Monitoring in Vivo Metabolism and Elimination of the Endogenous DNA Adduct, M_1dG {3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10(3*H*)-one}, by Accelerator Mass Spectrometry†

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Our laboratory is investigating the in vitro and in vivo metabolic processing of endogenously formed DNA adducts as a means of evaluating candidate urinary biomarkers. In particular, we have focused our studies on the metabolism and disposition of the peroxidation-derived pyrimidopurinone deoxyguanosine (dG) adduct, 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10(3*H*)-one (M_1dG), and its principal metabolite, 6-oxo-M_1dG. We now report the metabolic processing of M_1dG at concentrations 4−8 orders of magnitude lower in concentration than previously analyzed, by the use of accelerator mass spectrometry analysis. Administration of 2.0 nCi/kg [^{14}C]M_1dG resulted in 49% of the ^{14}C recovered in urine, whereas 51% was recovered in feces. In urine samples, approximately 40% of the ^{14}C corresponded to the metabolite, 6-oxo-M_1dG. Following iv administration of 0.5 and 54 pCi/kg [^{14}C]M_1dG, approximately 25% of the urinary recovery corresponded to the metabolite, 6-oxo-M_1dG. Thus, upon administration of trace amounts of M_1dG, a significant percentage of 6-oxo-M_1dG was produced, suggesting that 6-oxo-M_1dG maybe a useful urinary marker of exposure to endogenous oxidative damage.

Introduction

Oxidative damage to DNA is considered a contributing factor to the onset of several genetic diseases, including cancer (*1, 2*). Monitoring genomic exposure to oxidative damage may be useful in risk assessment studies (*3, 4*). Endogenously formed electrophiles arise from enzymatic and nonenzymatic sources and are involved in the etiology of disease. The principal mediators of endogenous damage are radicals. Radicals can directly damage DNA and other cellular macromolecules to disrupt native structure and function. Radical reactions (enzymatic and nonenzymatic) with polyunsaturated lipids are a precursor to the production of several highly reactive lipid hydroperoxides, 9- and 13-hydroperoxyoctadecadienoic acid, and aldehydic electrophiles, such as malondialdehyde, 4-hydroxy-nonenal, 4-oxo-nonenal, and others (*5*). When radicals or aldehydes react with DNA, modifications result that can lead to mutation. DNA adducts formed from endogenous sources are generally considered end points of reaction; thus, monitoring their formation may provide a useful index of genomic exposure to oxidative damage.

M_1dG[1] {3-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10(3*H*)-one} is a major endogenous, peroxidation-derived adduct of 2′-deoxyguanosine (dG) formed in reactions with either malondialdehyde (a product of lipid peroxidation, DNA peroxidation, and prostaglandin synthesis) or base propenal (a product of DNA peroxidation) (*6–9*). M_1dG is a substrate for the nucleotide excision repair pathway, which accounts for its presence in human urine as the deoxynucleoside (*10, 11*). Urinary elimination of DNA adducts is a common approach used to assess exposure to genetic insult from endogenous sources. Several laboratories have implemented strategies for the noninvasive quantification of endogenous adducts (*12–16*). An increasing body of evidence suggests that factors affecting the appearance of DNA adducts in urine (i.e., metabolism, biliary elimination, etc.) may limit their direct detection. The metabolic stability of M_1dG has been the subject of several investigations (*17–19*).

Results from in vitro and in vivo metabolism experiments have demonstrated that M_1dG is subject to enzymatic oxidation to a single metabolite, 6-oxo-M_1dG (*17, 19*). In vivo metabolism and elimination studies were performed with μCi quantities of carbon-14 (^{14}C) labeled M_1dG {[^{14}C]M_1dG, 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10(3*H*)-one-2-^{14}C}. Following iv administration of [^{14}C]M_1dG, 45% of the isotope was recovered as the metabolite, 6-oxo-M_1dG, with the balance of radioactivity attributed to [^{14}C]M_1dG (*19*). No other

† Dedicated to the memory of Anne Karpay, a graduate student in the Department of Biochemistry at Vanderbilt University, who left this world far too early.

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[1] Abbreviations: M_1dG, 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10(3*H*)-one; ^{14}C, carbon-14; AMS, accelerator mass spectrometry; dG, 2′-deoxyguanosine; [^{14}C]M_1dG, 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10(3*H*)-one-2-^{14}C.

