Metabolism and Elimination of the Endogenous DNA Adduct, 3-(2-Deoxy-β-D-erythropentofuranosyl)pyrimido[1,2-α]purine-10(3H)-one, in the Rat

Charles G. Knutson, Hao Wang, Carmelo J. Rizzo, and Lawrence J. Marnett

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Endogenously occurring damage to DNA is a contributing factor to the onset of several genetic diseases, including cancer. Monitoring urinary levels of DNA adducts is one approach to assess genomic exposure to endogenous damage. However, metabolism and alternative routes of elimination have not been considered as factors that may limit the detection of DNA adducts in urine. We recently demonstrated that M1dG is subject to enzymatic oxidation in vivo resulting in the formation of a major metabolite, 6-oxo-M1dG. Based on the administration of [14C]M1dG (22 μCi/kg) to Sprague-Dawley rats (n = 4), we now report that 6-oxo-M1dG is the principal metabolite of M1dG in vivo representing 45% of the total administered dose. When [14C]6-oxo-M1dG was administered to Sprague-Dawley rats, 6-oxo-M1dG was recovered unchanged (97% stability). These studies also revealed that M1dG and 6-oxo-M1dG are subject to biliary elimination. Additionally, both M1dG and 6-oxo-M1dG exhibited a long residence time following administration (>48 h), and the major species observed in urine at late collections was 6-oxo-M1dG.

Endogenously produced DNA damage is a contributing factor to the progression of several genetic diseases, including cancer (1, 2). Furthermore, there is a strong association between chronic inflammation and cancer risk (3). Assessing exposure to endogenously produced electrophiles and oxidants may have an impact in risk assessment (4). Monitoring the levels of DNA adducts in urine is a common approach used to assess exposure to genomic damaging agents. However, factors that may limit the detection of DNA adducts in urine (metabolism, routes of elimination, etc.) have not previously been investigated.

3-(2-Deoxy-β-D-erythropentofuranosyl)pyrimido[1,2-α]purine-10(3H)-one (M1dG) is an endogenous pyrimidopurinone adduct formed by reacting deoxyguanosine with malondialdehyde, a product of enzymatic and nonenzymatic lipid peroxidation reactions, or base propagation, a DNA peroxidation product (5–11). This adduct is mutagenic in bacteria and mammalian cells (12–15) and is a substrate for nucleotide excision repair (16, 17). It is also one of the first endogenously occurring DNA damage products to be detected in DNA of healthy humans (17). Prior attempts to quantify the levels of M1dG in human urine demonstrated an excretion rate of 12 fmol/kg/24 h (18). The rate of M1dG elimination in rats could not be determined, because the levels were below the limit of detection for the analytical method (19). The low rate of elimination in human populations and the absence of observed material in the urine of rats led us to hypothesize that factors such as metabolism (oxidation, conjugation, etc.) or alternative routes of elimination may limit the appearance of M1dG in urine.

We recently demonstrated that M1dG is subject to oxidative metabolism in the rat to a principal metabolite, 6-oxo-M1dG (20). However, the total recovery and extent of metabolism in these studies could not definitively be determined. To unambiguously monitor the metabolism and elimination of M1dG in vivo, we synthesized M1dG containing a carbon-14 incorporated into the purine ring for use in animal studies. This tracer allowed us to quantitatively monitor the metabolism and elimination of M1dG and its principal metabolite, 6-oxo-M1dG, in the rat. The results of this investigation are described herein.

**EXPERIMENTAL PROCEDURES**

All chemicals were obtained from commercial sources and used as received. Solvents were of HPLC grade purity or higher. [8-14C]2’-Deoxyguanosine was purchased from Sigma. Purified bovine xanthine oxidase was purchased from Calbiochem. Catheterized male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). HPLC-UV β-RAM separations were performed on a Waters 2695 autosampler and binary pump with a Waters 2487 dual-wave...
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length UV detector (Milford, MA) and an IN/US Systems model 2 β-RAM (Tampa, FL). Liquid scintillation counting was performed with a Beckman LS 6500 multipurpose scintillation counter (Fullerton, CA).

