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Rapid Report

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Molecular Dosimetry of Endogenous and Exogenous O^6-Methyl-dG and N7-Methyl-G Adducts Following Low Dose [D_3]-Methylnitrosourea Exposures in Cultured Human Cells

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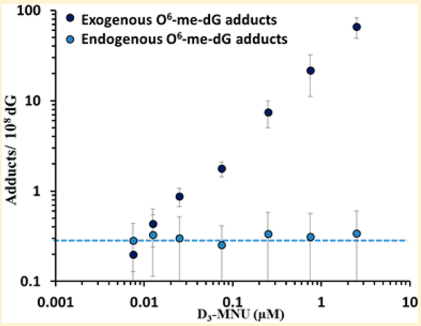
Supporting Information

ABSTRACT: For DNA-reactive chemicals, a low dose linear assessment of cancer risk is the science policy default. In the present study, we quantitated the endogenous and exogenous N7-methyl-G and O^6-methyl-dG adducts in human lymphoblastoid cells exposed to low dose [D_3]-methylnitrosourea. Endogenous amounts of both adducts remained nearly constant, while the exogenous adducts showed linear dose-responses. The data show that O^6-methyl-dG adducts $\geq 1.8/10^8$ dG correlated with published studies that demonstrated significant increases of mutations under these conditions. The combined results do not support linear extrapolations to zero when data are available for science-based regulations.



Cancer is caused by both DNA-reactive and non-DNA-reactive chemicals through the induction and accumulation of mutations in critical cellular genes.[1] An accurate assessment of the risks associated with such chemicals is necessary to understand their carcinogenic potential on human populations. One of the earliest events in chemical carcinogenesis by DNA-reactive chemicals is the formation of DNA adducts. These DNA adducts serve as biomarkers of exposure to carcinogens and an early indicator of cancer risk. In contrast to biomarkers of effects (e.g., mutations), the DNA adducts are produced linearly down to very low doses unless identical adducts are formed endogenously.[2] In contrast, a steady state background of endogenous DNA lesions is present, which contribute to nonlinear dose-responses for mutations.[3] However, for DNA-reactive chemicals, a linear assessment of risk, especially at low doses is the science policy default that is supported by several regulatory agencies.[4] This results in a high degree of uncertainty in the low dose range for exposure-response relationships of cancer risk assessments. Some scientists have challenged this linear risk assessment and have demonstrated thresholds for biomarkers of effect, such as mutations, micronuclei, and chromosome abnormalities for direct-acting genotoxins.[5,6] Alkylating agents like methylnitrosourea (MNU) have been prominent models used for developing such low dose mutation data.[5,6] The background mutations observed in these studies can result from identical and background endogenous DNA damage. However, there remain knowledge gaps regarding the formation of DNA adducts due to carcinogen exposure and the contribution of endogenous DNA adducts to the total adduct load and the extent of DNA adduction required for effective mutagenesis. Distinguishing the formation of endogenous and exogenous DNA adducts can provide a biologically relevant understanding of these biomarkers of exposure. This information will improve the scientific basis needed for the risk assessment process by providing better understanding of exposure through the use of biomarkers.

Therefore, in the present study, we have quantitated the endogenous and exogenous N7-methyl-G and O^6-methyl-dG adducts in human lymphoblastoid cells exposed to low doses of the model alkylating agent, MNU. MNU was selected as it represents alkylating agents, an important class of DNA-reactive genotoxins. Human exposure to exogenous alkylation can happen in everyday life, either directly or indirectly through chemotherapeutic agents, cigarette smoke, pharmaceutics, and occupational and dietary sources. Alkylating agents exert their mutagenic and genotoxic effects by forming adducts with the N- and O-atoms in DNA bases. Similar DNA adducts are also formed endogenously, possibly by *S*-adenosylmethionine (SAM), which is a key compound in transmethylation, aminopropylation, and transsulfuration metabolic pathways within cells.[7] MNU is a potent mutagen (low s value of 0.42) and alkylates O-atoms quite efficiently, yielding O^6-methyl-dG, along with N7-methyl-G adducts. While N7-methyl-G destabilizes the N-glycosidic bond, resulting in the formation of abasic sites, O^6-methyl-dG is biologically more relevant due to its greater mutagenic potential.[8]