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significant metabolites were observed in bile or urine samples. 6-Oxo-M$_1$dG accounted for 20% of the total radioactivity in the early time point urine samples; however, at late time points, there was an approximate equal ratio of M$_1$dG to 6-oxo-M$_1$dG. On the sole basis of the in vitro-determined K_m for M$_1$dG metabolism in rat liver cytosol (K_m = 370 μM), this observed increase in metabolite production may not be expected (*17*). Additionally, because endogenously formed adducts are produced in very low amounts, we were curious to know if M$_1$dG would be metabolized when administered at very low quantities that might approximate physiological levels. To address this question, we employed nCi and pCi dosing procedures with [^{14}C]M$_1$dG and monitored metabolism and elimination. Analysis of biological samples from these studies was possible by the use of accelerator mass spectrometry (AMS).

Experimental Procedures

Materials. All chemicals were obtained from commercial sources and used as received. Solvents were of HPLC grade purity or higher. [8-^{14}C]dG was purchased from Sigma (St. Louis, MO). [^{14}C]M$_1$dG was synthesized as previously described (*20*). HPLC-UV separations were performed with either a Waters 2695 autosampler and binary pump or a Waters 1585 binary pump and 717plus autosampler and a Waters 2487 dual wavelength UV detector (Milford, MA).

Administration of [^{14}C]M$_1$dG to Rats. Animal protocols were performed under approval of Vanderbilt University and in accordance with the Institutional Animal Care and Use Committee policies. Male Sprague−Dawley rats (225−250 g), with vascular catheters surgically implanted in the jugular vein, were obtained from Charles River Laboratories (Wilmington, MA) and housed in shoebox cages. Animals were transferred to metabolism cages prior to dosing and allowed to feed ad libitum throughout the experiment. The [^{14}C]M$_1$dG dosing solution (>99% pure) was prepared in sterile saline to the desired specific activity (3.0, 0.1, and 0.001 dpm/μL) and administered via the jugular catheter in approximately 0.3 mL of total volume over 45 s. All doses were followed by 0.4 mL of sterile heparinized saline to flush the catheters.

One cohort of four animals was used for the 2.0 nCi/kg (11 ng/kg) dosing. Three animals received the [^{14}C]M$_1$dG dosing solution (3.0 dpm/μL), and one animal received a vehicle control (sterile saline). Urine was collected over intervals (predose, 0−4, 4−8, 8−12, 12−16, 16−20, and 20−24 h). All samples were stored at −20 °C following collection.

A second cohort of four animals was used for the 0.5 (3.0 pg/kg) and 54 pCi/kg (300 pg/kg) studies. Three animals received the 0.5 pCi/kg [^{14}C]M$_1$dG dosing solution (0.001 dpm/μL), and one animal received the vehicle control (saline). After 12 h, the animals were removed from the metabolism cages and returned to shoebox cages overnight. Metabolism cages were thoroughly washed following the 0.5 pCi/kg dose. Approximately 24 h after receiving the 0.5 pCi/kg dose, the same three experimental animals received 54 pCi/kg [^{14}C]M$_1$dG dosing solution (0.1 dpm/μL), and the same control animal received a vehicle administration (saline). Urine was collected over intervals (predose, 0−2, 2−4, and 4−8). All samples were stored at −20 °C. Repeat dosing was implemented in this experiment to minimize the pain and distress of laboratory animals and to comply with the institutional guidelines for the use and care of animals in research.

Fecal samples were dried on the benchtop overnight. All pellets from a given time point were pooled, placed in a sealable plastic bag, crushed with a rubber mallet, and stored at −20 °C. For AMS analysis, fecal samples were weighed, diluted in 1 N NaOH, and stored at −20 °C.

HPLC Fraction Collection. Urine samples were centrifuged for 10 min at 14000*g*. Supernatants were removed and directly injected onto a Phenomenex Luna C18(2) column (4.6 nm × 250 nm, 5 μm) equilibrated with 90% solvent A (10 mM potassium phosphate in H$_2$O, pH 7.8) and 10% solvent B (Methanol) at a flow rate of 1.0 mL/min. The solvent was programmed as follows: a linear gradient from the equilibration conditions to 20% B in 10 min, holding at 20% B for 10 min, increasing to 90% B in 0.1 min, holding for 5 min, decreasing to 10% B in 0.1 min, and re-equilibrating to initial conditions. Standards (spiked into solvent A) applied to this gradient eluted at 12.7 (6-oxo-M$_1$dG) and 15.8 min (M$_1$dG). Standards spiked into blank urine samples demonstrated slight shifts in retention to 11.5 (6-oxo-M$_1$dG) and 15.7 min (M$_1$dG). The shift in 6-oxo-M$_1$dG retention time is likely due to ionization effects (pK_a = 7.0, data not reported). Injections (300 μL) were made for the 2.0 nCi/kg study, and 200 μL injections were made for the 54 and 0.5 pCi/kg studies. Fractions were collected from 10.5 to 12.5 min for 6-oxo-M$_1$dG and from 15 to 17 min for M$_1$dG. Samples were evaporated to dryness under a stream of nitrogen in a heated water bath and stored at −20 °C.

