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Quantification of Azaserine-induced Carboxymethylated and Methylated DNA Lesions in Cells by Nanoflow Liquid Chromatography-Nanoelectrospray Ionization Tandem Mass Spectrometry Coupled with the Stable Isotope-Dilution Method

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Abstract

Humans are exposed to *N*-nitroso compounds through environmental exposure and endogenous metabolism. Some *N*-nitroso compounds can be metabolically activated to yield diazoacetate, which is known to induce DNA carboxymethylation. DNA lesion measurement remains one of the core tasks in toxicology and in evaluating human health risks associated with carcinogen exposure. In this study, we developed a highly sensitive nano-flow liquid chromatography-nanoelectrospray ionization-multi-stage tandem mass spectrometry (nLC-nESI-MS³) method for the simultaneous quantification of *O*⁶-carboxymethyl-2'-deoxyguanosine (*O*⁶-CMdG), *O*⁶-methyl-2'-deoxyguanosine (*O*⁶-MedG), and *N*⁶-carboxymethyl-2'-deoxyadenosine (*N*⁶-CMdA). We were able to measure the levels of these three lesions with the use of low-microgram quantities of DNA from cultured human skin fibroblasts and human colorectal carcinoma cells treated with azaserine, a DNA carboxymethylating agent. Our results revealed that the levels of *O*⁶-CMdG and *O*⁶-MedG increased when the dose of azaserine was increased from 0 to 450 μ M. We, however, did not observe an apparent dose-dependent induction of *N*⁶-CMdA, suggesting the presence of repair mechanism(s) for the rapid clearance of this lesion in cells. This is the first report about the application of nLC-nESI-MS³ technique for the simultaneous quantification of *O*⁶-CMdG, *O*⁶-MedG and *N*⁶-CMdA. The method reported here will be useful for the future investigations about the repair of the carboxymethylated DNA lesions and about the implications of these lesions in carcinogenesis.

Graphical Abstract

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Supporting Information

Limits of quantitation for the three modified nucleosides, the levels of the three modified nucleosides in azaserine-treated cells, proposed major fragmentation pathways for product ions observed in MS/MS and MS³, representative HPLC trace for the enrichment of the three lesions, calibration curves, and a second set of representative SICs for quantification of the three lesions. The supporting Information is available free of charge on the ACS Publication website.

INTRODUCTION

Human genome is continuously assaulted by a variety of endogenous and exogenous toxicants which can give rise to DNA damage and compromise genomic integrity.¹ In this vein, *N*-nitroso compounds (NOCs) are present in food, tobacco, and pharmaceutical products.^{2,3} Despite the prevalence of exogenous sources of NOCs, it was estimated that endogenous sources constitute 45–75% of total NOC exposure in humans.² In a widely accepted model for the etiology of gastric cancer, DNA damage emanating from intragastric nitrosation is considered an important factor contributing to early stages of disease development.⁴ In support of this hypothesis, results from a recent European Prospective Investigation into Cancer and Nutrition, which encompassed more than 500,000 individuals and 314 incident cases of gastric cancer that had occurred after an average of 6.6 years of follow-up, showed a significant association between endogenous NOC level and the risk of non-cardia gastric cancer.⁵ In this vein, elevated red meat intake in humans was found to elicit a 3-fold increase in fecal NOC levels.⁶

After metabolic activation, some NOCs can lead to carboxymethylation, and to a lesser degree, methylation of DNA.³ *N*-nitrosoglycocholic acid (NOGC), an *N*-nitroso bile acid conjugate, was previously found to be mutagenic in *Salmonella typhimurium* and in diploid human lymphoblasts,⁷ and carcinogenic to laboratory animals.⁸ Incubation of calf thymus DNA with NOGC *in vitro* was shown to induce the carboxymethylation of the N3 of adenine as well as the N7 and O⁶ of guanine, which is accompanied with the methylation of the O⁶ of guanine.⁹ In addition, *N*-(*N*⁶-acetyl-L-prolyl)-*N*-nitrosoglycine was found to induce O⁶-carboxymethyl-2'-deoxyguanosine (O⁶-CMdG) and, to a lower degree, O⁶-methyl-2'-deoxyguanosine (O⁶-MedG, Scheme 1) in DNA.^{10,11} Aside from NOCs, DNA carboxymethylation can occur in cells upon exposure to azaserine,^{10–12} a pancreatic carcinogen.¹³ DNA carboxymethylation induced by the aforementioned agents is thought to arise from a common reactive intermediate, diazoacetate (Scheme 1).^{3,14} Diazoacetate can decompose to yield carboxymethyldiazonium and methyldiazonium ions, which result in the carboxymethylation and methylation of DNA, respectively.^{3,14} In addition to guanine and adenine, cytosine and thymine in DNA can also be carboxymethylated by diazoacetate.^{15,16}

