Molecular Dosimetry of DNA Adducts in Rats Exposed to Vinyl Acetate Monomer

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ABSTRACT

Vinyl acetate monomer (VAM) is heavily used to synthesize polymers. Previous studies have shown that inhaled VAM, being metabolized to acetaldehyde, may form DNA adducts including N2-ethylidene-deoxyguanosine (N2-EtD-dG), which may subsequently cause mutations and contribute to its carcinogenesis. Currently, there is little knowledge on the molecular dosimetry between VAM exposure and DNA adducts under dosages relevant to human exposure. In this study, 0.02, 0.1, 1, 10, 50, 200, and 600 ppm VAM were exposed to rats by inhalation for 14 days (6 h/day). The use of [13C2]-VAM allows unambiguous differentiation and quantification of the exogenous and endogenous N2-EtD-dG by highly sensitive LC-MS/MS. Our data indicate that VAM-induced exogenous DNA adducts were formed in a non-linear manner. Exogenous DNA adducts were only detected in the nasal epithelium of rats exposed to 10, 50, 200, and 600 ppm VAM, whereas endogenous adducts were found in all nasal and other tissues analyzed. In addition, ratios of exogenous/endogenous DNA adducts were less than 1 with the dose up to 50 ppm, indicating that endogenous DNA adducts are predominant at low VAM concentrations. Moreover, differential dose-response in terms of exogenous DNA adduct formation were observed between nasal respiratory and olfactory epithelium. Furthermore, the lack of exogenous DNA adducts in distant tissues, including peripheral blood mononuclear cells, liver, brain, and bone marrow, indicates that VAM and/or its metabolite do not distribute systemically to cause DNA damage in distant tissues. Together, these results provided new molecular dosimetry to improve science-based cancer risk assessments of VAM.

Key words: cancer; vinyl acetate monomer; DNA adduct; risk assessment; LC-MS/MS; mass spectrometry; molecular dosimetry.

Vinyl acetate monomer (VAM) is an important industrial intermediate, and synthesized polymers have versatile applications including adhesives, coatings, paints, and other end-products. However, whether VAM is carcinogenic in human remains debatable. The International Agency for Research on Cancer (IARC) listed VAM as group 2B, which indicates possibly carcinogenic but lack of evidence in animal and human studies (IARC, 1987). Human exposure to VAM can happen for workers involved in VAM production and polymerization, and for general population when polymerized end-products release residual VAM (Albertini, 2013).

Previous in vitro and in vivo toxicological studies have suggested the potential mechanisms underlying VAM carcinogenicity (Albertini, 2013; Bogdanffy, 1999; Bogdanffy et al., 1994; Kuykendall et al., 1993; Lambert et al., 1985). Figure 1A illustrates how the key event in cancer development, that is, formation of...
DNA adducts, can occur after exposure to VAM. Absorbed VAM is first metabolized into acetaldehyde and acetic acid by the carboxylesterase (CE). VAM-derived acetaldehyde, if not deactivated by the aldehyde hydrogenase (ALDH) family, attacks nucleotides and forms DNA adducts including \(N^2\)-ethylidene-deoxyguanosine (\(N^2\)-EtD-dG) and \(1, N^2\)-propano-dG, with \(N^2\)-EtD-dG being the primary DNA adducts as shown in our previous study (Liu et al., 2021). For convenience, DNA adducts, such as \(N^2\)-EtD-dG, induced by inhaled VAM rather than from endogenous acetaldehyde are referred as “exogenous adducts” in this study.

DNA adducts, if not repaired efficiently and correctly, may lead to mutations, contributing to cancer development (Chatterjee and Walker, 2017). Thus, the formation of DNA adducts serves as a key event in carcinogenesis. DNA adducts have been frequently used as biomarkers of exposure to evaluate the cancer risk of chemicals (Beland et al., 2005; Rundle, 2006). Thorough review on genotoxicity assessments related to VAM has been previously published (Albertini, 2013). DNA damage such as adduct formation and DNA-DNA crosslink have been reported for both VAM and its metabolite acetaldehyde in vitro assays (Budinsky et al., 2013; Jantunen et al., 1986). In vivo studies have shown that nasal tumors were induced by VAM in rodents at high doses (Bogdanoff et al., 1994; Owen, 1988). However, science-based toxicological assessment of VAM still suffers from the lack of high quality dose-response data. Molecular dosimetry between VAM exposure and corresponding DNA adducts remains unclear. One of the challenges in assessing VAM carcinogenicity is the existence of endogenous acetaldehyde. Acetaldehyde from VAM metabolism and endogenous physiological processes can both result in formation of \(N^2\)-EtD-dG, which is undistinguishable in conventional assays. We have been developing methods by introducing stable isotope labeled chemicals for exposure to address this long-standing issue of the availability to reliable data in risk assessment of compounds with both endogenous and exogenous sources, such as VAM. As presented in this study, exogenous and endogenous \(N^2\)-EtD-dG can be differentiated by mass spectrometry when stable isotope labeled \(^{13}\text{C}_2\)-VAM is used for exposure, which is illustrated in Figure 1B.