AMS Analysis. MS analyses were conducted at the MIT BEAMS Laboratory using the instrument and procedures described elsewhere (*21–24*). Urine samples and fecal extracts were diluted as required to bring the total carbon and ^{14}C concentrations within the dynamic range of the AMS system and analyzed without further processing. HPLC fractions were reconstituted with appropriate measured volumes of H$_2$O. Aliquots of urine, fecal, and HPLC samples (1.50 μL) were absorbed into pellets of packed CuO powder that had previously been exposed for 30 min to an atmosphere of O$_2$ at 720 °C. After they were dried briefly in a vacuum oven, the sample-loaded CuO pellets in their holders were transferred to a laser-induced combustion interface (*4*) for subsequent AMS analysis. Each run of samples was organized to include quantitation standards (0.0030 dpm/μL) and blanks (1 μg/μL albumin). Quantitation was performed by integrating peaks produced in the continuous trace of ^{14}C detector count rate vs time generated during operation of the combustion interface, which produces and delivers the CO$_2$ of combustion to the AMS ion source, and by taking the product of the sample:standard peak area ratio multiplied by the standard concentration as the concentration of the sample. All samples were analyzed in duplicate.

Results

nCi Dosing. [^{14}C]M$_1$dG was synthesized as previously described (*19, 20*) and administered iv to male Sprague−Dawley rats (2.0 nCi/kg). Animals were housed in metabolism cages to collect urine and feces at intervals over the course of the experiment. Urine and fecal samples were subject to AMS analysis for total ^{14}C recovery (Table 1). Forty-nine percent of the recovered ^{14}C was found in urine, while 51% was found in

Table 1. Total ^{14}C Recovery in Urine and Feces Following Administration of 2.0 nCi/kg [^{14}C]M$_1$dG to Male Sprague−Dawley Rats (n = 3)[a]

	AMS analysis
dose (dpm)	1027.00 ± 25.51
urine	584.11 ± 91.10
feces	603.97 ± 29.01
recovery (%)	115.70 ± 3.67

[a] Expressed as means ± SD.

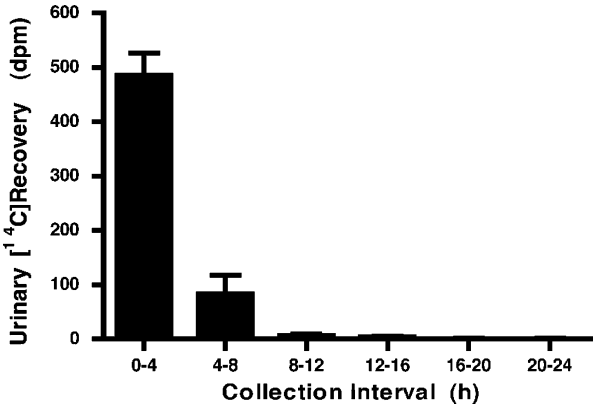


Figure 1. ^{14}C recovery in urine following administration of 2.0 nCi/kg [^{14}C]M_1dG to male Sprague−Dawley rats ($n = 3$).

