$HOCH_2$-dG DNA adducts are used as the biomarker of FA exposure.

The data generated in studies described above provide strong scientific evidence for the assessment of risk resulting from FA exposure through inhalation. Low doses of exogenous FA exposure (<0.7 ppm) are within the range of normal human exposure and, thus, relevant; however, it is highly

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Table 4. FA-Induced dG-CH_2-Cys in nasal tissue, PBMCs, and Bone Marrow of Rats Exposed to Air Control versus 15 ppm of [$^{13}CD_2$]-FA for 1 Day (6 h/day)[a]

tissue	[$^{13}CD_2$]-FA concentration (ppm)	endogenous (cross-links/10^8 dG)	exogenous (cross-links/10^8 dG)
nasal	air control (n = 5)	6.50 ± 0.30	ND
	15.0 (n = 6)	4.42 ± 1.10	5.52 ± 0.80
PBMC	air control (n = 5)	4.98 ± 0.61	ND
	15.0 (n = 4)	3.26 ± 0.73	ND
bone marrow	air control (n = 4)	1.64 ± 0.49	ND
	15.0 (n = 4)	1.80 ± 0.47	ND

[a]ND indicates not detectable at a quantitation level of ~4 DPCs/10^9 dG.

challenging to examine the formation of DNA adducts induced by low doses of FA. Taking advantage of ultrasensitive nanoLC-ESI-MS/MS, both exogenous and endogenous DNA monoadduct (N^2-HOMe-dG) and DPC (dG-CH_2-Cys) were measured to assess the formation of DNA adducts arising from the inhalation of 0, 1, 30, or 300 ppb [$^{13}CD_2$]-FA in rats for 28 days in our recent study.[59] The limits of detection for N^2-HOMe-dG and dG-CH_2-Cys were ~0.5 and ~5 attomole on the column, respectively. This translates to ~0.5 and ~5 adducted dG/10^{10} nonadducted dG. Tables 5 and 6 summarize the amounts of FA-induced DNA monoadducts and DPCs in different tissues that were analyzed, including nasal epithelium, bone marrow, trachea, liver, brain, and lungs. Endogenous FA-induced DNA adducts and DPCs were detected across different tissues, but no exogenous FA-induced DNA adducts were detected in any tissue, including nasal epithelium. In addition, exogenous FA did not induce statistically significant changes in the amounts of endogenous FA monoadduct or DPC in any dosing groups compared to the air control. These findings show that against a readily detected naturally occurring background, no exogenous DNA adducts were detectable in rats exposed to 300 ppb or lower FA in the 28 day exposure study.

As discussed above, studies in rats have provided extensive information regarding FA-derived DNA damage, but the structure of nasal passages and the disposition of inhaled chemicals between rodents and primates, including humans, are different. These differences and oral/nasal breathing patterns make primates a more appropriate model of human exposure. We extended our studies to examine both exogenous and endogenous FA-induced DNA adducts in nonhuman primates.[156,158] Two groups (n = 4 animals/group) of cynomolgus macaques (*Macaca fascicularis*) were whole body exposed to either 1.9 or 6.1 ppm [$^{13}CD_2$]-FA for 2 consecutive days (6 h per day), followed by measurement of FA-induced DNA adducts. Table 7 summarizes endogenous and exogenous N^2-$HOCH_2$-dG levels found in both tissues. In the nasal maxilloturbinate DNA, exogenous N^2-$HOCH_2$-dG was present at 0.26 ± 0.04 and 0.41 ± 0.05 adducts/10^7 dG following the 1.9 and 6.1 ppm exposures, respectively.[158] Interestingly, the numbers of exogenous DNA adducts at these exposure concentrations are at least 2-fold lower those observed in rats, suggesting that monkeys and humans may be less susceptible than rats to a given airborne concentration in terms of adduct formation and FA-induced DNA damage. Endogenous N^2-$HOCH_2$-dG adducts were present in the nasal DNA of all animals studied, with an average of 2.24 ± 0.50 adducts/10^7 dG. In bone marrow, no exogenous adducts were detected, even though ~10-fold higher amounts of DNA were analyzed, providing a 10-fold higher capability to detect exogenous DNA adducts. Endogenous N^2-$HOCH_2$-dG adducts were present at 17.5 ± 2.6 and 12.4 ± 3.6 adducts/10^7 dG in bone marrow DNA from the 1.9 and 6.1 ppm exposures, respectively. In another monkey study using a high concentration (6 ppm for 1 day), exogenous FA-induced DPC (dG-CH_2-Cys) were present at 1.36 ± 0.2 cross-links/10^8 dG in nasal epithelium, while endogenous FA DPC were detected at 3.76 ± 1.5 cross-links/10^8 dG, as shown in Table 8.[156,158] No exogenous FA DPC were detected in PBMC or bone marrow. Taken together, results from the primates are consistent with those from rats. Exogenous FA induces DNA adducts only at the portal of entry (nasal epithelium), with no systemic distribution of inhaled FA in the body. In addition, endogenous FA-induced DNA adducts predominate, especially at low doses of FA exposure.

5.2. Vinyl Acetate Monomer. VAM, a synthetic organic ester, is widely used in the production of polyvinyl acetate, poly(vinyl alcohol), and other polymers. The polymers derived from VAM have a variety of applications including drug delivery, adhesives, paint coatings, wire and cable insulation, and other end-products.[159] VAM has been shown to induce nasal tumors in rodents at high air concentrations (> 600 ppm). Mechanistic studies have been conducted to understand the genotoxicity and cytotoxicity of VAM, and the results show that the carcinogenic effects of VAM are likely attributed to its metabolites, with AA as a DNA damaging agent and acetic acid as a cytotoxicant.[60,160,161] Similar to FA and VAM, AA