In our recent study, we have demonstrated the formation of exogenous \(N^2\)-Et-dG in respiratory and olfactory epithelia of rat exposed to high doses of \(^{13}\text{C}_2\)-VAM (50, 200, and 400 ppm) for 6 h (Liu et al., 2021). However, the molecular dosimetry of DNA adducts over a wide dose range critical for risk assessment of VAM remains unknown. In addition, our previous study used 1-day exposure (6 h) (Liu et al., 2021), whereas DNA adducts would approach the steady state from repeated exposure. Therefore, the objective of this study was to establish the molecular dosimetry of DNA adducts in rats exposed to VAM for 14 days (6 h/day). Specifically, 0.02, 0.1, 1, 10, 50, 200, and 600 ppm VAM were exposed to rats for 6 h/day for 14 consecutive days. The doses we used ranged from highly human relevant low doses to the high concentrations that induced nasal tumors in rodent cancer bioassays. Proximal tissues including respiratory and olfactory epithelia in nasal cavity, and distant tissues including peripheral blood mononuclear cells (PBMCs), liver, brain, and bone marrow were all collected and analyzed for endogenous and

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Figure 1. A, DNA adduct formation by exposure to VAM and B, differentiation of endogenous \(N^2\)-EtD-dG and exogenous \(^{13}\text{C}_2\)-\(N^2\)-EtD-dG by using \(^{13}\text{C}_2\)-VAM for exposure. Note: \(N^2\)-EtD-dG is not stable and requires chemical reduction into stable \(N^2\)-ethyl-dG (\(N^2\)-Et-dG) for detection. VAM, vinyl acetate monomer; CE, carboxylesterase; ALDH, aldehyde dehydrogenase; \(N^2\)-EtD-dG, \(N^2\)-ethylidene-deoxyguanosine; AA, acetaldehyde.
MATERIALS AND METHODS

Chemicals and materials. All reagents and chemicals, unless otherwise specified, were obtained from Sigma-Aldrich (St Louis, Missouri). High-performance liquid chromatography (HPLC) or Optimal LC-MS grade water, methanol, acetonitrile, and 2-propanol were purchased from Thermo Fisher Scientific (Rockford, Illinois). NucleoBond AXG 20 and AXG 100 anion-exchange columns and corresponding buffer kits were purchased from Macherey-Nagel (Bethlehem, Pennsylvania). Proteinase K was purchased from WVR International LLC (Atlanta, Georgia); stainless steel beads (5 mm) were from QIAGEN (Germantown, Maryland); and Nanosep centrifugal devices 3K were obtained from Pall Life Sciences (Port Washington, New York).

Rat exposure to VAM. Test atmospheres of VAM were generated by vaporizing [$^{13}$C$_2$]-VAM (CAS No. 106139-40-6, P/N: 493481, from Pall Life Sciences (Port Washington, New York)). Maryland); and Nanosep centrifugal devices 3K were obtained from Pall Life Sciences (Port Washington, New York).

HPLC fractionation and purification of DNA adducts. The hydrolyzed DNA solutions were injected onto an Agilent 1200 Series UV-HPLC that contained a fraction collector. The DNA adducts of interest were separated from the abundant common nucleosides and collected by reversed-phase liquid chromatography with an Atlantis C$_{18}$ T3 column (150 x 4.6 mm, 3 µm, Waters) under 30°C column temperature. The mobile phases consisted of water with 10 mM ammonium acetate (A) and methanol (B) with the gradient set as the following: 5% B from 0 to 5 min, 5% to 15% B from 5 to 12 min, 15% to 22% B from 12 to 34 min, 22% to 80% B from 34 to 35 min, and a 5-min hold at 80% B for re-equilibration. N$^2$-dG and N$^2$-Et-dG were collected between 20.5 to 22.5 min and 27.0 to 31.3 min, respectively. The signal area of UV absorption at wavelength 254 nm in each of the HPLC fractionation was used to calculate the amount of dG in each of the loaded samples by a calibration curve (Supplementary Figure 1).

Nano-LC–ESI-MS/MS analysis. An Ultimate 3000 RSLCnano system coupled to a Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer through an EASY-Spray ion source for nanoelectrospray ionization (Thermo Fisher Scientific) was used to perform nano-LC–ESI-MS/MS analysis for the detection of DNA adducts. Fractions collected from the HPLC system were dried in vacuo and reconstituted in 0.1% formic acid for analysis. The samples were first loaded into a C$_{18}$ trapping cartridge (5 µm particle, 0.5 cm x 300 µm i.d., catalog no. 160454, Thermo Fisher Scientific) using 0.1% formic acid in water as loading solvent at 5 µl/min for 3 min. The trapped analytes were subsequently eluted to a PepMap C$_{18}$ analytical column (2 µm particle, 25 cm x 75 µm i.d., catalog no. E802A, Thermo Fisher Scientific) for separation prior to the detection of MS. The binary solvent system for mobile phase was consisted of water (A) and acetonitrile (B) both with 0.1% formic acid at a flow rate of 300 nl/min. The gradient was set as the following: 5% from 0 to 3 min (trapping time), 5% to 40% B from 3 to 16 min, 40% to 90% B from 16 to 16.1 min, 90% B to 100% B for 0.1 min and then to 5% B for 0.1 min.
from 16.1 to 26 min, 90% to 5% B from 26 to 26.1 min, and 5% B from 26.1 to 40 min as a final re-equilibration.