If not repaired, carboxymethylated DNA lesions may compromise the flow of genetic information by inhibiting DNA replication and transcription, and induce mutations in these processes. Gottschalg *et al.*¹⁴ observed that the replication of diazoacetate-treated, human *p53* gene-containing plasmid in yeast cells could give rise to remarkably similar mutation profiles (types and frequencies of mutations) at non-CpG sites as those of *p53* gene found in human stomach and colorectal cancers. In particular, diazoacetate could induce nearly equal frequencies of transition and transversion mutations, which differ from the predominant GC→AT transition mutations (>80%) induced by a DNA methylating agent (i.e. *N*-methyl-*N*-nitrosourea).¹⁴ Preferential formation of *O*⁶-CMdG over *O*⁶-MedG was observed for isolated DNA treated with various DNA carboxymethylating agents and for human cells treated with diazoacetate.^{10,17} These findings, along with the fact that *O*⁶-MedG induces predominantly GC→AT transition mutation,¹⁸ indicate that DNA carboxymethylation, rather than methylation, may contribute to the mutations of *p53* gene found in gastrointestinal cancer.¹⁴ Recent replication and transcription studies with the use of plasmids housing a site-specifically inserted DNA lesion showed that *O*⁴- and *N*³-carboxymethylthymidine could substantially stall DNA replication and transcription in cells, and induce mutations in these processes.^{19–21} On the other hand, *N*⁴-carboxymethyl-2'-deoxycytidine and *N*⁶-carboxymethyl-2'-deoxyadenosine don't constitute strong blocks to DNA replication in *Escherichia coli* cells, and replication across these lesions is highly accurate.²⁰

The potential involvement of NOCs and the ensuing DNA carboxymethylation in human gastrointestinal carcinogenesis calls for robust analytical methods for the unambiguous identification and accurate quantification of carboxymethylated DNA lesions. In this context, ³²P-postlabeling assay,²² immunoslot blot assay²³ and immunoaffinity enrichment coupled with HPLC with fluorescence detection^{10,11} were employed for measuring the levels of *O*⁶-CMdG and *O*⁶-MedG *in vitro* and *in vivo*. Owing to advances in instrumentation and sample preparation methods, mass spectrometry has become widely employed for the detection of DNA adducts by providing structural and quantitative information.^{24,25} In this regard, solid-phase extraction followed by LC-MS/MS analysis on a triple-quadrupole mass spectrometer was previously employed for the quantification of *O*⁶-CMdG and *O*⁶-MedG, directly or after converting them to the corresponding modified nucleobases with acid hydrolysis at elevated temperature.^{17,26} Although neither approach provided adequate sensitivity for detecting these modified nucleosides in human urine samples,²⁶ LC-MS/MS analysis of the acid-hydrolyzed samples facilitated the successful detection of *O*⁶-CMdG and *O*⁶-MedG in DNA isolated from cultured human cells treated with diazoacetate.¹⁷

In this study, we developed a highly sensitive nano-flow liquid chromatography-nano electrospray ionization tandem mass spectrometry (nLC-nESI-MS³) coupled with the stable isotope-dilution method for the simultaneous quantification of *O*⁶-CMdG, *O*⁶-MedG and *N*⁶-CMdA (Scheme 1) in genomic DNA extracted from cultured human cells treated with azaserine. We decided to assess the formation of *O*⁶-CMdG and *O*⁶-MedG because their measurements will allow for the comparison of the levels of DNA lesions formed from *in-vivo* carboxymethylation and methylation. We chose to include *N*⁶-CMdA in our analysis

because our previous LC-MS/MS analysis suggests that this lesion is produced at a much higher level than O^6 -CMdG in diazoacetate-treated calf thymus DNA.¹⁶

EXPERIMENTAL SECTION

Materials & cell lines

All chemicals and enzymes, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO) or New England Biolabs (Ipswich, WA). Stable isotope-labeled compounds were obtained from Cambridge Isotope Laboratories (Cambridge, MA). *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) hydrochloride was from Tocris Bioscience (Ellisville, MO). GM04429 human skin fibroblasts deficient in xeroderma pigmentosum complementation group A (XPA) were kindly provided by Prof. Gerd P. Pfeifer. The HCT-116 human colorectal carcinoma cells were purchased from ATCC (Manassas, VA).