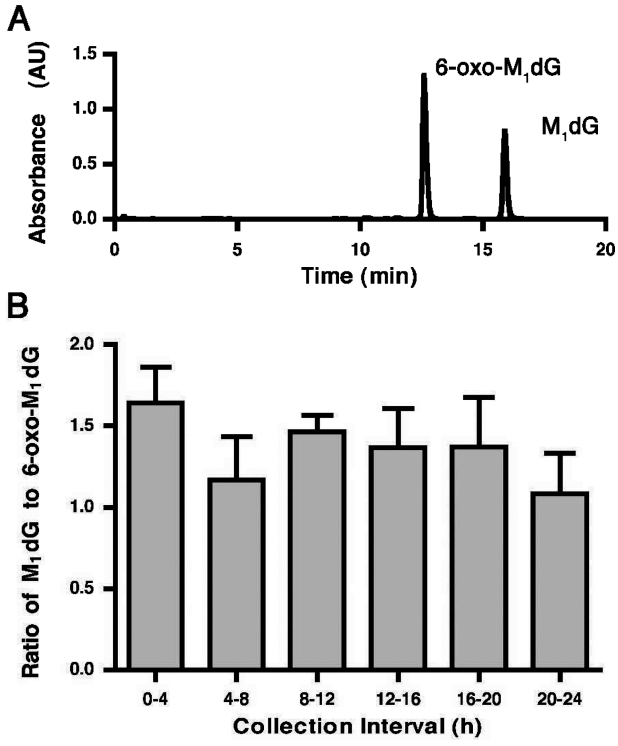


Figure 2. (A) Reverse phase HPLC separation of M_1dG and 6-oxo-M_1dG standards on a C18 column with a potassium phosphate, pH 7.8, and methanol gradient. (B) Ratio of M_1dG to 6-oxo-M_1dG in rat urine over time following administration of 2.0 nCi/kg [^{14}C]M_1dG to male Sprague−Dawley rats ($n = 3$).

feces. Approximately 84% of the urinary radioactivity was collected in the first 4 h, and isotope was observed out to 24 h of collection (Figure 1). This elimination profile was very similar to the previously observed time course from the μCi dosing study (*19*). No ^{14}C was detected in any biological samples from the vehicle control animal at amounts measurably above the background levels of ^{14}C (standard isotopic abundance) present in biological samples.

To determine the source of ^{14}C in the urine, samples were separated by HPLC, and fractions corresponding to M_1dG to 6-oxo-M_1dG were collected (Figure 2A) and analyzed by AMS (Figure 2B). The average observed ratio of M_1dG to 6-oxo-M_1dG over the 24 h collection was 1.4:1, which demonstrated that 40% of the urinary radioactivity corresponded to 6-oxo-M_1dG. The mean ^{14}C recovery from fraction analysis was 82% with a standard deviation of 12%.

pCi Dosing. On the basis of the high ^{14}C signal-to-noise observed in each urine fraction following administration of 2.0 nCi/kg [^{14}C]M_1dG, we reasoned that the administration of [^{14}C]M_1dG could be reduced and still be reliably detected. Therefore, we lowered the iv dosing of Sprague−Dawley rats to 0.5 and 54 pCi/kg [^{14}C]M_1dG, which approached our AMS limit of detection for ^{14}C in urine. Urine was collected at intervals during the experiment to assess ^{14}C recovery. The total ^{14}C content in urine samples was assessed directly by AMS analysis. The data from the 54 pCi/kg dosing are summarized in Figure 3: Approximately 42% of the administered ^{14}C was recovered in urine. Direct AMS analysis of urine samples from the 0.5 pCi/kg dose did not offer significant signal above background, and no total urinary ^{14}C recovery could be determined. Significant ^{14}C enrichment was observed in all fractions following HPLC purification. Analyte was detected in some urine samples following AMS analysis of the 0.5 pCi/kg samples, albeit at a low signal-to-noise. Only M_1dG and 6-oxo-M_1dG fractions that displayed a signal-to-noise ≥3 were reported. The average observed ratio of M_1dG and 6-oxo-M_1dG was approximately 3:1 following administration at either 54 or 0.5 pCi/kg [2-^{14}C]M_1dG (Table 2). Thus, 25% of the urinary ^{14}C was recovered as 6-oxo-M_1dG following administration of M_1dG at the AMS limit of detection. The mean ^{14}C recovery from fraction analysis performed on the 54 pCi/kg urine samples was 55% with a standard deviation of 20%. A similar decrease in recovery was observed with late time point urine collections in the nCi dosing study. Recoveries of nearly 86−99% were obtained during the first 8 h of collection, and recoveries of 56−78% were obtained during the final 8 h of collection following administration of 2.0 nCi/kg M_1dG. Thus, because of the very low analyte levels, the lower recoveries in the pCi dosing studies were attributed to nonspecific loss.

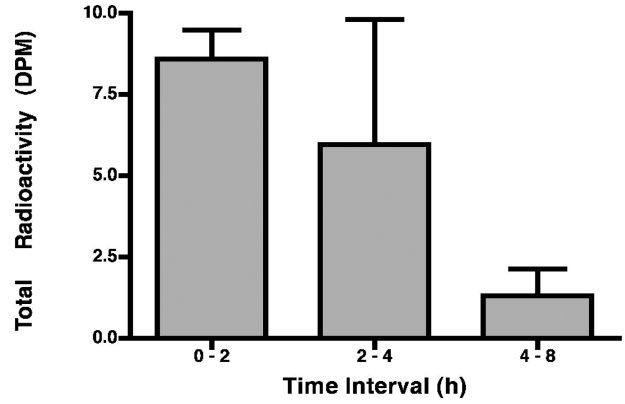


Figure 3. ^{14}C recovery in urine following administration of 54 pCi/kg [^{14}C]M_1dG to male Sprague−Dawley rats ($n = 3$).