Targeted parallel reaction monitoring (PRM) mode was applied to collect LC-MS/MS raw data. For the detection of N²-Et-dG, an inclusion list composed of m/z 296.1353 (endogenous N²-Et-dG), m/z 298.1420 (exogenous [13C2]-N²-Et-dG), and m/z 301.1205 (internal standard [15N5]-N²-Et-dG) was respectively targeted at m/z of 338.1450, 340.1526 (+2 form exogenous), and 342.1593 (+4 form exogenous) with co-monitoring of the internal standard [13C2][15N4]-N²-Et-dG at m/z 353.1651 for fragmentations. The inclusion list for the detection of N²-ε-dG was comprised of m/z 292.1040 (endogenous N²-ε-dG) and m/z 302.1375 (internal standard [13C2][15N4]-N²-ε-dG). The automatic gain control target and the maximum fill time were set at 3 x 10² and 250 ms, respectively. The resolution of the Orbitrap was set at 60 000. The isolation width was set to 1.4 for the selection of precursors, and the following higher-energy collisional dissociation (HCD) was set to a normalized collision energy of 25 for all targets. The quantification analysis was done by extracting the major product ion with a neutral loss of deoxyribose moiety from each corresponding precursor (Supplementary Table 2). Quantitation of the analytes was achieved by fitting their respective signal ratio against their corresponding internal standard to a prepared calibration curve to obtain the amount ratio, which was then multiplied by the known amount of spiked internal standards and normalized by the amount of deoxyguanosine (dG) measured from the HPLC-UV system. The calibration curve converting signal ratio to amount ratio in the LC-MS/MS analysis was based on our previous studies (Hsiao et al., 2020; Liu et al., 2021).

Statistical analysis. Kruskal-Wallis nonparametric test was applied to determine the statistical significance of DNA adduct amounts between the different exposure groups. Dunn’s test with the Benjamini-Hochberg p-value adjustment procedure was done to identify group difference, and an adjusted p-value was reported. The significant level of tests was set at 0.05. Statistical tests and figures were made on R (ver. 4.0.3) combined with RStudio (ver. 1.4.1103, Boston, MA) or Prism 8 (GraphPad).

RESULTS

Animal Exposure
The animal exposure setup is illustrated in Figure 2. Briefly, rats were exposed to 0.02, 0.1, 1, 10, and 50 ppm stable isotope labeled [13C2]-VAM; or 200 and 600 ppm unlabeled [12C2]-VAM for 14 consecutive days (6 h/day). Unlabeled VAM was used for the 200 and 600 ppm exposure groups due to the high cost of using [13C2]-VAM for 14 days at high concentrations. Acetone was the vehicle chemical used to vaporize VAM in this study, and rats exposed to air/acetone served as the control group and was annotated as group 1. Other groups exposed to VAM were annotated as the exposure groups 2–8 with the dose ranging from 0.02 to 600 ppm. It should be noted that analytically measured concentrations of VAM were generally higher than target ones and used in dose-response analysis in the study, as shown in Table 1.

Analytical Assay for DNA Adduct Quantification
The primary goal of this study was to establish the molecular dosimetry of N²-Et-dG DNA adducts in rats exposed to VAM. Various doses of stable isotope labeled [13C2]-VAM were exposed to rats through nose-only inhalation units to distinguish exogenous [13C2]-N²-Et-dG adducts from endogenous adducts. Exogenous [13C2]-N²-Et-dG and endogenous N²-Et-dG in DNA were chemically reduced to [13C2]-N²-Et-dG and N²-Et-dG, respectively (Matsuda et al., 2007; Mizumoto et al., 2017; Moeller et al., 2013). The mass spectrometry-based analytical platform for accurate quantification of N²-Et-dG, N²-ε-dG, and 1, N²-prop-ano-dG adducts has been demonstrated in our previous studies (Hsiao et al., 2020; Liu et al., 2021).