Chemical Synthesis of $[2-^{14}C]3-(2$-Deoxy-$β$-d-erythropentofuranosyl)-pyrimido[1,2-a]purine-10(3H)-one ($[2-^{14}C]M_1$dG)—$[2-^{14}C]M_1$dG synthesis was carried out as described elsewhere (21). Briefly, $[8-^{14}C]2$-deoxyguanosine ($^{14}C$-dG) was obtained from Sigma (0.2 mCi, 55 mCi/mm) as a solution in ethanol/water (1:1). Iodoacrolein was freshly prepared from ethyl cis 3-iodoacrylate as described previously and dissolved in anhydrous $N$,N-dimethylformamide (22). Freshly prepared iodoacrolein was analyzed by $^1$H NMR and determined to be >95% pure. $^{14}$C-dG (0.2 mCi, 1 mg) was diluted with excess ethanol and evaporated to dryness under reduced pressure. The remaining residue was dissolved in $N$,N-dimethylformamide (0.4 ml) in the presence of 1.5 eq of K$_2$CO$_3$ and heated to 65 °C. To this solution, 1 eq of freshly prepared iodoacrolein was added each hour over 5 h of reaction (Fig. 1). The resulting solution was evaporated under reduced pressure and dissolved in 1.0 ml of 0.01 M potassium phosphate. The mixture was purified by HPLC using a Phenomenex Luna C18 (2) column (250 × 4.6 mm, 5 μm) equilibrated with 100% solvent A (0.01 M potassium phosphate, pH 7.8) at a flow rate of 1.0 ml/min. The following gradient elution was applied: 100% solvent A with 0% solvent B (methanol) for 5 min followed by a linear increase to 20% B over 30 min, holding at 20% B for 5 min and increasing to 85% B in 1 min, holding at 85% B for 5 min, decreasing to 0% B in 1 min, and re-equilibrating at initial conditions for 5 min. Fractions corresponding to $M_1$dG (~35 min) were collected, pooled, and concentrated to a minimal volume. Purity was assessed by HPLC-UV with β-RAM detection. The radiochemical purity was assessed to be >95%, with a minor impurity of residual $^{14}$C-dG (supplemental Fig. S1). A final yield of 15% was obtained. Electrospray ionization-mass spectrometry was m/z 306.4.

Chemical Synthesis of $[2-^{14}C]3-(2$-Deoxy-$β$-d-erythropentofuranosyl)-pyrimido[1,2-a]purine-10(3H)-one diiones ($[2-^{14}C]6$-oxo-$M_1$dG)—A solution of $[2-^{14}C]M_1$dG was diluted in 0.6 ml of 0.1 M potassium phosphate, pH 7.8, and allowed to incubate with 2 units of purified bovine xanthine oxidase for 1 h; an additional 2 units was then added and allowed to incubate for an additional hour (Fig. 1). The resulting solution was purified by HPLC as described for $[2-^{14}C]M_1$dG. Fractions containing $[2-^{14}C]6$-oxo-$M_1$dG (~26 min) were collected, pooled, and concentrated to a minimal volume. The resulting sample exhibited >99% radiochemical purity (supplemental Fig. S2), and a final yield of 10% was obtained. Electrospray ionization-mass spectrometry was m/z 322.4.

Administration of $[2-^{14}C]M_1$dG and $[2-^{14}C]6$-oxo-$M_1$dG to Male Sprague-Dawley Rats—Animal protocols were performed under the approval of Vanderbilt University and in accordance with the Institutional Animal Care and Use Committee policies. Male Sprague-Dawley rats (225–250 g) with catheters surgically implanted into the jugular vein and bile duct were obtained from Charles River Laboratories and housed in shoebox cages. Prior to the experiment the animals were transferred into metabolism cages and allowed to feed ad libitum. The dosing solution was prepared in sterile saline solution, administered through the jugular vein over 45 s in an approximate volume of 0.4 ml, and flushed with 0.4 ml of sterile heparinized saline. Four animals were used in each experiment (two animals with and two animals without bile catheters). $[2-^{14}C]M_1$dG was dosed at 22 μCi/kg (123 μg/kg), and $[2-^{14}C]6$-oxo-$M_1$dG was dosed at 15 μCi/kg (83 μg/kg) in separate experiments. All animals were housed in metabolism cages through the duration of the experiment to collect urine and feces over the following intervals: pre-dose, 0–4, 4–8, 8–12, 12–16, 16–20, 20–24, 24–32, 32–40, 40–48, and 48–64 h. Bile was also collected (from two animals) as outlined above, but the first interval was broken down as follows: 0–0.5, 0.5–1, 1–2, and 2–4 h. All samples were collected into pre-tared tubes and weighed following the collection interval to determine the mass of sample collected (feces was first dried on the bench top). Samples were stored at −20 °C until analysis.