Table 5. Levels of Endogenous and Exogenous N^2-HOMe-dG (adducts/10^7 dG) in Rat Tissues Exposed to [$^{13}CD_2$]-Formaldehyde (1, 30, 300 ppb) for 28 Days[a]

	air control		1 ppb		30 ppb		300 ppb		
tissues	endogenous	exogenous	endogenous	exogenous	endogenous	exogenous	endogenous	exogenous	n
nasal mucosa	3.23 ± 0.85	ND	3.59 ± 0.90	ND	3.27 ± 0.76	ND	3.48 ± 0.83	ND	8
bone marrow	4.83 ± 1.54	ND	4.32 ± 1.21	ND	5.03 ± 1.71	ND	4.42 ± 0.69	ND	8
PBMC	2.64 ± 1.03	ND	2.72 ± 0.73	ND	2.80 ± 1.11	ND	2.94 ± 1.15	ND	8
trachea	3.14 ± 0.61	ND	3.23 ± 1.02	ND	3.34 ± 0.75	ND	3.23 ± 0.47	ND	6
liver	2.48 ± 0.21	ND	2.57 ± 0.31	ND	2.44 ± 0.34	ND	2.60 ± 0.76	ND	6
hippocampus	2.35 ± 0.56	ND	2.62 ± 0.74	ND	2.52 ± 0.82	ND	2.86 ± 0.76	ND	5
olfactory bulbs	2.51 ± 0.62	ND	2.74 ± 1.05	ND	2.84 ± 0.45	ND	2.59 ± 0.38	ND	5
cerebellum	2.45 ± 0.76	ND	2.62 ± 0.67	ND	2.46 ± 0.43	ND	2.35 ± 0.57	ND	5
lung	5.25 ± 3.23	ND	3.72 ± 2.20	ND	4.79 ± 3.22	ND	5.06 ± 2.51	ND	7

[a]ND indicates not detectable at a limit of detection of ~0.5 adducts/10^{10} dG.

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Table 6. Levels of Endogenous and Exogenous dG-Me-Cys (adducts/10^8 dG) in Rat Tissues Exposed to [$^{13}CD_2$]-Formaldehyde (1, 30, 300 ppb) for 28 Days[a]

	air control		1 ppb		30 ppb		300 ppb		
tissues	endogenous	exogenous	endogenous	exogenous	endogenous	exogenous	endogenous	exogenous	*n*
nasal mucosa	2.66 ± 0.54	ND	2.77 ± 0.61	ND	3.01 ± 0.85	ND	2.85 ± 0.74	ND	8
bone marrow	2.19 ± 0.46	ND	2.28 ± 0.55	ND	1.98 ± 0.42	ND	2.45 ± 0.48	ND	8
PBMC	1.96 ± 0.66	ND	2.08 ± 0.56	ND	1.88 ± 0.64	ND	1.93 ± 0.85	ND	8
trachea	1.52 ± 0.70	ND	2.30 ± 1.03	ND	2.41 ± 0.83	ND	1.99 ± 0.57	ND	8
liver	7.27 ± 1.66	ND	8.03 ± 1.46	ND	7.93 ± 1.58	ND	7.13 ± 1.58	ND	8
hippocampus	1.81 ± 0.46	ND	1.87 ± 0.41	ND	1.63 ± 0.51	ND	1.94 ± 0.39	ND	5
olfactory bulbs	1.69 ± 0.37	ND	2.55 ± 0.40	ND	1.89 ± 0.34	ND	2.04 ± 0.42	ND	5
cerebellum	2.71 ± 0.87	ND	2.37 ± 0.68	ND	2.39 ± 1.60	ND	2.33 ± 0.73	ND	5
lung	4.07 ± 1.11	ND	3.99 ± 0.61	ND	3.34 ± 0.67	ND	3.48 ± 0.65	ND	8

[a]ND indicates not detectable at limit of detection of ~5 DPCs/10^{10} dG.

Table 7. FA-Induced DNA Adducts (N^2-$HOCH_2$-dG) in Nose and Bone Marrow of Primates Exposed to 2 and 6 ppm of [$^{13}CD_2$]-FA for 2 Consecutive Days (6 h/day)[a]

tissue	[$^{13}CD_2$]-FA concentration (ppm)	exogenous adducts/10^7 dG	endogenous adducts/10^7 dG
nasal maxilloturbinate	1.9	0.26 ± 0.04	2.50 ± 0.40
	6.1	0.41 ± 0.05	2.05 ± 0.54
bone marrow	1.9	ND	17.5 ± 2.6
	6.1	ND	12.4 ± 3.6

[a]ND indicates not detectable at a detection level of ~2 adducts/10^9 dG.

Table 8. FA-Induced DPC (dG-CH2-Cys) in the Nose, PBMCs, and Bone Marrow of Primates Exposed to Air Control versus 6 ppm of [13CD2]-FA for 2 Consecutive Days (6 h/day)[a]

tissue	[$^{13}CD_2$]-FA concentration (ppm)	endogenous (cross-links/10^8 dG)	exogenous (cross-links/10^8 dG)
nose	air control	3.59 ± 1.01 (n = 5)	ND
	6	3.76 ± 1.50 (n = 5)	1.36 ± 0.20
PBMC	air control	1.34 ± 0.25 (n = 5)	ND
	6	1.57 ± 0.58 (n = 4)	ND
bone marrow	air control	2.30 ± 0.30 (n = 4)	ND
	6	1.40 ± 0.46 (n = 5)	ND

[a]ND indicates not detectable at a quantitation level of ~4 DPCs/10^9 dG.

exposures at high air concentrations (i.e., ≥ 200 ppm) result in a highly nonlinear dose−response for rat nasal tumors. AA can interact with DNA to form mutagenic N^2-EtD-dG and 1,N^2-PdG.[55,60,61,162−164] Figure 6 illustrates the metabolism of VAM and how its metabolite, AA, interacts with DNA to form these adducts. Inhaled VAM is metabolized to AA by a CE-catalyzed reaction, in which vinyl alcohol is an intermediate.[81,164,165] However, AA is also an endogenous metabolite in the body. The microbial or hepatic metabolism of threonine and pyruvate can produce AA.[164,166−168] In addition, ethyl alcohol is metabolized to AA, which can result in systemic tissue damage in heavy drinkers. ALDH is responsible for the detoxification of AA by oxidizing it to acetic acid.[165,169] AA reacts with DNA to form N^2-EtD-dG as a major DNA adduct. A secondary reaction can also happen between N^2-EtD-dG with an additional molecule of AA to form 1,N^2-PdG DNA adducts.[61] To date, informing the cancer risk assessment of VAM using DNA adducts as potential biomarkers of exposure has been impeded due to the inability to evaluate separately the contribution of inhaled VAM when the identical endogenous DNA adducts are always present.