Preparation of standards

O^6 -CMdG was prepared following published procedures,¹¹ and $[D_2]$ - O^6 -CMdG was synthesized by using the same procedures except that $[D_2]$ -methyl glycolate, synthesized from the commercially available $[D_2]$ -glycolic acid, was used in lieu of methyl glycolate. Exact mass measurement yielded m/z 328.1231 for the $[M + H]^+$ ion of the $[D_2]$ - O^6 -CMdG, which is in keeping with the calculated m/z of 328.1226.

O^6 -MedG was synthesized according to a previously reported method,²⁷ and the similar procedures were employed for the synthesis of $[D_3]$ - O^6 -MedG except that CD_3OD was used for incorporating the $-OCD_3$ group. Exact mass measurement gave m/z 285.1397 for the $[M + H]^+$ ion of the $[D_3]$ - O^6 -MedG, which is consistent with the calculated m/z of 285.1391.

N^6 -CMdA was synthesized previously.¹⁶ $[^{13}C_2, ^{15}N]$ - N^6 -CMdA was prepared following the same procedures as those for the preparation of the unlabeled counterpart except that glycine ethyl ester was replaced with $[^{13}C_2, ^{15}N]$ -glycine ethyl ester, and the latter was synthesized from the commercially available $[^{13}C_2, ^{15}N]$ -glycine. Exact mass measurement (Agilent 6210 ESI-TOF MS) gave m/z 313.1185 for the $[M + H]^+$ ion of the $[^{13}C_2, ^{15}N]$ - N^6 -CMdA, which is consistent with the calculated m/z of 313.1189.

Cell culture and azaserine treatment

XPA-deficient GM04429 human skin fibroblast cells and HCT-116 human colorectal carcinoma cells were cultured at 37°C in 5% CO_2 atmosphere in ATCC-recommended medium containing 10% fetal bovine serum and 100 IU/ml of penicillin. Cells ($1 \sim 1.5 \times 10^6$) were seeded in 75 cm^2 flasks in complete medium. After 24 h, the cells were treated with different concentrations of azaserine (i.e. 0, 50, 150 and 450 μM). After 4 h of treatment, the cells were detached by using trypsin-EDTA and harvested by centrifugation. The cell pellets were subsequently washed twice with phosphate-buffered saline (PBS) to remove residual medium and azaserine.

Extraction and enzymatic digestion of nuclear DNA

The experimental procedures for DNA extraction, enzymatic digestion, and off-line HPLC enrichment of modified nucleosides were similar as previously described.²⁸ In this respect, a high-salt method was employed for the extraction of genomic DNA.²⁹ A lysis buffer (70 μ L) containing 20 mM Tris (pH 8.1), 20 mM EDTA, 400 mM NaCl, 1% SDS (w/v), and 10 μ L of proteinase K (20 mg/mL) was added to the cell pellet and incubated at 55°C overnight. Saturated NaCl solution (0.5 volume) was subsequently added to the digestion mixture, and the mixture was vortexed briefly and incubated at 55°C for another 15 min. The resulting mixture was centrifuged at 13,000 rpm for 30 min, and the nucleic acids were precipitated from the supernatant with 2 volumes of ethanol. The RNA in the nucleic acid mixture was removed by digestion with 3 μ L RNase A (10 mg/mL) and 2 μ L RNase T1 (25 units/ μ L) at 37°C overnight followed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). The DNA was then precipitated from the aqueous layer with ethanol and centrifuged at 13,000 rpm for 10 min. The resulting DNA pellet was washed with 70% cold ethanol twice, allowed to air-dry at room temperature, re-dissolved in doubly-distilled water, and quantified by UV absorption measurement at 260 nm. Typically approximately 15 μ g DNA could be extracted from $1\sim 1.5 \times 10^6$ cells.