Figure 3 shows representative nano-LC-ESI-MS/MS PRM chromatograms of endogenous N²-Et-dG, exogenous [13C2]-N²-Et-dG, and [15N5]-N²-ε-dG internal standard in nasal respiratory epithelium of rats exposed to air control and 50 ppm [13C2]-VAM. Clearly, in the control rats, exogenous [13C2]-N²-Et-dG adducts have been demonstrated in our previous studies (Hsiao et al., 2020; Liu et al., 2021).
Table 1. Endogenous and Exogenous N2-Et-dG Adduct Amount (adducts/10^8 dG) in Nasal Tissues Including Respiratory and Olfactory Epithelia of Rats Exposed to Various Concentrations of VAM for 6 h/day and 14 Consecutive Days

| Tissue     | Exposure Group | Exposure Type | Target Concentration (ppm) | Analytically Measured Concentration (ppm) | n | ^13C2^-N2-Et-dG Level (adduct/10^8 dG) | ^13C2^-N2-Et-dG Level (adduct/10^8 dG) | Calculated Exogenous N2-Et-dG Level (adduct/10^8 dG) | Exogenous-Endogenous Ratio |
|------------|----------------|--------------|----------------------------|------------------------------------------|---|-------------------------------------|-------------------------------------|------------------------------------------------|---------------------------|
| Respiratory| 1 —            | —            | 0                          | 0                                        | 7 | 26.03 ± 16.44                       | N.A. b                               | <0.022                                         | —                         |
|            | 2 ^13C2^-VAM   | 0.02         | 0.04                       | 7                                        | 22.49 ± 5.69                           | N.D. c                               | <0.022                                         | <0.00097                            |
|            |                |              |                            | 3^a                                      | 31.87 ± 3.31                           | N.D. c                               | <0.0018                                         | <0.000056                           |
|            | 3 ^13C2^-VAM   | 0.1          | 0.23                       | 7                                        | 23.82 ± 4.10                           | N.D. c                               | <0.022                                         | <0.00092                            |
|            |                |              |                            | 3^a                                      | 38.94 ± 2.34                           | N.D. c                               | <0.0018                                         | <0.000046                           |
|            | 4 ^13C2^-VAM   | 1            | 2.5                        | 7                                        | 23.01 ± 4.82                           | N.D. c                               | <0.022                                         | <0.00095                            |
|            |                |              |                            | 3^a                                      | 19.18 ± 4.15                           | N.D. c                               | <0.0018                                         | <0.00093                            |
|            | 5 ^13C2^-VAM   | 10           | 21                         | 7                                        | 26.60 ± 8.25                           | 2.16 ± 0.58                          | 2.16 ± 0.85                                     | 0.09 ± 0.05                         |
|            |                |              |                            |                                          |                                          | 15.00 ± 6.02                        | 15.00 ± 6.02                                    | 0.67 ± 0.31                         |
|            | 6 ^13C2^-VAM   | 50           | 54                         | 7                                        | 23.43 ± 4.93                           | N.A. b                               | 240.32 ± 56.17                                   | 9.23 ± 2.15                         |
|            |                |              |                            |                                          |                                          | 190.49 ± 32.41                      | 577.79 ± 190.49                                 | 22.19 ± 7.31                         |
|            | 7 Unlabeled VAM| 200          | 210                        | 7                                        | 266.36 ± 56.17                         | N.A. b                               | 577.79 ± 190.49                                   | 9.23 ± 2.15                         |
|            | 8 Unlabeled VAM| 600          | 626                        | 7                                        | 603.83 ± 190.49                        | N.A. b                               | 577.79 ± 190.49                                   | 9.23 ± 2.15                         |
| Olfactory  | 1 —            | —            | 0                          | 0                                        | 7                                        | 38.21 ± 14.63 | N.A. b       | <0.022                                         | —                         |
|            | 2 ^13C2^-VAM   | 0.02         | 0.04                       | 7                                        | 40.54 ± 14.51                           | N.D. c                               | <0.022                                         | <0.00054                            |
|            |                |              |                            | 3^a                                      | 43.76 ± 5.74                           | N.D. c                               | <0.0018                                         | <0.000041                           |
|            | 3 ^13C2^-VAM   | 0.1          | 0.023                      | 7                                        | 39.14 ± 17.27                           | N.D. c                               | <0.022                                         | <0.00056                            |
|            |                |              |                            | 3^a                                      | 43.70 ± 2.21                           | N.D. c                               | <0.0018                                         | <0.000041                           |
|            | 4 ^13C2^-VAM   | 1            | 2.5                        | 7                                        | 38.64 ± 19.10                           | N.D. c                               | <0.022                                         | <0.00057                            |
|            |                |              |                            | 3^a                                      | 31.20 ± 1.87                           | N.D. c                               | <0.0018                                         | <0.000058                           |
|            | 5 ^13C2^-VAM   | 10           | 21                         | 7                                        | 38.88 ± 18.87                           | N.D. c                               | <0.022                                         | <0.00057                            |
|            |                |              |                            | 5^a                                      | 20.97 ± 1.96                           | 0.08 ± 0.001                         | 0.08 ± 0.001                                    | 0.003 ± 0.007                       |
|            | 6 Unlabeled VAM| 50           | 54                         | 7                                        | 36.08 ± 18.83                           | 1.27 ± 0.33                          | 1.27 ± 0.33                                     | 0.046 ± 0.027                       |
|            | 7 Unlabeled VAM| 200          | 210                        | 7                                        | 81.78 ± 23.20                           | N.A. b                               | 43.57 ± 23.19                                   | 1.140 ± 0.607                       |
|            | 8 —            | 600          | 626                        | 7                                        | 371.09 ± 101.26                         | N.A. b                               | 332.88 ± 101.26                                  | 8.712 ± 2.650                       |

Data are presented as mean ± standard deviation.