SILMS was used to distinguish and quantify both endogenous and exogenous DNA adducts in rats exposed to VAM. Rats were exposed to 0, 50, 200, or 400 ppm [$^{13}C_2$]-VAM for 6 h, followed by DNA adduct analysis in both nasal epithelium tissues and PBMC samples (Liu et al., 2021). Both respiratory and olfactory epithelia in the nasal cavity were collected for DNA adduct analysis in this study, as both regions had tumors, albeit at different incidences in the available cancer bioassays.[81,82] DNA purification, enrichment, and LC-ESI-MS/MS procedures were similar to the FA studies discussed above, except that the MS analysis was performed with a high-resolution Orbitrap MS using the PRM mode to take advantage of the high mass accuracy of the Orbitrap MS (<3 ppm).

Figure 7 shows the typical nanoLC-ESI-MS/MS PRM chromatograms for endogenous, exogenous, and the internal standard for N^2-Et-dG in rats exposed to [$^{13}C_2$]-VAM. There is a clear dose-dependent increase of exogenous N^2-Et-dG in rats exposed to 50, 200, and 400 ppm. The results for endogenous and exogenous N^2-Et-dG adducts in the rat nasal respiratory and olfactory epithelia are summarized in Table 9. No exogenous DNA adducts were detected in nasal samples of control rats. Dose-dependent increases of exogenous N^2-Et-dG were observed in both nasal respiratory and olfactory epithelia. However, there was no statistically significant difference in endogenous N^2-Et-dG adducts among the exposure groups. In addition, endogenous N^2-Et-dG DNA adducts predominate at low doses, with the ratio of exogenous/endogenous DNA adducts being only 0.13 ± 0.07 in rats exposed for a single day to 50 ppm.

Interestingly, the amounts of exogenous N^2-Et-dG in the nasal olfactory epithelium were significantly lower than those in the nasal respiratory epithelium collected from the same rats exposed to [$^{13}C_2$]-VAM. The location-specific difference in DNA adduct amount may result from multiple factors, such as diffusion and vapor deposition efficiency of VAM in the nasal cavity, activity/expression of CE and ALDH, heterogeneity between nasal tissues, and differences in DNA repair between respiratory and olfactory epithelium, as was discussed elsewhere.[170]

We next examined whether exogenous N^2-Et-dG can be formed in tissues other than those at the portal of entry due to potential systemic distribution of VAM. To do this, PBMC

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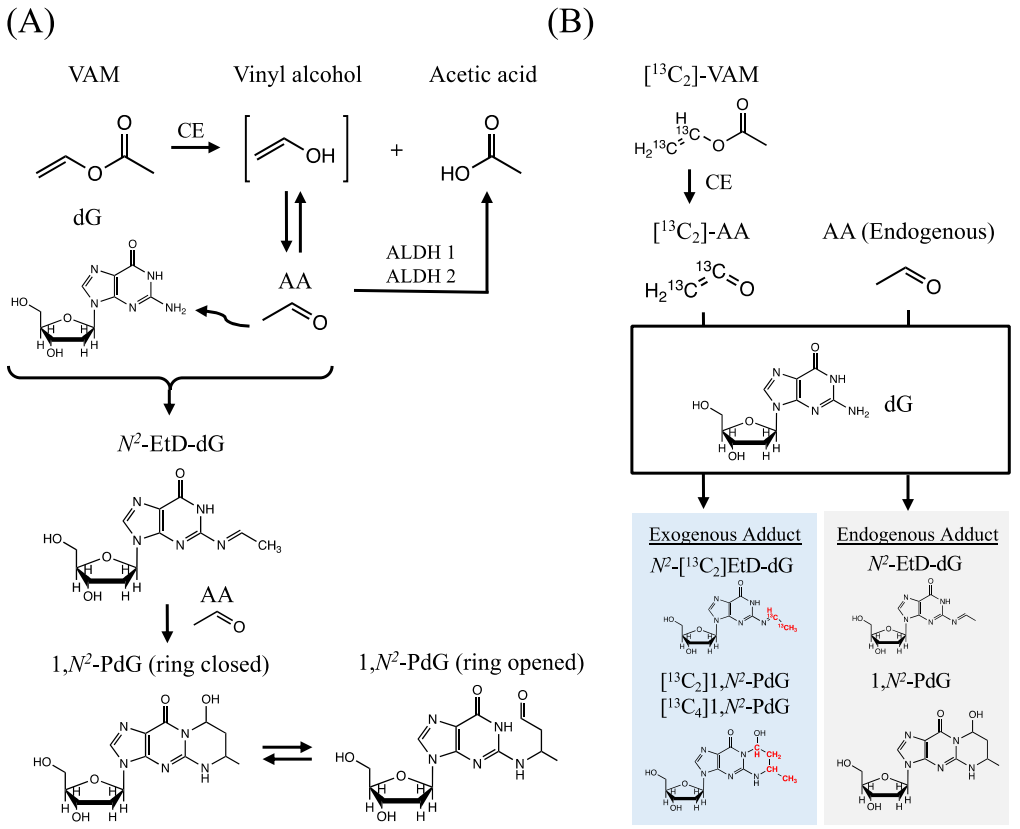

Figure 6. Metabolism of VAM and the formation of DNA adducts by its metabolite, AA (A). Differentiation of exogenous N^2-Et-dG adducts from endogenous ones when [$^{13}C_2$]-VAM is used for exposure (B).