The extracted cellular DNA (10 μ g) was digested with 1 unit of nuclease P1 and 0.00125 unit of phosphodiesterase II in a 15- μ L buffer containing 300 mM sodium acetate (pH 5.6), 10 mM ZnCl₂, and 2.5 nmol of EHNA, and to the mixture were added 80 fmol [D₂]-*O*⁶-CMdG, 8 fmol [D₃]-*O*⁶-MedG, and 12 fmol [¹³C₂,¹⁵N]-*N*⁶-CMdA. EHNA served as an inhibitor for adenine deaminase and minimized the deamination of dA to 2'-deoxyinosine (dI). The above mixture was incubated at 37°C for 48 h. To the mixture were then added 1.0 unit of alkaline phosphatase, 0.0025 unit of phosphodiesterase I, and a 20- μ L buffer containing 0.5 M Tris-HCl (pH 8.9). After incubation at 37°C for 2 h, the digestion mixture was neutralized with 9.5 μ L 1.0 M formic acid. The enzymes in the digestion mixture were subsequently removed by extraction with an equal volume of chloroform. The aqueous layer was concentrated to 100 μ L and subjected to off-line HPLC enrichment.

HPLC enrichment

An Alltima HP C18 column (4.6 $\times$ 250 mm, 5 μ m in particle size, 300 Å in pore size, Grace Inc., Deerfield, IL) was employed for the enrichment of *O*⁶-CMdG, *O*⁶-MedG and *N*⁶-CMdA from the enzymatic digestion mixture. A solution of 10 mM ammonium formate (mobile phase A) and acetonitrile (mobile phase B) were used, and the gradient comprised of 0–2% B in 5 min, 2% B for 40 min and 2–40% B in 30 min, with the flow rate being 1 mL/min. The HPLC fractions in the retention time range of 22.5–27.5 min were pooled for *O*⁶-CMdG and *N*⁶-CMdA, and those fractions in 54.5–57.5 min were combined for *O*⁶-MedG (Figure S1). The eluents were dried in a Speed-vac, reconstituted in doubly distilled water and subjected to nLC-nESI-MS³ analysis. The lack of analyte carry-over during enrichment was confirmed by the failure in detecting the modified nucleosides in the corresponding fractions collected from the enrichment of a blank inserted between two sample enrichment runs.

nLC-nESI-MS³ analysis

Online nLC-nESI-MS³ analyses were conducted on an LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ionization source and coupled with an EASY-nLC II system (Thermo Fisher Scientific), as described previously.²⁸ HPLC separation was conducted by employing a homemade trapping column (150 $\mu\text{m} \times 40\text{ mm}$) and an analytical column (75 $\mu\text{m} \times 200\text{ mm}$), both packed with Magic C18 AQ (200 $\text{\AA}$, 5 μm , Michrom BioResource, Auburn, CA). Mobile phases A and B were 0.1% formic acid in doubly distilled H₂O and acetonitrile, respectively. Initially, the sample was loaded onto the trapping column with mobile phase A at a flow rate of 2.5 $\mu\text{L}/\text{min}$ in 6 min. The modified nucleosides of interest were then eluted by using a 30-min linear gradient of 0–20% mobile phase B at a flow rate of 300 nL/min. The LTQ-XL mass spectrometer was operated in the positive-ion mode, the voltage for electrospray was 2.0 kV, and the temperature for the ion transfer tube was 275°C. In MS³ analysis, the widths for precursor ion selection were 3 and 2 m/z units in MS/MS and MS³ modes, respectively. The normalized collision energy, activation Q and activation time were 35, 0.3, and 30 ms, respectively, in both MS/MS and MS³. The limit of quantitation (LOQ), defined as the amount of analyte generating a signal-to-noise ratio (S/N) of 10 in the selected-ion chromatograms (SICs) plotted for the transitions used for quantification, were determined via three independent measurements conducted in three separate days (Table S1). The intra- and inter-day precision and accuracy (Table 1) were evaluated by analyzing quality control samples of *O*⁶-CMdG, *O*⁶-MedG and *N*⁶-CMdA at two different concentrations. The quality control samples were generated from calf thymus DNA spiked with unlabeled standard solutions of the three modified nucleosides, following the same sample preparation procedure as those described above for the cellular DNA samples (DNA digestion, chloroform extraction and offline HPLC enrichment). The precision was expressed as relative standard deviation (RSD), and the mean accuracy was expressed as percent recovery. The amounts of the carboxymethylated and methylated DNA lesions (in moles) in the nucleoside mixtures were calculated from the peak area ratios found in the SICs for the analytes over their corresponding stable isotope-labeled standards, the amounts of the labeled standards used (in moles) and the calibration curves (Figure S2). The final DNA lesion levels, in terms of numbers of lesions per 10⁷ nucleosides, were calculated by dividing the amount of the DNA adducts with the amount of total nucleosides (in moles) in the digested DNA. In this regard, it is worth noting that the purities of unlabeled standards were assessed by using ¹H-NMR, and their concentrations were accurately determined. However, the purities of the stable isotope-labeled standards, owing to their limited quantities, were not examined by ¹H-NMR, which may lead to their inaccurate quantification and result in calibration curves with a slope deviating from 1.0. Since the same aliquots of the stable isotope-labeled standards were employed for sample analyses and for constructing the calibration curves, we believe that the lack of accurate quantification of the internal standards should not interfere with the accurate measurements of the modified nucleosides in cellular DNA samples.