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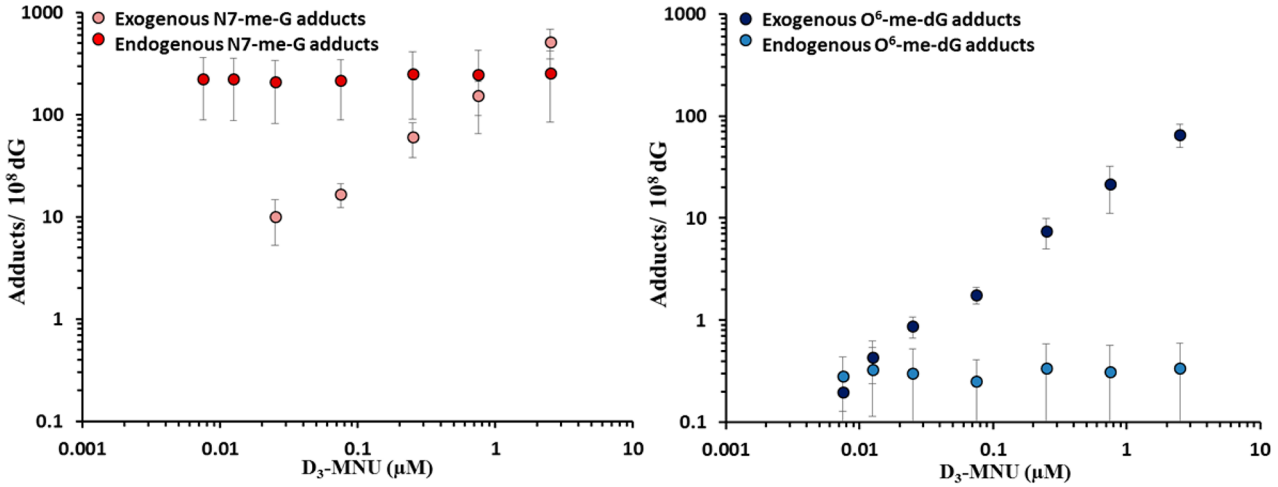

Figure 1. Endogenous versus exogenous adducts in AHH-1 cells exposed to D3-MNU (0.0075 μM to 2.5 μM) for 1 h. The endogenous and exogenous O^6-me-dG and N7-me-G adducts at each exposure concentration are plotted on a log versus log scale. Data represent the mean ± SD.

The evaluation of DNA adducts was done at low doses, similar to those used to collect mutation data in the same cell type, as has been reported previously.[5,6] This offers a possibility for the comparison of thresholds for biomarkers of exposure versus biomarkers of effect. This is the first study to distinguish between endogenous and exogenous formation of MNU induced N7-methyl-G and O^6-methyl-dG adducts following low dose exposures to stable isotope MNU ([D_3]-methylnitrosourea). The data generated provide a data-driven understanding of the relative contributions of identical endogenous and exogenous DNA adducts to the overall amount of methylated DNA. It also provides an explanation for low dose thresholds.

Briefly, human lymphoblastoid cells (AHH1) were exposed to [D_3]-MNU (0.0075 μM to 2.5 μM) for 1 h (for details, see Supporting Information). DNA was extracted and subjected to neutral thermal hydrolysis to remove N7-methyl-G followed by enzymatic hydrolysis. The fraction containing O^6-methyl-dG was collected by HPLC. Both endogenous and exogenous N7-methyl-G and O^6-methyl-dG were analyzed using sensitive LC-MS/MS methods employing selected reaction monitoring with detection limits of one N7-methyl-G/10^7 dG and one O^6-methyl-dG/10^9 dG per sample (for details, see Supporting Information). The presence of both endogenous (166 → 149 m/z) and exogenous (169 →152 m/z) N7-methyl-G, and endogenous (282 →166 m/z) and exogenous (285 → 169 m/z) O^6-methyl-dG with the respective internal standards was monitored, as shown in Figures S1−S4 (Supporting Information).