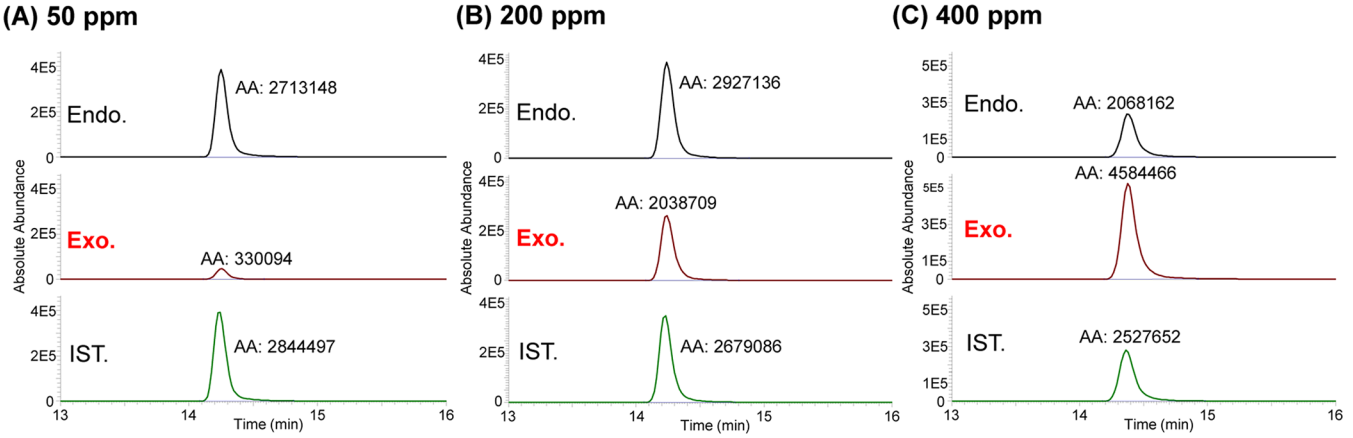

Figure 7. Typical nanoLC-ESI-MS/MS PRM chromatograms of endogenous (Endo.) and exogenous (Exo.) N^2-Et-dG in nasal respiratory tissues of rats exposed to 50 ppm (A), 200 ppm (B), and 400 ppm (C) of [$^{13}C_2$]-VAM for 6 h.

Table 9. Endogenous and Exogenous N^2-Et-dG Amounts (adducts/10^8 dG) in Respiratory and Olfactory Epithelia of Rats Exposed to [$^{13}C_2$]-VAM for 6 h (n = 5−7)[a]

	respiratory			olfactory		
dose group	endogenous	exogenous	exo/endo ratio	endogenous	exogenous	exo/endo ratio
air control	47.97 ± 3.86	ND	0.00	45.07 ± 7.00	ND	0.00
50 ppm	55.29 ± 12.53	6.82 ± 2.09	0.13 ± 0.07	42.56 ± 6.07	1.92 ± 0.38	0.05 ± 0.01
200 ppm	43.15 ± 14.26	60.05 ± 23.84	1.50 ± 0.72	40.45 ± 9.45	24.52 ± 11.7	0.69 ± 0.51
400 ppm	60.76 ± 8.50	122.35 ± 23.62	2.02 ± 0.31	44.76 ± 21.47	27.51 ± 17.5	0.66 ± 0.29

[a]ND indicates not detectable at a detection limit of ~2 N^2-Et-dG/10^{10} dG.

samples were collected at 6 h postexposure. The endogenous and exogenous N^2-Et-dG adducts from those PBMCs samples are summarized in Table 10. Exogenous N^2-Et-dG adducts were present at 0.21 ± 0.03, 0.42 ± 0.09, and 0.74 ± 0.08

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Table 10. Endogenous and Exogenous N^2-Et-dG Amounts (adducts/10^8 dG) in the PBMC of Rats Exposed to [$^{13}C_2$]-VAM for 6 h[a]

dose group	endogenous	exogenous	exo/endo ratio
control	46.90 ± 5.57	ND	–
50 ppm	75.91 ± 16.82	0.21 ± 0.03	0.0029 ± 0.0003
200 ppm	89.95 ± 24.23	0.42 ± 0.09	0.0047 ± 0.0005
400 ppm	105.45 ± 65.51	0.74 ± 0.08	0.0087 ± 0.0040

[a]DNA was pooled (n = 3) to improve sensitivity with 120 μg DNA used in total. ND indicates not detectable at a detection limit of ~0.2 N^2-Et-dG/10^{10} dG.

adducts/10^8 dG adducts in the PBMC of rats exposed for 6 h to 50, 200, and 400 ppm [$^{13}C_2$]-VAM, respectively. There were no significant differences in the endogenous adduct levels across exposure groups. The ratios of exogenous adduct versus endogenous adduct were <1% in all groups, suggesting only an extremely small percentage of [$^{13}C_2$]-VAM or its metabolite may enter into systemic circulation to potentially affect tissues beyond the nasal epithelium. The slightly greater distribution of VAM (measured as AA-DNA adducts in PBMCs) when compared to FA was expected, as higher concentrations of VAM were studied, and VAM needs to be metabolized (no extracellular binding) to AA before DNA binding can occur.

5.3. Other Chemicals. As demonstrated above with FA and VAM, SILMS is a powerful methodology for distinguishing between endogenous and exogenous DNA adducts and improving the understanding of MOA and quantitative risk assessment. SILMS is versatile and not limited to FA or VAM and has also been used to study other chemicals that form exogenous DNA adducts identical to endogenous adducts. For example, vinyl chloride (VC) has been classified as a group 1 carcinogen by IARC, causing hepatic tumors. VC is metabolized to chloroethylene oxide, which reacts with DNA to generate adducts such as 7-OE-dG and several mutagenic etheno adducts. Since the etheno adducts can also result from endogenous lipid peroxidation, the Swenberg lab administered [$^{13}C_2$]-VC to rats and successfully differentiated the exogenous and endogenous VC-induced adducts.[79] Likewise, EO, a widely used industrial chemical, has been classified as a known human carcinogen by IARC, and it can also be formed endogenously by metabolic oxidation of ethylene. Both inhaled and endogenous EO can result in the formation of adducts, such as N^7-HOEt-dG.[171−173] Marsden et al. conducted exposures by administrating [$^{14}C_2$]-EO to rats.[171] Endogenous and exogenous N^7-HOEt-dG adducts were distinguished by MS. In addition, they found that [$^{14}C_2$]-EO exposure resulted in a significant increase in endogenous N^7-HOEt-dG at the two highest doses. These studies were conducted to assess adduct formation at relatively high doses, and further research is needed to define the dose−response at low concentrations and better inform the risk assessment of these chemicals.