RESULTS

Syntheses of unlabeled and stable isotope-labeled standards

We set out to establish an nLC-nESI-MS³ coupled with the stable isotope-dilution method for the quantification of carboxymethylated dG and dA lesions in cellular DNA. To this end, we first synthesized standards for the unlabeled and stable isotope-labeled *O*⁶-CMdG, *O*⁶-MedG, and *N*⁶-CMdA, as described in the Experimental Section. In this regard, we did not observe any appreciable H/D exchange of [D₂]-*O*⁶-CMdG at room temperature over a month, indicating that [D₂]-*O*⁶-CMdG is a suitable internal standard for quantitation.

nLC-nESI-MS³ for the quantifications of *O*⁶-CMdG, *O*⁶-MedG, and *N*⁶-CMdA

With the above stable isotope-labeled standards, we developed a sensitive LC-MS³ method for the accurate quantification of these lesions. In this method, we first digested the cellular DNA with a cocktail of 4 enzymes to liberate the lesions of interest as mononucleosides (See Experimental Section). The isotope-labeled *O*⁶-CMdG, *O*⁶-MedG, and *N*⁶-CMdA were added to the DNA digestion mixture prior to the first step of DNA digestion and chloroform extraction, which corrected for the potential analyte loss during the subsequent steps of sample preparation. We then assessed the LOQ of the nLC-nESI-MS³ method prior to the analyses of cellular DNA samples. When pure unlabeled standards were analyzed, the LOQs for *O*⁶-CMdG, *O*⁶-MedG, and *N*⁶-CMdA were determined to be 73.4, 6.2 and 11.5 amol, respectively (Table S1). We also assessed the intra- and inter-day precision and accuracy by analyzing calf thymus DNA spiked with different concentrations of standard solutions of *O*⁶-CMdG, *O*⁶-MedG and *N*⁶-CMdA. It turned out that our method provides reasonably good precision (with relative standard deviations of 5.4–20.5%) and accuracy (with recoveries of 70.0–92.3%) for measuring the three lesions (Table 1).

Relatively large amounts of unmodified nucleosides can significantly interfere with the detection of low levels of DNA lesions. Hence, we employed offline HPLC to enrich *O*⁶-CMdG, *O*⁶-MedG, and *N*⁶-CMdA from the DNA digestion mixtures prior to nLC-nESI-MS³ analysis. In this context, due to the better sensitivity achieved in the positive- than the negative-ion mode, we quantified the carboxymethylated and methylated DNA lesions by using nLC-nESI-MS³ in the positive-ion mode, where 0.1% formic acid (v/v) was added to the mobile phases to promote the protonation of the analytes. Upon collisional activation, the [M + H]⁺ ions of *O*⁶-CMdG, *O*⁶-MedG, and *N*⁶-CMdA readily eliminate a neutral 2-deoxyribose to yield the protonated nucleobase as the most abundant fragment ion in MS², which is subsequently isolated and collisionally activated for MS³ analysis. In this vein, the cleavages of the [M + H]⁺ ions of the nucleobase portion of *O*⁶-CMdG and [D₂]-*O*⁶-CMdG (*m/z* 210 and 212) led the formation of the major fragment ion of *m/z* 152, which stems from the neutral loss of the carboxymethyl functionality (Figure 1). The selected-ion chromatograms (SICs) for monitoring the *m/z* 326→210→152 and *m/z* 328→212→152 transitions revealed a peak at very similar retention time (21.7 min). This observation, together with the similar MS³ for the analyte and the internal standard, provides unambiguous identification of *O*⁶-CMdG. The excellent S/N observed in the SICs facilitates the accurate quantification of the level of this lesion.