Radiochemical Analysis of Biological Samples—Biological samples were processed as suggested by PerkinElmer Life Sciences. Urine (0.1–1.0 ml) and bile (0.1–0.3 ml) samples were added directly to 10 ml of Pico-Fluor™ and analyzed by liquid scintillation counting (10-min counts). Fecal samples were dried on the bench top for a minimum of 24 h. The fecal material was broken into small pieces and mixed, and ~20 mg of feces (dry weight) was transferred to scintillation vials (n = 4) and re-hydrated with 0.2 ml of water. To each sample 0.5 ml of SOLUEN®-350 was added, mixed, and heated to 40 °C for 1.5 h. A 0.5-ml aliquot of isopropyl alcohol was added and swirled, followed by dropwise addition of 0.2 ml of H$_2$O$_2$. The resulting mixture was heated to 40 °C for 2 h and cooled, and 10 ml of Hionic-Fluor™ scintillation mixture was added. Samples were stored in the dark for a minimum of 36 h prior to counting by liquid scintillation counting (10-min counts). Urine and bile samples were profiled by HPLC-UV with β-RAM detection, and centrifuged at 200,000 × g for 1 h prior to analysis. Injections (0.025–0.3 ml) were made onto a Phenomenex Luna C18 (2) column (250 × 4.6 mm, 5 μm) equilibrated with 100% solvent A (0.5% formic acid in H$_2$O) at a flow rate of 1.0 ml/min. The following gradient elution was applied: 100% solvent A with 0% solvent B (0.5% formic acid in methanol) for 5 min followed by a linear increase to 20% B over 30 min, holding at 20% B for 5 min, increasing to 85% B in 1 min, holding at 85% B for 5 min, decreasing to 0% B in 1 min, and re-equilibrating at initial conditions for 5 min. Column effluent first passed through the UV detector (254 nm) and onto the β-RAM, where it mixed with IN-FLOW™ 2.1 (IN/US Systems) at a ratio of 1:1.1 (HPLC effluent: mixture) and was analyzed in 0.5-ml flow cell with a dwell time of 0.2 s.

LC-MS/MS Analysis of Biological Samples—Tandem mass spectrometry employing selected reaction monitoring was used to verify the molecular ions attributed to the main source or radioactivity in the biological samples. Approximately 500–1000 dpm were analyzed per injection. Urine and bile samples were diluted to an approximate concentration of 50,000 dpm/ml and injected onto a Phenomenex Luna C18 (2) col-
umn (250 × 2.0 mm, 5 μm) equilibrated with 100% solvent A (0.5% formic acid in H₂O) at a flow rate of 0.3 ml/min. The following gradient elution was applied: 100% solvent A with 0% solvent B (0.5% formic acid in methanol) for 5 min followed by a linear increase to 20% B over 30 min, holding at 20% B for 5 min, increasing to 85% B in 1 min, holding at 85% B for 5 min, decreasing to 0% B in 1 min, and re-equilibrating at initial conditions for 5 min. A ThermoElectron Quantum triple-quadrupole instrument with an electrospray source operated in positive ion mode was used during the analysis.

The following instrument parameters were set: spray voltage 4.3 kV; capillary temperature = 250 °C; sheath gas = 33 p.s.i.; auxiliary gas = 25; source CID off; collision pressure 1.5 mTorr.