^aDNA from every 3–4 rats were pooled for analysis (n = 3 or 5) to improve sensitivity for low dosing groups of 0.02, 0.1, 1, and 10 ppm in addition to individual rat samples analyzed (n = 7). In total, DNA from approximately 20 rats were used in each dosing group for adduct analysis.

^bN.A.: not exposed to ^13C2^-VAM. 13C adducts were not expected and detected in these samples.

^cN.D.: exposed to ^13C2^-VAM but 13C adducts not detected (ie, below the LOD).
adduct was not detected. Signal ratios between the analyte (endogenous or exogenous adduct signals) and internal standard were used to calculate the exact amounts of target analytes based on the known amount of spiked internal standard. The final amounts of target analytes were normalized to the dG amounts of samples used for HPLC purification after DNA digestion. The quantification of N2-Et-dG and 1, N2-propano-dG followed the same strategy in calculation. The on-column limit of detection for N2-Et-dG, N2-Et-dG, and 1, N2-propano-dG on the LC-MS/MS platform were 1.2, 4.7, and 6.0 amol, respectively. Our previous study demonstrated that N2-EtD-dG was the primary exogenous DNA adducts induced by VAM exposure, with exogenous N2-Et-dG only being detected in rats exposed to a single exposure of 400 ppm [13C2]-VAM for 6 h (Liu et al., 2021). Herein, exogenous 1, N2-propano-dG adducts were not detected in nasal epithelium of rats exposed to 50 ppm [13C2]-VAM for 14 days (Supplementary Figure 2), whereas exogenous N2-Et-dG adducts were readily detected in the same exposed rats, again highlighting N2-Et-dG as the primary exogenous DNA adducts of VAM. Consequently, the focus of our analysis and discussion has been placed on N2-Et-dG adducts.

**Figure 3.** Representative nano-LC-ESI-MS/MS PRM chromatograms of endogenous N2-Et-dG (upper panel), exogenous [13C2]-N2-Et-dG (middle panel), and [15N5]-N2-Et-dG spiked in samples as internal standard (bottom panel) in nasal respiratory epithelium of rats exposed to acetone (as control vehicle) (A) and 50 ppm [13C2]-VAM (B). Chemical structures of N2-Et-dG, [13C2]-N2-Et-dG, and [15N5]-N2-Et-dG and their quantifying transition were annotated in each panel. Quantifying product ion of each precursor was the major fragment ion with a neutral loss of deoxyribose moiety after fragmentation.

**Figure 4.** Endogenous (12C) and exogenous (13C) N2-Et-dG in nasal tissues including respiratory epithelium (A) and olfactory epithelium (B). Data were shown in means and standard deviations. Endogenous N2-Et-dG and exogenous [13C2]-N2-Et-dG from nasal tissues are analyzed by LC-MS/MS. Olfactory epithelium under exposure to 1 ppm and 10 ppm [13C2]-VAM were analyzed for 120 μg DNA with n = 3 or 5 replicates. Other tissues are analyzed for 10 μg with n = 7 replicates. Nondetected groups were annotated with “N.D.”
adduct measurement for the dosing groups of 0.02, 0.1, and 1 ppm. However, exogenous \( ^{13}\text{C}_2\)-N\(^2\)-Et-dG were still not detected despite 12 times more DNA being used. Under such scenario, the limits of detection were used to estimate the normalized exogenous DNA adducts, as listed in Table 1.

Figure 4 illustrates the levels of endogenous and exogenous N\(^2\)-Et-dG in nasal respiratory and olfactory epithelia of rats exposed to 0.02 to 50 ppm \( ^{13}\text{C}_2\)-VAM. Endogenous N\(^2\)-Et-dG was detected in all samples analyzed and showed no difference among groups (\( p = .77 \)). This indicates that exposure to \( ^{13}\text{C}_2\)-VAM even up to 50 ppm for 14 days did not alter endogenous N\(^2\)-Et-dG. Exogenous \( ^{13}\text{C}_2\)-N\(^2\)-Et-dG was only detected in rats exposed to 10 and 50 ppm \( ^{13}\text{C}_2\)-VAM. In addition, the amounts of exogenous N\(^2\)-Et-dG were less than endogenous DNA adducts, especially at low concentrations. For example, the ratio of exogenous/endogenous DNA adducts was approximately 0.046 and 0.67 in nasal olfactory and respiratory epithelia of rats exposed to 50 ppm \( ^{13}\text{C}_2\)-VAM. Likewise, the ratio of exogenous/endogenous DNA adducts was only approximately 0.0038 and 0.09 in nasal olfactory and respiratory epithelia of rats exposed to 10 ppm \( ^{13}\text{C}_2\)-VAM. These data indicate that endogenous DNA adducts were predominant in rats exposed to up to 50 ppm VAM. The exogenous adducts induced by VAM only accounted for a very small portion of total DNA adducts in exposed rats at low doses.