6. APPLICATION AND UTILITY OF DNA ADDUCTS FOR QUANTITATIVE RISK ASSESSMENT

6.1. Background on Cancer Risk Assessment. For most of the history of cancer risk assessment, evaluation of a chemical's carcinogenic potential has been driven by the philosophy that there is no safe dose of a carcinogen. This was motivated by assumptions that chemical agents acted in a fashion similar to ionizing and other radiation, which was itself assumed to cause its effects through direct interaction with DNA to produce cancer-initiating mutations. For example, it was widely accepted that "carcinogens are mutagens" as proposed in Ames et al.[174]

Contemporary concepts of carcinogenesis acknowledge that not all chemicals produce cancer through direct interaction with DNA and that cancer induction is a multistep process involving mutations and cell proliferation.[175] Mutation produced by chemicals is also accepted to be a multistep process.[176,177] Likewise, contemporary studies of mutation caused by chemicals (including DNA reactive carcinogens) show departures from linearity in the low-dose range.[81,83,177−179] Studies indicate that even in some situations where DNA adduct formation appears linear at low doses, mutation frequency has been observed to depart from linearity at these doses.[180] It is also important to note that many carcinogens act in other ways than by direct interaction with DNA, such as receptor binding leading to cell proliferation or toxicity leading to compensatory cell proliferation.[181−187] It is clear that one adduct is not equivalent to one mutation and that one mutation in the genome does not equal one tumor. Mutations must be in critical genes, and the cell must survive and proliferate, escape immune surveillance and move through the remaining steps of a multistep process that will result in tumor formation. Thus, risk assessors are moving to low-dose modeling procedures for chemical carcinogens that better reflect our understanding of carcinogenesis as complex multifactorial process rather than a single molecular event.

After nearly 20 years of discussion and revision, the US EPA published revised Guidelines for Carcinogen Risk Assessment.[188] The guidelines built on the substantial increases in knowledge of carcinogenic processes since publication of the original guidance and acknowledged the need to continue to incorporate new information and processes as they become applicable.[189] To this end, the cancer guidelines emphasized understanding MOA as critical to the assessment of an agent's carcinogenic potential. This was coupled in the hazard characterization with a description of the conditions under which a chemical was likely to present a carcinogenic risk. These conditions could include high-dose-only effects, route-specific effects, effects dependent on duration of exposure, and consideration of animal tumors produced by a MOA not relevant to humans. Knowledge of MOA was to be used in directing choices of low-dose extrapolation procedures rather than a constant reliance on any particular model and specifically the linear no threshold (LNT) model. The 2005 cancer guidelines contain an explicit preference for the use of toxicokinetic modeling to estimate dose and toxicodynamics (biologically based dose response models) when such are supported by adequate data and modeling procedures. As discussed in this review and various original research papers, FA-adduct formation is highly nonlinear in the nasal epithelium DNA of rats exposed to airborne concentrations of FA ranging from 0.7 to 15 ppm. Furthermore, a recent study shows that DNA adducts associated with exogenous inhaled FA were absent at after exposures of 0.001, 0.03, and 0.3 ppm (i.e., nondetectable at detection limits that are orders of magnitude lower than the concentration of endogenous adducts).[59] Inhaled FA toxicokinetics thus indicate that at air concentrations ≤ 300 ppb, FA metabolism and distribution are not saturated and there may be a *de minimus* exposure of DNA to FA.[190] Studies focusing on effect biomarkers such as exogenous DNA adduct revealed that 700 ppb is the lower boundary for the nonlinear dose−response relationship for

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FA.[59] This may suggest that at at least 700 ppb, metabolism begins to saturate as evidenced by a highly nonlinear dose response for these biomarkers of exposure.

The 2005 US EPA cancer guidelines specifically noted that there was not to be a standard requirement for data in order to depart from an accepted default (such as LNT). In other words, data are to be used before defaults in all aspects of the risk assessment. As presented in this paper, the FA dose−response for DNA adduct formation is highly nonlinear. This provides a sound toxicokinetic basis for a nonlinear response at high doses and an absence or *de minimiz* level of DNA adduct formation at low doses (i.e., $\leq$ 0.3 ppm FA) in rats. Per the EPA guidelines, linear extrapolation should be used when data indicating that the dose−response curve has a linear component below the point of departure (POD) or as a default the mode of action is not established for a tumor site. Neither of these cases apply to FA-induced nasal tumors.

6.2. Consideration of Endogenous Compounds in Risk Assessment. Conducting a risk assessment for a compound that is present endogenously poses several challenges. Besides methods needed to quantify endogenous production and to differentiate DNA damage arising from exogenous exposure, the additional challenge to the risk assessor is determining how to best interpret the results and incorporate those results into an appropriate dose−response assessment. The risk assessor must also attempt to determine whether exogenous exposures can significantly contribute to the adduct level and can sufficiently create biologically relevant perturbations that culminate in detectable adverse effects.

FA provides a good example of a small reactive aldehyde that is present endogenously in all living cells. It also has numerous exogenous sources including vehicle emissions, off-gassing from building materials, and tobacco smoke; it arises as well as from the metabolism of foods, chemicals, and drugs. In conducting a FA dose−response assessment for inhaled FA, there are several questions that need to be addressed, which are relevant both in determining how to conduct a dose−response assessment as well as interpreting the results of that assessment

- How can we accurately assess the risk from exogenous FA in the presence of a substantial background of endogenous FA?
- What is needed to conduct a dose−response assessment considering the "background" concentrations that are always present in biological systems?
- If a specific marker is capable of differentiating endogenous from exogenous exposure, can this be a biomarker of exposure and/or effect to directly inform the mode of action?

6.3. Use of DNA Adducts as Biomarkers of Exposure in Risk Assessment. The studies discussed herein employed stable isotope-labeled [$^{13}CD_2$]-FA for exogenous exposure, coupled with highly sensitive MS detection methods. Results from these studies provide a tissue and molecular target level characterization of exposure that can be incorporated into dose−response assessments of the potential carcinogenicity of FA. There are substantial advantages to relying upon the use of specific DNA adducts in dose−response assessments, as these biomarkers of exposure are more informative regarding the critical targets and tissue concentrations that drive carcinogenesis than are the corresponding external exposure concentrations. As with most pharmacokinetic dose metrics, molecular dosimeters such as DNA adducts can be used in traditional dose−response modeling; however, the challenge of incorporating endogenous production of DNA adducts still remains. To address this challenge, a novel "bottom up" approach was developed by Starr and Swenberg.[191,192]