RESULTS

[2-14C]M₁dG was synthesized from [8-14C]2’-deoxyguanosine in a reaction with iodoacrolein in the presence of a mild base (K₂CO₃) (Fig. 1) (21). The material was purified by HPLC, and the radiochemical purity was determined to be >95% (supplemental Fig. S1). A small amount (<5%) of unreacted starting material ([8-14C]dG) was observed in the dosing solution. [2-14C]M₁dG was diluted in sterile saline solution and administered intravenously (22 μCi/kg, 123 μg/kg) to male Sprague-Dawley rats (n = 4) via catheters surgically implanted in the jugular vein. Two animals contained additional catheters surgically implanted into the bile duct. The animals were housed in metabolism cages throughout the duration of the experiment to collect urine, feces, and bile (where applicable). The collected biological samples were analyzed for total radioactivity by liquid scintillation counting, and the data are summarized in Table 1. Approximately 50% of the radioactivity was recovered in the urine of both bile-catheterized and non-bile-catheterized animals. Greater than 90% of the urinary recovery was collected during the first 8 h (Fig. 2A). The remaining radioactivity was recovered in the feces of non-bile-catheterized animals and in the bile of bile-catheterized animals (Fig. 2B and C).

Urine and bile samples were profiled by HPLC with radiochemical detection. Representative chromatograms from urine and bile are shown in Fig. 3. The urine profiles from both the bile-catheterized and non-bile-catheterized rats were identical. The major metabolite observed in all radiochemical profiles eluted with the authentic retention time for 6-oxo-M₁dG and accounted for 20% of the radioactivity recovered in urine. The analysis of bile samples revealed that 70% of the radioactivity in bile was attributed to the metabolite 6-oxo-M₁dG. Selected reaction monitoring analysis of these samples revealed the expected transitions for M₁dG.
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(306.4 → 190.4) and 6-oxo-M₁dG (322.4 → 206.4), thereby confirming their identity. The bile and urine HPLC profiles demonstrated that 90% of the recovered radioactivity could be attributed to the combination of M₁dG and 6-oxo-M₁dG. The remaining 10% of the radioactivity was distributed among five distinct peaks in the radiochemical profiles, part of which (~5%) was attributed to the [8-14C]dG in the dosing solution. Thus, metabolites other than 6-oxo-M₁dG collectively accounted for ~6% of the total radioactivity. 6-Oxo-M₁dG accounted for 45% of the total recovered radioactivity.

Analysis of late time point urine collections (post 24 h) from both non-bile-catheterized and bile-catheterized animals suggested 6-oxo-M₁dG was present at the same or higher abundance than M₁dG (Fig. 6). This was especially apparent in the LC/MS-MS analysis of the bile-catheterized animals where the radioactivity present in the sample could almost entirely be attributed to 6-oxo-M₁dG.

Based on the long residence time of the [2-14C]M₁dG dose and the significant conversion of M₁dG to 6-oxo-M₁dG, we continued experiments to study the metabolism and elimination of the principal metabolite 6-oxo-M₁dG. We hypothesized that 6-oxo-M₁dG would have significant biliary clearance and a long residence time similar to the parent molecule M₁dG. Furthermore, this experiment would clarify the ultimate source of the minor metabolites observed in the [2-14C]M₁dG study (i.e. derived from M₁dG or 6-oxo-M₁dG).

[2-14C]6-Oxo-M₁dG was prepared from [2-14C]M₁dG (described above) by enzymatic oxidation at the 6-position.

FIGURE 3. HPLC-radiochemical and -MS/MS profiles of urine and bile samples following administration of [2-14C]M₁dG. Male Sprague-Dawley rats received a single intravenous dose of [2-14C]M₁dG (22 μCi/kg). A, representative urine sample (0–4 h) from a non-bile-catheterized animal was profiled by radiochemical detection and separately by LC-MS/MS using selected reaction monitoring following the transitions for the parent molecule M₁dG (m/z 306.4 → 190.4) and the principal metabolite 6-oxo-M₁dG (m/z 322.4 → 206.4). B, representative urine sample (0–4 h) from a bile-catheterized animal was profiled by radiochemical detection and separately by LC-MS/MS using selected reaction monitoring following the transitions for the parent molecule M₁dG (m/z 306.4 → 190.4) and the principal metabolite 6-oxo-M₁dG (m/z 322.4 → 206.4). C, representative bile sample (0–0.5 h) from a bile-catheterized animal was profiled by radiochemical detection and separately by LC-MS/MS using selected reaction monitoring following the transitions for the parent molecule M₁dG (m/z 306.4 → 190.4) and the principal metabolite 6-oxo-M₁dG (m/z 322.4 → 206.4).
of the pyrimido ring with purified bovine xanthine oxidase (Fig. 1). The material was purified by HPLC and exhibited radiochemical purity of >99% (supplemental Fig. S2). Freshly prepared [2-14C]6-oxo-M$_1$dG was diluted in sterile saline solution and intravenously administered (15 μCi/kg, 83 μg/kg) to male Sprague-Dawley rats ($n = 4$) via the jugular catheter. Two animals also contained catheters surgically implanted into the bile duct. The animals were housed in metabolism cages throughout the duration of the experiment to collect urine, feces, and bile (where available) at intervals. All samples were collected into pre-tared tubes and weighed following collection to determine the mass of sample collected. The biological samples were processed by liquid scintillation counting (Fig. 4), and the results from the elimination data are summarized in Table 2. The non-bile-catheterized rats eliminated 30 ± 5% of the dose in the urine, whereas 70 ± 5% was recovered in the feces. In bile-catheterized animals, 45 ± 7% was recovered in urine, 1% in feces, and 54 ± 2% in bile.