Table 2. Percent of [^{14}C]M_1dG Converted to [^{14}C]6-oxo-M_1dG in Urine Following Administration of 0.5 or 54 pCi/kg [^{14}C]M_1dG to Male Sprague−Dawley Rats ($n = 3$)[a]

	collection interval (h)	percent 6-oxo-M_1dG
54 pCi/kg	0−2	24.5 ± 3.0
	2−4	26.1 ± 2.4
0.5 pCi/kg	0−2	25.4 ± 10.5
mean and SD		25.4 ± 5.9

[a] Expressed as means ± SD.

Discussion

Noninvasive analysis of endogenously occurring DNA damage is of interest for use in clinical and animal studies. Accordingly, several laboratories have developed analytical methodologies to assess adduct levels in biological matrixes

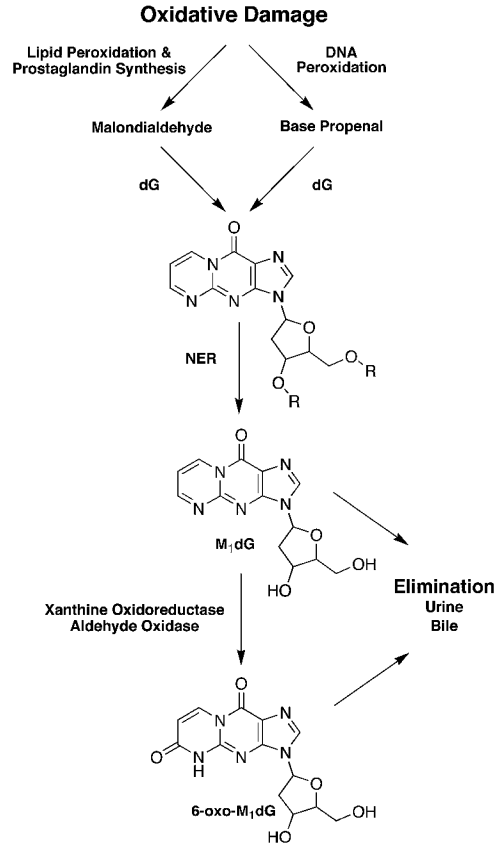
(*12–16*). The appearance of adducts in urine is typically attributed to repair-dependent removal from DNA (*12, 16, 25*). However, it should be noted that urinary analysis is an indirect method of assessment, and the true origins of the adducts are unknown. One cannot rule out the possibility that adducts arising from either the nucleotide pool or the biological processing of nucleic acids during apoptotic or necrotic cell death mechanisms likely contribute to the steady-state elimination rates of DNA adducts in urine. Another important and largely unstudied factor is the bioavailability of DNA adducts present in the diet. Clearly, a comprehensive assessment of the origins of endogenous DNA adducts appearing in urine is a prerequisite to their clinical use as biomarkers of endogenously occurring DNA damage.

Independent of the route of formation, adducts are potentially subject to metabolic processing within the tissue of origin. Alternatively, adducts may be released from tissues and traverse through the systemic circulation prior to elimination. It stands to reason that DNA adducts in the circulation would be subject to similar metabolism and elimination pathways as other purine derivatives. Purine-based therapeutics such as acyclovir, penciclovir, zaleplon, and others readily undergo oxidative metabolism and active transport (*26–34*). Our results on the oxidative metabolism of M_1dG and M_1G suggest that metabolism and alternate routes of elimination may also contribute to the complexity of DNA adduct analysis in biological matrixes (*18, 19*).

The use of ^{14}C in DNA adduct analysis has been limited, principally due to the poor yields in chemical synthesis and low rates of adduct formation in DNA, which is coupled to the need of sufficient amounts of material for traditional liquid scintillation counting. The advent of AMS analysis has greatly reduced the required isotope levels for experimental analysis, by altering the mode of detection from decay counting to mass analysis (*24*). Our study suggests that less than 1000 dpm per animal is required for sound in vivo metabolism and elimination studies involved in evaluating candidate urinary biomarkers. Results from this type of analysis provide, at a minimum, recovery and elimination data and, when coupled to fraction collection analysis, offers an estimation of percent metabolism of the administered material. Thus, AMS analysis may be applicable to many toxicology problems and significantly impact the development and evaluation of biomarkers.