The principal concept underlying the bottom-up approach to bounding human low-dose carcinogenic risks is that endogenous exposures to specific carcinogenic substances are causally associated with at least some, if not all, of the background risk of cancer development in specific target tissues.[191,192] If low-dose exogenous exposures to chemicals give rise to the same carcinogenic substances in the same target tissues, those exposures simply add incrementally to the background exposure in those target tissues (C_0), leading to incremental increases in cancer risk over and above the background risk (P_0). After adjusting these two background parameters that characterize endogenous exposure and background cancer risk for statistical uncertainty by replacing their central, that is, maximum likelihood, estimates with corresponding upper (P_{0U}) and lower (C_{0L}) confidence bounds, the ratio P_{0U}/C_{0L} provides a conservative cancer risk "slope factor" estimate that can be used to bound the added risk (AR_U) associated with incremental steady-state exogenous exposures (C_{xss}) as given by the equation:

$$AR_U = \frac{P_{0U}}{C_{0L}} \times C_{xss}$$

Strengths of the bottom-up approach are that it: (1) requires only information on exposure from endogenous sources, the associated background cancer risk, and additional steady-state exposures from exogenous sources; (2) yields linear upper-bound estimates of the added risk; and (3) is consistent with the "additivity to background" concept. Furthermore, because it does not rely on high-dose tumor data to make inferences regarding the shape of the true dose−response relationship at lower concentrations, it provides an independent "reality check" on upper bound added risk estimates that are derived with the typical top-down approach of fitting empirical dose−response models to high-dose tumor data acquired in epidemiologic studies of exposed workers or long-term carcinogenicity studies using laboratory animals.

To illustrate the application of this novel approach, Starr and Swenberg estimated the upper bound added human nasopharyngeal cancer risk from lifetime continuous inhalation exposure to 1 ppm airborne FA for comparison with the corresponding US EPA top-down estimate that had been derived from human epidemiologic data (US EPA 2010).[191−193] First, Starr and Swenberg extrapolated downward linearly to 1 ppm from a monkey nasal tissue estimate of C_{xss} for 6 ppm FA that was obtained with a simple one-compartment linear model of the production and elimination of dual isotope-labeled N^2-$HOCH_2$-dG adducts.[57,192] This yielded a value of 1.32 N^2-$HOCH_2$-dG adducts per 10^7 dG (see Table 1 of the Starr and Swenberg article).[192] They similarly obtained a lower 95% confidence bound on the endogenous background exposure in the same cynomolgus monkey nasal tissues (C_{0L}) of 3.55 unlabeled N^2-$HOCH_2$-dG adducts per 10^7 dG. They then assumed that the human values for C_{xss} at 1 ppm and for C_{0L} would be the same as those that were obtained with cynomolgus monkeys, and they utilized US national cancer statistics to estimate the human background lifetime risk of death from nasopharyngeal cancer (P_0) as 7.25 per 10,000.[193]

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Because the sample size for this estimate is so large, the upper 95% confidence bound estimate of the background cancer risk, P_{0U}, is essentially the same as this central estimate, so they utilized the central estimate in calculating the bottom up upper bound slope factor for human nasal pharyngeal cancer. The corresponding upper 95% confidence bound on the added risk for nasal pharyngeal cancer mortality associated with lifetime continuous exposure to 1 ppm airborne FA was $AR_U = 2.69 \times 10^{-4}$, over 40-fold lower than the corresponding US EPA top down estimate of 1.1%.[193]

Starr and Swenberg similarly estimated the upper bound on added risk for leukemia mortality from lifetime continuous exposure to 1 ppm airborne FA.[192] The concentration of exogenous N^2-$HOCH_2$-dG adducts in scraped bone marrow from cynomolgus monkeys (the presumptive target tissue for leukemia) immediately following two daily 6 h exposures to 6 ppm airborne FA was found to be $< 2.19 \times 10^{-4}$ adducts per 10^7 dG (see Table 1 of the Starr and Swenberg article).[192] Since no exogenously derived bone marrow adducts were detected in this experiment, the adduct concentration arising from airborne FA exposure had necessarily to be no greater than the method's limit of detection (2.19×10^{-4} adducts per 10^7 dG).

Using the same pharmacokinetic model of adduct formation and elimination, this upper limit on the adduct concentration in bone marrow was converted to an estimate of the steady-state value, C_{xss}, that would arise from continuous 24 h exposure to the same airborne concentration of 6 ppm FA, giving $C_{xss} < 4.79 \times 10^{-3}$ adducts per 10^7 dG.[57] Downward linear extrapolation of this value to that expected for 1 ppm airborne FA yielded $C_{xss} < 7.98 \times 10^{-4}$ adducts per 10^7 dG. Starr and Swenberg (2016) similarly obtained a lower 95% confidence bound on the endogenous background exposure in cynomolgus monkey bone marrow (C_{0L}) of 9.48 N^2-$HOCH_2$-dG adducts per 10^7 dG (see Table 1 of the Starr and Swenberg article).[192]

Finally, they utilized the lifetime risk of developing leukemia provided in Table 13.20 of the "SEER" Cancer Statistics Review 1975−2012, namely 1.47×10^{-2}, for the endogenous background risk parameter P_0.[194] Again, because the lower 95% confidence bound estimate (P_{0L}) was essentially the same as the central estimate (P_0) due to the very large sample size, they used this central estimate (P_0) in the upper bound added risk calculation, giving $AR_U < 1.24 \times 10^{-6}$ for lifetime continuous exposure to 1 ppm for FA. This is more than 4 orders of magnitude (> 45,000-fold) smaller than the corresponding US EPA estimate of 5.7×10^{-2}, that is, 5.7%.

The marked discrepancy between the upper 95% confidence bound bottom up and US EPA top down estimates of added leukemia risk is attributable to two factors. First, no exogenous N^2-$HOCH_2$-dG adducts were detected in the bone marrow collected in the cynomolgus monkey experiment despite the method's extraordinarily sensitive limit of detection, more than 10,000-fold lower than the endogenous N^2-$HOCH_2$-dG adduct concentration in bone marrow. Indeed, no such exogenous adducts were detected in any of the measured monkey tissues other than the nasal cavity respiratory epithelium.

Second, the US EPA upper bound added risk estimate for leukemia is derived from epidemiologic data that are extremely weak.[191] Thus, the confidence interval on the top down central estimate of added leukemia risk is more than wide enough to include zero risk; that is, no statistically significant dose−response relationship was found between leukemia risk and cumulative exposure to airborne FA in the epidemiologic study relied upon by USEPA in developing its added risk estimate for leukemia. It is well worth noting here that the bottom up approach does not suffer from this limitation that arises necessarily from weak (or even nonexistent) high dose data.