Our results show a clear dose-dependent linear increase in the formation of exogenous adducts with increasing [D_3]-MNU exposure concentrations, whereas the endogenous level of both O^6-methyl-dG and N7-methyl-G adducts remained nearly constant (Figure 1). This is likely to represent steady-state concentrations of these adducts. Average endogenous amounts of O^6-methyl-dG and N7-methyl-G were 0.30 ± 0.04 and 232 ± 16.67 (mean ± SD) adducts/10^8 dG, respectively. Exogenous N7-methyl-G was significantly lower than the corresponding endogenous adducts at [D_3]-MNU concentrations ≤0.75 μM, with statistically significant (Student's t test, p value <0.05) differences at 0.075 μM and 0.025 μM. At a concentration of 2.5 μM, the exogenous N7-methyl-G became higher than the corresponding endogenous adducts. Unlike the N7-methyl-G, O^6-methyl-dG adducts started showing significant increases in the exogenous compared to corresponding endogenous adducts at much lower concentrations (≤0.025 μM). The relative ratio between O^6-methyl-dG and N7-methyl-G adducts is dependent upon the chemical, and for MNU, it is reported as 0.1, which was observed in the present study.[5,8] Another important observation was the ratio between endogenous O^6-methyl-dG and N7-methyl-G adducts, which was constant across different doses and experiments and found to be approximately 0.001.

The sum of both the endogenous and exogenous N7-methyl-G, as well as that of the total O^6-methyl-dG adducts was significantly increased (Student's t test, p value <0.05) above the average endogenous adducts at [D_3]-MNU concentrations ≥0.75 μM and ≥0.025 μM, respectively (Table 1). The exogenous N7-methyl-G adducts did not drive the total number of adducts until very high concentrations were present. In contrast, the O^6-methyl-dG adducts resulted in a combined increase in adducts at all but the two lowest exposures. The thresholds for biomarkers of exposure and effect do not necessarily lie in the same range. Thomas et al.[6] found nonlinear responses in point mutation induction with a no-observed genotoxic effect level (NOGEL) of ~0.075 μM and

Table 1. Total N7-me-G and O^6-me-dG Adducts in AHH1 Cells Exposed to [D_3]-MNU[a]

[D_3]-MNU (μM)	N7-me-G (adducts/10^8 dG)	O^6-me-dG (adducts/10^8 dG)
0	224 ± 182 (n = 4)	0.25 ± 0.14 (n = 5)
0.0075	226 ± 136 (n = 4)	0.37 ± 0.18 (n = 5)
0.0125	226 ± 135 (n = 4)	0.48 ± 0.28 (n = 5)
0.025	221 ± 122 (n = 4)	1.0 ± 0.19*** (n = 4)
0.075	235 ± 127 (n = 4)	1.8 ± 0.36*** (n = 6)
0.25	314 ± 160 (n = 4)	7.5 ± 2.4*** (n = 4)
0.75	401 ± 179 (n = 4)	22 ± 11*** (n = 4)
2.5	773 ± 205** (n = 4)	66 ± 17*** (n = 4)

[a]Data represent the mean ± SD. Statistical comparison between the sum of exogenous and endogenous adducts and the endogenous mean was conducted using a t test (**p < 0.01; ***p < 0.005) to determine doses when the amount of total adducts become significantly higher than the identical average endogenous adducts.

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lowest observed genotoxic effect level (LOGEL) of ~0.1 μM. When correlating the present O^6-methyl-dG adduct data with published mutation data,[5,6] we can conclude that only O^6-methyl-dG adducts $\geq 1.8/10^8$ dG are effective in producing significant increases in mutations in AHH1 cells.

The present study supports the use of such science-based data for low dose risk assessment for many genotoxicants that induce DNA damage lesions that are identical to those produced from endogenous sources. [D_3]-MNU allowed us to perform molecular dosimetry studies at very low doses to understand the relative formation of different adduct types (N7-methyl-G and O^6-methyl-dG).

This information is important for determining the thresholds of both biomarkers of exposure and effect. The N7-methyl-G adduct data reveals that at low exogenous exposure conditions, the amount of endogenous DNA adducts dominated over the exogenous adducts. Our data clearly shows that we can expect exogenous adducts to be linear through zero. However, such data can only be demonstrated through the use of stable isotope exposures and ultrasensitive analytical methods. When the combined total of exogenous and endogenous DNA adducts in our study are examined for thresholds for the total methyl biomarkers of exposure and compared with published thresholds for mutations using the same system, the data strongly support the use of such data for science-based regulation of genotoxic chemicals, but rejects the use of default linear extrapolations when based on scientific data.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and a calibration curve. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MNU, methylnitrosourea; SAM, *S*-adenosyl-methionine; NOGEL, no-observed genotoxic effect level; LOGEL, lowest-observed genotoxic effect level

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