Incorporation of a stable ^{14}C tracer into M_1dG for use in vivo in rats has allowed us to investigate the metabolic processing of M_1dG at very low concentrations (Scheme 1). Our findings revealed that when [^{14}C]M_1dG was administered at 2.0 nCi/kg (approximately 2.5 ng per animal), approximately 40% of the urinary ^{14}C was recovered as 6-oxo-M_1dG. When the administration level of [^{14}C]M_1dG was further lowered to 0.5 and 54 pCi/kg (approximately 750 fg and 75 pg per animal, respectively), approximately 25% of the urinary ^{14}C was recovered as 6-oxo-M_1dG. On the basis of only the calculated K_m for M_1dG metabolism in rat liver cytosol (370 μM) (*17*), the in vivo oxidation at very low concentrations may not be expected. However, in vivo, the high K_m is likely mitigated by the efficiency of enzyme turnover ($v_{max}/K_m = 0.001$ min^{-1} mg rat liver cytosol^{-1}) (*17*). The reasons for the observed differences in percent conversion at the different dosing levels are unknown. One possibility for the decreased conversion of M_1dG to 6-oxo-M_1dG may be related to the enzyme efficiency. It is possible that at the lower level of administration (0.5 and 54 pCi/kg), the effective concentration at the site of metabolism was not sufficient to promote turnover at saturating conditions. Thus, a decrease in the percent conversion may be observed at lower levels of abundance.

Scheme 1. Proposed Route of Metabolic Processing for M_1dG[a]



[a] dG, 2′-deoxyguanosine; NER, nucleotide excision repair.

The observed contribution of 6-oxo-M_1dG to the total M_1dG levels in rat urine approximated our previous estimations based on ^{14}C decay counting (*19*). The fact that 25−40% of the administered [^{14}C]M_1dG was recovered from urine as 6-oxo-M_1dG in the present studies strongly suggests that M_1dG metabolism is likely to occur under physiological conditions of M_1dG production. A prior estimate of the M_1dG elimination rate in human urine demonstrated that the adduct was eliminated at 12 fmol/kg/24 h (*10*). This elimination rate corresponds to approximately 3.6 pg/kg/24 h. Our administration of [^{14}C]M_1dG at 0.5 and 54 pCi/kg (750 fg and 75 pg, respectively) brackets the anticipated daily physiological clearance of M_1dG. The rate of elimination for M_1dG in the rat has not been determined. However, if we assume the average elimination rate to be similar in rats as observed in humans, then based on the elimination of 30 mL of urine per day, a total clearance of 100 fg may be anticipated in the rat.

It should be noted that our discussion of the percent conversion to 6-oxo-M_1dG following nCi and pCi dosing is limited to urinary analysis. Prior studies have shown that biliary elimination is a key route of excretion for 6-oxo-M_1dG (*19*). Following iv administration of [^{14}C]6-oxo-M_1dG to Sprague−Dawley rats, [^{14}C]6-oxo-M_1dG was cleared unchanged in urine and bile; 55−70% of the total dose was eliminated in either bile or feces (*19*). When [^{14}C]M_1dG was administered iv to Sprague−Dawley rats, approximately 50% of the dose was cleared in bile or feces; the majority of radioactivity (70%) cleared in bile corresponded to [^{14}C]6-oxo-M_1dG (*19*). If we apply the same biliary conversion to the nCi and pCi dosing

studies, then greater than 50% of the administered material in the current studies would have been converted to 6-oxo-M_1dG. This represents a significant portion of the total M_1dG.

On the basis of these and prior experiments, it is clear that iv-administered M_1dG is taken up and likely accumulates in the liver. The physiological and biochemical explanation for this is unknown. However, on the basis of significant biliary elimination of iv-administered M_1dG, it is reasonable to assume that transport proteins are involved in the disposition of M_1dG and 6-oxo-M_1dG into and out of the liver. Ultimately, this process leads to oxidative metabolism of exogenously administered M_1dG, even at concentrations that approach the analytical limit of sensitivity by AMS. Thus, there exists a distinct possibility that oxidation occurs at the projected rates of M_1dG formation in animals and humans. Our laboratory is currently developing analytical methods to probe for the appearance of M_1dG and 6-oxo-M_1dG in human elimination products.

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