The dose-response between inhaled concentrations of VAM and the amounts of resulted exogenous N\(^2\)-Et-dG in nasal tissues is presented in Figure 5. A clear nonlinear dose response was observed across the doses used in this study. Consistent with our previous 1-day exposure study (Liu et al., 2021), more exogenous N\(^2\)-Et-dG adducts were observed in respiratory than in olfactory epithelia. However, differential dose-response was observed between nasal respiratory and olfactory epithelia. For instance, the amount of exogenous DNA adducts increased.

Table 2. Endogenous and Exogenous N\(^2\)-Et-dG Adduct Amount (adducts/10\(^8\) dG) in PBMCs of Rats Exposed to Various Concentrations of VAM for 6 h/day and 14 Consecutive Days

| Exposure Group | Exposure Type | Target Concentration (ppm) | Analytically Measured Concentration (ppm) | n | \( ^{12}\text{C}_2\)-N\(^2\)-Et-dG Level (adduct/10\(^8\) dG) | \( ^{13}\text{C}_2\)-N\(^2\)-Et-dG Level (adduct/10\(^8\) dG) | Calculated Exogenous N\(^2\)-Et-dG Level (adduct/10\(^8\) dG) | Exogenous-Endogenous Ratio |
|----------------|---------------|-----------------------------|------------------------------------------|---|----------------|----------------|---------------------------------|---------------------------|
| 1              | —             | 0                           | 0                                        | 3 | 53.23 ± 3.98  | N.A.          | <0.0018                         | —                         |
| 5              | \( ^{13}\text{C}_2\)-VAM | 10                          | 21                                       | 3 | 68.88 ± 5.51  | N.D.          | <0.0018                         | <0.0000382                |
| 6              | \( ^{13}\text{C}_2\)-VAM | 50                          | 54                                       | 3 | 70.44 ± 16.78 | 0.2102        | 0.2102                          | 0.0019                    |
| 7              | Unlabeled VAM | 200                         | 210                                      | 3 | 37.17 ± 2.38  | N.A.          | N.D.                            | N.A.                      |
| 8              | Unlabeled VAM | 600                         | 626                                      | 3 | 50.63 ± 3.50  | N.A.          | N.D.                            | N.A.                      |

Data are presented in means ± standard deviation.

\(^*\)Sample size (n): For PBMC samples, 120 μg DNA was used, and 3 replicates were pooled from every 2–3 rats.

\(^\text{a}\)N.A.: not exposed to \( ^{13}\text{C}_2\)-VAM. \(^{13}\text{C} \) adducts were not expected and detected in these samples.

\(^\text{b}\)N.D.: exposed to \( ^{13}\text{C}_2\)-VAM but \(^{13}\text{C} \) adducts not detected (ie, below the LOD).
in Supplementary Figure 3. N²-ε-dG was successfully detected in all tissue samples in different exposure groups (Table 3). Levels of N²-ε-dG in the nasal tissues including respiratory and olfactory epithelia were similar among all groups, with p values of Kruskal-Wallis groupwise test in respiratory and olfactory epithelia being 0.57 and 0.99, respectively. PBMC was analyzed for N²-ε-dG in rats of the groups 1, 5, 6, 7, and 8 and showed no statistically significant difference (p value = .11). For liver, brain, and bone marrow, N²-ε-dG in groups 1, 5, 6 were analyzed and showed no statistically significant difference among exposure groups as well (p values were .46, .44, and .45, respectively). Taken together, N²-ε-dG was monitored as a biomarker of lipid peroxidation in this study, but no difference was observed among the exposure groups of all tissues analyzed.

**DISCUSSION**

In this study, stable isotope labeled [¹³C₂]-VAM was utilized for inhalation exposure in rats to distinguish exogenous and endogenous N²-Ed-dG adducts. Rat tissues exposed to various doses of VAM for 14 consecutive days (6 h/day) were analyzed to quantify exogenous and endogenous DNA adducts. Our objective in the study is to establish the molecular dosimetry of DNA adducts in rats exposed to VAM, which will provide critical new data to improve science-based cancer risk assessment of VAM exposure.

VAM is metabolized into acetaldehyde that can form different DNA adducts including N²-Ed-dG and 1, N²-propano-dG. In addition, acetaldehyde can cause lipid peroxidation, leading to the formation of etheno-adducts, such as N²-ε-dG. Therefore, all 3 DNA adducts were monitored in this study. As described in the Results, exogenous N²-Ed-dG adducts were detected in rats exposed to VAM at the concentrations of 10 ppm and above and a nonlinear dose response was observed. However, no exogenous N²-ε-dG and 1, N²-propano-dG were detected in the same animal samples.