As noted previously, the bottom up approach assumes that the upper bound on added risk is essentially linear in the exogenous DNA adduct concentration near the endogenous background concentration. In fact, Starr and Swenberg assumed approximate linearity of this bound and the underlying linearity of the relationship between exogenous DNA adduct concentrations in target tissues and airborne FA concentrations all the way up to 6 ppm airborne FA.[192] However, Lu et al. previously identified a highly nonlinear increase in N^2-$HOCH_2$-dG adducts in nasal DNA of rats exposed to 0.7, 2, 5.8, 9.1, or 15.2 ppm [$^{13}CD_2$]-FA for 6 h, as demonstrated by the fact that a 21.7-fold increase in exposure (0.7−15.2 ppm) produced 286-fold higher amounts of exogenous DNA adducts in rat nasal epithelium.[56] The most recent exogenous DNA adduct data collected by Leng et al. further demonstrate that exogenous adduct concentrations in rat epithelium are not proportional to inhaled concentrations, with no exogenous adducts being detected in any of the examined tissues, including nasal epithelium, following 6 h/day exposures for consecutive 28 days to 0.01, 0.1, or 0.3 ppm airborne FA.[59] The implied steady-state exogenous concentrations C_{xss} and the associated upper bound added risks arising from lifetime continuous exposures are, thus, expected to be far lower than is implied by Starr and Swenberg's downward linear extrapolation from the C_{xss} values estimated for higher airborne FA concentrations.[192]

7. DISCUSSION AND CONCLUSION

Potential cancer risk from exposure to environmental chemicals remains a driver of risk management decisions regarding exposure regulations. It has likewise spurred research into the ways in which exposures to chemicals are likely (or not likely) to result in tumor formation. Determining what a chemical does in the body, its toxicokinetics, and MOA, forms a scientifically sound basis for assessments as opposed to reliance on defaults and unrealistic concepts of carcinogenesis.

DNA adducts provide characterization, and quantitation of DNA adduct formation provides very informative and useful tools for improving our understanding of how inhaled chemicals act within the body. DNA adduct formation has been consistently used as a biomarker of chemical exposure and can be considered as a molecular initiating event or early key event in pathways leading to cancer as an adverse outcome. Methods for detecting and identifying adducts described in this paper have clearly become both more sensitive and selective, and this trend is expected to continue.

Notably, current methods employing SILMS enable the distinction of the DNA adducts that arise from endogenous and exogenous sources in tissues of interest. FA was used as the primary case study in this review, and the data demonstrate that low exposures to exogenous FA do not appreciably increase DNA adduct levels. Furthermore, at the higher (i.e., ≥0.7 ppm) exposures that produced detectable levels of exogenous DNA adducts, the high levels were found in rats only at the site of initial contact; exogenous DNA adducts were not found at distant site tissues outside the portal of entry. This is in contrast to results from vinyl acetate, which requires metabolism to an electrophilic intermediate, leading to

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systemic distribution of exogenous adducts in blood but not other tissues.[170]

The DNA adduct data reviewed herein provide quantifiable evidence to support MOA evaluations and directly inform risk assessment based on contemporary concepts of carcinogenic processes. The FA case study demonstrates the utility of applying a dose−response evaluation procedure that incorporates knowledge of DNA adducts arising from both endogenous and exogenous exposures. The dose−response evaluation of FA concluded that some but not all of the background risk for cancer was attributable to endogenously formed FA.[191] It also incorporated a conservative low-dose linear upper bound on the added cancer risk attributable to exogenous DNA adducts. This approach produced an upper bound estimate of added leukemia risk that was more than 4 orders of magnitude (> 45,000-fold) smaller than the US EPA's estimate of 5.7×10^{-2}, that is, 5.7%. Finally, the DNA adduct data also provide direct empirical evidence that exposure of rat nasal epithelium DNA to inhaled FA is highly nonlinear. Thus, as such, there are several aspects of FA carcinogenesis that are proportionally nonlinear, supporting the inference that the added cancer risk that arises from low-level exogenous FA exposure is nonlinearly related to that exposure.

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Notes

The authors declare no competing financial interest.

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Yun-Chung Hsiao received his B.S. in Public Health from National Taiwan University in 2019 and worked at the Institute of Information Science, Academia Sinica as a R programmer. He is currently pursuing his Ph.D. in Environmental Sciences and Engineering at the University of North Carolina at Chapel Hill. He is involved in research that identifies, characterizes, and quantitates DNA adducts from different stressors. His other interest includes how the gut microbiome facilitates xenobiotic metabolism and endogenous processes, exposome profiling on environmental pollutants such as e-cigarettes, and other biochemical analyses by mass spectrometry.

Chih-Wei Liu earned his Ph.D. degree in Molecular Biology from the National Chung Hsing University in Taiwan focusing on plant proteomics study using mass spectrometry. During postdoctoral research, his work focused on the development and applications of ambient ionization mass spectrometry and human type 1 diabetes protein biomarker discovery from human tissues and plasma samples. Dr. Liu currently holds the position of Postdoctoral Research Associate at the University of North Carolina at Chapel Hill, performing a variety of aldehyde-induced DNA adducts and DNA-protein cross-links biomarker detections as well as large-scale metabolomics studies in biological samples.

Dr. Rita Schoeny retired from the U.S. EPA after 30 years and is currently a consultant in risk assessment and science policy. Her positions included Senior Science Advisor, Office of Science Policy, and Director, Risk Assessment Forum. She has held academic positions and lectures internationally. Recent publications include frameworks for human health risk assessment, interpretation of DNA adduct data for risk assessment, contemporary approaches to dose−response, and adverse outcome pathways and mode of action. Her awards include EPA's Science Achievement Award, Gold, Silver, and Bronze Medals, an FDA award, and professional society awards for publications. She is a Fellow of the Society for Risk Analysis.

Dr. Robinan Gentry has over 30 years of experience in toxicological issues relevant in the determination of the potential safety or risk associated with exposure to chemicals in consumer products, pharmaceuticals, or the environment. Over her career, she has been a principal investigator or contributing author for numerous safety and risk assessments for both government and industry. The purpose for a number of these assessments has been to incorporate innovative quantitative approaches in the determination of acceptable levels of exposure of humans to chemicals in the environment, pharmaceuticals, and consumer products.