In all HPLC radiochemical profiles, the principal peak observed eluted with the retention time of 6-oxo-M$_1$dG (Fig. 5). No significant secondary metabolites of 6-oxo-M$_1$dG were observed during any collection interval. Therefore, 6-oxo-M$_1$dG was determined to be metabolically stable (>97%) and not subject to further metabolism in vivo. These data suggest that the minor metabolites observed in the [2-14C]M$_2$dG study arose directly from M$_1$dG and did not represent further metabolism of 6-oxo-M$_1$dG.

[2-14C]6-Oxo-M$_1$dG also exhibited significant biliary clearance (as seen in the [2-14C]M$_2$dG study), which suggested 6-oxo-M$_1$dG was absorbed by the liver and subject to transport into bile. As seen in the [2-13C]M$_2$dG study, [2-14C]6-oxo-M$_1$dG also exhibited a long residence time (>48 h) following intravenous administration. The identity of 6-oxo-M$_1$dG was verified by selected reaction monitoring analysis of the late time point urine collections (Fig. 6E).

**DISCUSSION**

The endogenous peroxidation-derived DNA adduct, M$_1$G, was recently found to undergo metabolism in vivo to a single oxidized metabolite, 6-oxo-M$_1$G (20). Our laboratory has now performed definitive in vivo analysis of the metabolism and excretion of M$_1$G and its major metabolite 6-oxo-M$_1$G by the incorporation of stable carbon-14 tracers into these molecules. Male Sprague-Dawley rats with ($n = 2$) and without ($n = 2$) bile catheters were intravenously dosed with [2-14C]M$_1$dG at 22 μCi/kg (123 μg/kg). Approximately 50% of the administered dose was recovered in the urine, whereas the remainder was recovered in the feces and bile (Fig. 7). 6-Oxo-M$_1$G accounted for 45% of the recovered radioactivity. The significant conversion of M$_1$G to 6-oxo-M$_1$G prompted additional metabolism and elimination studies on the metabolite. Upon dosing [2-14C]6-oxo-M$_1$G (15 μCi/kg, 83 μg/kg), 30 ± 6% of the radioactivity was recovered in the urine, whereas 71 ± 6% was excreted in the feces of non-bile-catheterized animals. Rats containing bile catheters deposited 45 ± 7% of the radioactivity into the bile, 54 ± 2% into the bile, and 1% into the feces. Profiling bile and urine samples revealed the metabolite, [2-14C]6-oxo-M$_1$G, was cleared unchanged (>97%), with no significant metabolism to secondary metabolites.

The simplicity of M$_1$G metabolism and elimination is noteworthy. A single metabolite is formed that is neither conjugated nor further metabolized. In our study, 6-oxo-M$_1$G represents a significant contribution (45% of the total dose) in the overall mass balance of M$_1$G. It was previously hypothesized that depurination reactions may give rise to the free base M$_1$G as an alternate metabolic pathway (23); however, no accumulation of this compound or its metabo-

![FIGURE 4. 14C recovery following administration of [2-14C]6-oxo-M$_1$G. Male Sprague-Dawley rats were dosed intravenously with [2-14C]6-oxo-M$_1$G (15 μCi/kg) and housed in metabolism cages to collect urine, bile, and feces during the experiment. A, 14C recovery in urine from bile-catheterized and non-bile-catheterized animals. B, 14C recovery in feces from bile-catheterized and non-bile-catheterized animals. C, 14C recovery in bile from bile-catheterized animals.](image)