For group 1, N²-propano-dG was only detected in rats exposed to 400 ppm [¹³C₂]-VAM (6 h/day, 1 day) in our previous study (Liu et al., 2021). However, was not detected in rats exposed to 50 ppm [¹³C₂]-VAM (6 h/day for 14 days) in this study, the highest dosing group using stable isotope labeled VAM for exposure. These results are consistent with our previous study and support that N²-Ed-dG is the primary DNA adducts induced by VAM exposure (Liu et al., 2021). Likewise, N²-ε-dG adducts were detected in all rat tissues we analyzed, but no statistical significance was found between the control and exposed rats. Taken together, N²-Ed-dG may serve as a more sensitive and suitable biomarker to evaluate the exposure and/or effects of VAM exposure. In addition, the nonlinear dose response of N²-Ed-dG observed in this study would be useful to model the formation of DNA adducts induced by VAM and in the risk assessment of VAM carcinogenicity.

In this study, rats were exposed to VAM at 0.02, 0.1, 1, 10, 50, 200, and 600 ppm for 14 days (6 h/day), followed by DNA adduct analysis. Exogenous N²-Ed-dG adducts were found in nasal tissues in rats at 10, 50, 200, and 600 ppm dosing groups (Table 1). Exogenous N²-Ed-dG was not detectable in nasal epithelium tissues of rats exposed to the doses of 0.02, 0.1, and 1 ppm. Despite 12-fold more DNA used for analysis to increase the chance of detecting exogenous DNA adducts in these low dosing group, no exogenous DNA adducts could be detected. Therefore, for the dosing groups of 0.02, 0.1, and 1 ppm, the limit of detection was used to calculate normalized exogenous DNA adducts and the ratio of exogenous/endogenous DNA adducts. If the exogenous DNA adducts were present in these samples, the

**Potential Systemic Distribution of VAM or Its Metabolite**

In addition to DNA adducts at respiratory and olfactory epithelia, VAM or its metabolite may potentially further distribute and result in adducts in distant organs. Other tissues including PBMC, liver, brain, and bone marrow were also analyzed for endogenous and exogenous N²-Ed-dG to examine potential systemic distribution of VAM. We particularly focused on the dosing groups in which exogenous DNA adducts were detected in the nasal epithelium. PBMC from group 1 (control) and groups 5–8 (10 and 50 ppm [¹³C₂]-VAM, 200 and 600 ppm [¹²C₂]-VAM) were examined. Livers, brains, and bone marrows of rats in group 1 (control), group 5 (10 ppm), and group 6 (50 ppm) were also analyzed. Table 2 summarized the results of endogenous and exogenous N²-Ed-dG in PBMC samples of rats. Exogenous [¹³C₂]-N²-Ed-dG was undetectable in the control group (group 1) and in rats exposed to 10 ppm [¹³C₂]-VAM (group 5). Exogenous N²-Ed-dG was detected in 1 of 3 pooled replicates of PBMC samples in the group 6 rats (50 ppm [¹³C₂]-VAM), with approximately 0.21 adducts/10⁸ dG. The level of exogenous adduct was only 0.19% of the endogenous adduct measured in the same sample. For group 7 (200 ppm [¹²C₂]-VAM) and group 8 (600 ppm [¹²C₂]-VAM), the measured N²-Ed-dG had levels of 37.17 ± 2.38 and 50.63 ± 3.50 adducts/10⁸ dG, which had no statistically significant different (p = .10 and 0.82, respectively) when compared with the controls. Likewise, no exogenous adduct was detected in liver, brain, and bone marrow samples of rats exposed to [¹³C₂]-VAM, and no statistically significant difference in endogenous adducts among exposure groups (Figure 6).

**Detection of N²-ε-dG Adducts Among Tissues**

The metabolite of VAM, acetaldehyde, may induce ROS that leads to lipid peroxidation, which synthesizes γ,δ-unsaturated aldehydes to attacks DNA and form the mutagenic N²-ε-dG (Mizumoto et al., 2017). Therefore, we further measured the levels of N²-ε-dG in the tissue samples of rats exposed to VAM. A representative LC-MS/MS chromatogram of N²-ε-dG was shown approximately 38.5- and 262-fold in nasal respiratory and olfactory epithelia respectively, when the dose was increased from 50 to 600 ppm.