Thomas B. Starr is an independent consultant on human health risk assessment for environmental chemicals. He also serves as an adjunct Associate Professor in the Department of Environmental Sciences and Engineering at the University of North Carolina-Chapel Hill and is an active emeritus member of the Society of Toxicology. His research and consulting interests have focused on incorporating knowledge of toxic mechanisms into the quantitative risk assessment process.

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ABBREVIATIONS

1,N^2-PdG, 1,N^2-propano-2′-dG; 1,N^2-ε-dG, 1,N^2-etheno-2′-dG; 1,N^6-ethano-dA, 1,N^6-ethano-2′-dA; 1,N^6-ε-dA, 1,N^6-etheno-2′-dA; 3,N^4-ε-dC, 3,N^4-etheno-dC; 4-ABP, 4-aminobiphenyl; 4-HNE, 4-hydoxy-2-nonenal; 5-Cl-dC, 5-chloro-dC; 5-OH-dC, 5-hydroxy-dC; 7-CH_3-dG, 7-methyl-dG; 7-OE-dG, 7-2′-oxoethyl-dG; 8-OH-dA, 8-hydroxy-dA; 8-OH-dG, 8-hydroxy-dG; 8-oxo-dG, 8-oxo-2′-dG; AA, acetaldehyde; AFB_1, aflatoxin B_1; ALDH, aldehyde dehydrogenase; AP, alkaline phosphatase; ATP, adenosine triphosphate; AαC, 2-amino-9H-pyrido[2,3-*b*]indole; AαC-HN^2-*O*-Gluc, *O*-(β-D-glucosidurony1)-2-hydroxyamino-9H-pyrido[2,3-*b*]indole; B*a*P, benzo-[*a*]-pyrene; BER, base excision repair; BPDE-dA, 7,8-diol-anti-9,10-epoxide-dA; BPDE-dG, 7,8-diol-anti-9,10-epoxide-dG; CBI, covalent binding index; CE, carboxylesterase; CEO, chloroethylene oxide; Cys, cysteine; dA, deoxyadenosine; dC, deoxycytodine; DDR, DNA damage response; dG, deoxyguanosine; dG-C8-AαC, *N*-(deoxyguanosin-8-yl)-2-amino-9H-pyrido[2,3-*b*]indole; dG-C8-4-ABP, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl; dG-C8-IQ, *N*-(deoxyguanosin-8-yl)-2-amino-3-methylimidazo[4,5-*f*]quinoline; dG-CH_2-Cys, deoxyguanosine-cysteine monoadduct cross-link; dG-N^2-IQ, 5-(deoxyguanosin-N^2-yl)-2-amino-3-methylimidazo[4,5-*f*]-quinoline; dG-C8-MelQx, N^2-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; DNA, DNA; DNase, deoxyribonuclease; dR, deoxyribose; dT, deoxythymidine; ECHA, European Chemical Agency; EO, ethylene oxide; EPA, Environmental Protection Agency; ESI, electrospray ionization; FA, formaldehyde; GC, gas chromatography; HOCl, hypochlorous acid; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; IARC, International Agency for Research on Cancer; IQ, 2-amino-3-methylimidazo-[4,5-*f*]quinoline; IS, internal standard; LC-MS, liquid chromatography-mass spectrometry; LNT, linear no threshold; M1-dA, N^6-(3-oxoprenyl)-deoxyadenosine; M1-dC, N^4-(3-oxoprenyl)-deoxycytidine; M1-dG, malondialdehyde-2′-deoxyguanosine; MDA, malondialdehyde; MelQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MS, mass spectrometry; MS^2, double stage tandem mass spectrometry; *m*/*z*, mass to charge ratio; N^2-CE-dG, N^2-carboxyethyl-2′-deoxyguanosine; N^2-CH_3-dG, N^2-methyl-deoxyguanosine; N^2-Et-dG, N^2-ethyl-deoxyguanosine; N^2-EtD-dG, N^2-ethylidene-deoxyguanosine; N^2-$HOCH_2$-dG, N^2-hydroxylmethyl-deoxyguanosine; N^3-CH_3-dC, N^3-methyl-deoxyguanosine; *N*3-Et-dT, N^3-ethyl-deoxyguanosine; N^6-CH_3-dA, N^6-methyl-deoxyadenosine; N^6-Et-dA, N^6-ethyl-deoxyadenosine; N^6-EtD-dA, N^2-ethylidene-deoxyadenosine; N^6-$HOCH_2$-dA, N^6-hydroxyl-methyl-deoxyadenosinel; N^7-HOEt-dG, N^7-(2-hydroxyethyl)-deoxyguanosine; nanoLC, nanoscale liquid chromatography; $NaCNBH_3$, sodium borocyanohydride; NBA, 3-nitrobenzathrone; NER, nucleotide excision repair; *N*-OH-ABP, *N*-hydroxy-4-aminobiphenyl; *N*-OH-IQ, *N*-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoline; *N*-OH-MelQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; O^2-CH_3-dT, O^2-methyl-deoxythymidine; O^2-Et-dT, O^2-ethyl-deoxythymidine; O^2-POB-dT, O^2-[4-(3-pyridyl-4-oxobut-1-yl]thymidine; O^4-CH_3-dT, O^4-methyl-deoxythymidine; O^4-Et-dT, O^4-ethyl-deoxythymidine; O^6-CH_3-dG, O^6-methyl-deoxythymidine; O^6-Et-dG, O^6-ethyl-deoxyguanosine; O^6-Me-dG, O^6-methyl-deoxyguanosine; PAH, polycyclic aromatic hydrocarbon; PBMC, peripheral blood mononuclear cell; POD, point of departure; Pol, DNA polymerase; PRM, parallel reaction monitoring; QqQ, triple quadrupole; Q-orbitrap, quadrupole-orbitrap; ROS, reactive oxygen species; SEER, Surveillance, Epidemiology, and End Results; SVP, snake venom phosphodiesterase; UHPLC, ultrahigh-performance liquid chromatography; VC, vinyl chloride; α-OH-PdG, α-hydroxy-propano-deoxyguanosine

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