**TABLE 2**

| Bile catheter | No bile catheter |
|---------------|------------------|
| Dose (μCi)    | 3.5 ± 0.05       | 4.15 ± 0.05     |
| Urine         | 1.84 ± 0.26      | 1.25 ± 0.39     |
| Feces         | 0.03 ± 0.01      | 2.90 ± 0.16     |
| Bile          | 2.22 ± 0.34      | NA              |
| Recovery (%)  | 114.6 ± 4.5      | 101.2 ± 15.2   |
was observed in our analysis. Furthermore, our investigation of 6-oxo-M$_1$dG metabolism revealed that it was exceptionally stable \textit{in vivo} ($>97\%$) and not metabolized to additional products. It should be noted that 6-oxo-M$_1$dG contains a possible Michael acceptor on the pyrimido ring (at C8), but no evidence for nucleophilic addition or conjugation (glutathione addition) was observed in the recovered samples.

Considering the main metabolite of M$_1$dG is a singly oxidized species, the extent to which M$_1$dG and 6-oxo-M$_1$dG were cleared through biliary excretion was unexpected. The intravenous administration of M$_1$dG and 6-oxo-M$_1$dG, and subsequent appearance of these products in bile and feces, strongly suggests the role of transporters in the disposition of M$_1$dG and 6-oxo-M$_1$dG \textit{in vivo}. Nucleosides and nucleoside derivatives are reported substrates for transport proteins, such as organic anion transporters (24) and the concentrative, Na$^+$-dependent (CNT) and equilibrative, Na$^+$-independent nucleoside transporters (ENT) (25). Indeed, ENT isoforms appear to be involved in nucleoside transport into hepatocytes (26), whereas the CNT isoforms are expressed along the bile canalicular membrane and are likely involved in biliary transport (27). It would be interesting to evaluate their involvement in DNA adduct disposition in future studies.

The notion that DNA adducts were subject to biliary elimination was first suggested by the work of Wang and Hecht (28). Following an intravenous administration of $N^7,C$-8 N-nitrosopyrrolidine guanine adduct to F344 rats, 52.2\% of the administered radioactivity was recovered in the urine.
Ninety percent of the urinary radioactivity was attributed to the parent molecule. The remaining 47.8% of the radioactivity was either retained in the animal or cleared through the biliary elimination pathway. However, neither biliary elimination nor fecal elimination was measured in their study. It is tempting to speculate that either the N7,C-8-N-nitrosopyrrolidine guanine adduct was a direct substrate for biliary transport or that the adduct underwent metabolism that rendered the molecule a substrate for transport into bile.

Our findings, in addition to those of Wang and Hecht (28), suggest that disposition of adducts may be a key limiting factor in the analysis of urinary DNA adducts and their metabolites.

The amount of M1dG administered in our study (~31 μg per animal) was lower than our previous investigation (~500 μg per animal) (20). However, the administered dose is still higher than the anticipated physiological levels of M1dG in the rat (19). Administration of microgram levels of M1dG and 6-oxo-M1dG in these studies was necessary to obtain a reliable mass balance and metabolite profile from which to proceed with future physiological determinations.

Administration of [2-14C]M1dG and [2-14C]6-oxo-M1dG resulted in rapid elimination of the administered material in the first 4 h, followed by an extended slow release of the remaining dose. During the LC-MS/MS analysis of late time point urine collections from the [14C]M1dG administration study, we observed that 6-oxo-M1dG was as abundant or more abundant than the parent molecule M1dG. Considering the likely involvement of transporters in the disposition of M1dG, it remains possible that transport may afford an effectively high concentration at the site of metabolism to facilitate the production of 6-oxo-M1dG even at low concentrations. Inspection of the relative urinary elimination rates for M1dG and 6-oxo-M1dG suggests this increase cannot simply be explained by a slower rate of elimination for 6-oxo-M1dG, because the elimination profiles for M1dG and 6-oxo-M1dG are nearly identical. Ongoing efforts in our laboratory are directed toward assessing the contribution of 6-oxo-M1dG to the overall population of M1dG in human and animal studies.

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