**Figure 6.** Measurement of endogenous N²-Et-dG in peripheral blood mononuclear cells (PBMC), liver (LV), brain (BR), and bone marrow (BM) of rats exposed to air control, 10 ppm [¹³C₂]-VAM, and 50 ppm [¹³C₂]-VAM. Data were shown in means and standard deviations. Sample size (n) were annotated in the beside the types of tissue. Levels of endogenous N²-Et-dG levels in each tissue show no statistical significance of difference by Kruskal-Wallis tests.
Table 3. Additive Amount (Adducts/10^8 dG) in Tissues of Rats Exposed to Various Concentrations of VAM for 60-day and 14 Consecutive Days Exposure

| Exposure Type | Concentration (ppm) | Target Tissue | Adducts/10^8 dG |
|---------------|--------------------|---------------|----------------|
| Respiratory Epithelium | 0.02 | Liver | 6.02 |
| | 0.04 | Brain | 2.44 |
| | 0.06 | Bone Marrow | 0.26 |
| | 0.08 | Skin | 0.83 |
| | 0.1 | Skin | 0.91 |
| | 0.2 | Skin | 1.65 |
| | 0.4 | Skin | 2.68 |
| | 1 | Skin | 5.45 |
| | 10 | Skin | 21.00 |
| Olfactory Epithelium | 0.02 | Liver | 0.33 |
| | 0.04 | Brain | 0.33 |
| | 0.06 | Bone Marrow | 0.33 |
| | 0.08 | Skin | 0.83 |
| | 0.1 | Skin | 0.91 |
| | 0.2 | Skin | 0.91 |
| | 0.4 | Skin | 0.91 |
| | 1 | Skin | 0.91 |
| | 10 | Skin | 0.91 |

aData are presented as "mean ± standard deviation (number of biological replicates)." A total of 10 mg DNA from each of the tissue was used for analysis if not specified. Tissues not analyzed are annotated with "—."
respectively). However, in rats exposed to 600 ppm VAM, the amount of exogenous N²-Et-dG in nasal respiratory epithelium is only approximately 1.7-fold higher than olfactory epithelium (577.79 ± 190.49 and 332.88 ± 101.26 adducts/10⁶ dG, respectively). The significant reduced difference in the amounts of exogenous DNA adducts between nasal olfactory and respiratory epithelium at high doses may be impacted by diffusion and vapor deposition efficiency along nasal cavity and potential saturation of metabolism enzymes such as CE and ALDH in nasal respiratory epithelium at high VAM concentrations.

It has been reported that chronic inhalation exposure induced nasal tumor in rodents at high doses (such as 600 ppm), with nasal olfactory epithelium being more sensitive than respiratory epithelium (Albertini, 2013; Bogdanffy et al., 1994, 1997; Budinsky et al., 2013; Morris, 1997; Stanek and Morris, 1999). Herein, we noticed that a significant amount of exogenous N²-Et-dG was formed in nasal olfactory epithelia of rats exposed to 600 ppm VAM, which may contribute to the increased tumor incidence in nasal olfactory epithelium. The olfactory epithelium may be more susceptible to tumorigenesis due to its higher rate in cell proliferation. Bogdanffy et al. observed the cellular adaptation by evaluating the cell proliferation in the rat nasal respiratory and olfactory epithelia exposed to 0, 50, 200, 600, or 1000 ppm VAM for 1, 5, or 20 days (6 h/day) (Bogdanffy et al., 1997). The trends of cell proliferation were similar between respiratory and olfactory epithelia after 1 and 5 day exposure, however, much more cell proliferation was observed in the olfactory epithelium after 20 days exposure, resulting in the cellular damages mainly confined in the olfactory epithelium. The formation of high amount of exogenous DNA adducts (approximately 8.7-fold higher than endogenous ones), coupled with enhanced cell proliferation, may account for the sensitivity of nasal olfactory in VAM-induced nasal cancer.

There are several limitations associated with the study. First, we were not able to use [13C₂]-VAM for all dosing groups, as [13C₂]-VAM is cost prohibitive for a 14-day high dose exposure. In the dosing groups of 50 ppm and below, [13C₂]-VAM was used, and endogenous and exogenous DNA adducts were unambiguously differentiated and determined by mass spectrometry. The exogenous adducts in the groups of 200 and 600 ppm were calculated by subtracting the amount of endogenous DNA adducts of the controls from the total adducts of rats exposed to 200 and 600 ppm unlabeled VAM. Although such a strategy may not be optimal, it may still offer a reasonable method to estimate exogenous DNA adducts induced by exposure, as our data indicate that VAM exposure does not alter the amount of endogenous DNA adducts. Second, systemic distribution of VAM can exist, especially under high concentrations of exposure. We observed insignificant difference for N²-Et-dG in the PBMC samples in the 200 and 600 ppm groups compared with nonexposed controls. However, the lack of isotopic labels hindered us from differentiating endogenous and exogenous N²-Et-dG, especially when the exogenous adducts existed in a trace amount thus were veiled by the abundant endogenous adducts. Third, we exposed rats to VAM for 14 days (6 h/day). The exposure period may not be enough to approach the steady state of exogenous DNA adducts yet and a longer exposure time, such as 28 days, would be more appropriate. However, we have generated data on DNA adducts from 1 day exposure and 14-day exposure, coupled with the kinetics of DNA adduct repair/half-life from our previous study (Liu et al., 2021), which would allow the estimation of the steady state of exogenous DNA adducts to improve science-based cancer risk assessment of VAM.

**SUPPLEMENTARY DATA**

Supplementary data are available at Toxicological Sciences online.

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**DECLARATION OF CONFLICTING INTERESTS**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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