Similar LC-MS³ analysis was employed for the quantification of *O*⁶-MedG and *N*⁶-CMdA. In this context, MS³ arising from the fragmentation of protonated nucleobase portions of *O*⁶-MedG and [D₃]-*O*⁶-MedG led to neutral loss of an NH₃ molecule to yield product ions of *m/z* 149 and *m/z* 152 for *O*⁶-MedG and [D₃]-*O*⁶-MedG in MS³, respectively (Figure 2). On the other hand, the collisional activation of the protonated nucleobase of *N*⁶-CMdA (*m/z* 194) and [¹³C₂,¹⁵N]-*N*⁶-CMdA (*m/z* 197) led to the facile neutral loss of an HCOOH and H¹³COOH molecule from the carboxymethyl side chain to yield the ions of *m/z* 148 and *m/z* 150 in MS³, respectively (Figure 3). The proposed fragmentation pathways for the three aforementioned DNA lesions are displayed in Supplementary Figure S3. Another set of representative SICs for the quantification of the three lesions were provided in the Supporting Information (Figures S4–S6).

Levels of *O*⁶-CMdG, *O*⁶-MedG, and *N*⁶-CMdA in azaserine-treated GM04429 cells

With the above-described nLC-nESI-MS³ method, we quantified the levels of *O*⁶-CMdG, *O*⁶-MedG and *N*⁶-CMdA in genomic DNA of GM04429 human skin fibroblasts and HCT-116 human colorectal carcinoma cells treated with different concentrations of azaserine. The quantification data revealed a dose-dependent formation of *O*⁶-CMdG and *O*⁶-MedG in both cell lines (Figure 4). The levels of *O*⁶-CMdG and *O*⁶-MedG in GM04429 cells increased from 0.054 to 4.95 and from 0.092 to 0.264 lesions per 10⁷ nucleosides, respectively, when the dose of azaserine was increased from 0 to 450 μM (Figure 4, Table S2). Similarly, the levels of *O*⁶-CMdG and *O*⁶-MedG in HCT-116 cells changed from 0.247 to 9.12 and from 0.07 to 0.52 lesions per 10⁷ nucleosides, respectively, with the dose of azaserine increased from 0 to 450 μM (Figure 4, Table S2). Thus, azaserine treatment could predominantly induce DNA carboxymethylation rather than methylation in cultured human cells, as reflected by the magnitude of increase in the levels of *O*⁶-CMdG (by 4.9 and 8.9 lesions per 10⁷ nucleosides in GM04429 and HCT-116 cells, respectively) and *O*⁶-MedG (by 0.172 and 0.45 lesions per 10⁷ nucleosides in GM04429 and HCT-116 cells, respectively. Figure 4A & 4B, Table S2). These results are in agreement with the previously reported findings made for isolated DNA treated with various DNA carboxymethylating agents,¹⁰ suggesting the more efficient generation and/or less efficient repair of the *O*⁶-CMdG than the *O*⁶-MedG. Moreover, all three lesions were detectable in control samples without azaserine treatment, indicating the existence of endogenous agents that can induce DNA carboxymethylation and methylation. The magnitude of increase in the levels of *N*⁶-CMdA (by 0.18 lesions per 10⁷ nucleosides) in GM04429 cells arising from azaserine treatment, however, was very minimal compared to that of *O*⁶-CMdG (Figure 4A & 4C, Table S2). Moreover, no dose-dependent induction of *N*⁶-CMdA was observed in genomic DNA extracted from azaserine-treated HCT-116 cells (Figure 4A & 4C, Table S2). These results, along with the lower levels of this lesion (Table S2) than *O*⁶-CMdG, indicated the preferential formation of *O*⁶-CMdG over *N*⁶-CMdA and/or the presence of repair mechanism(s) for the facile removal of *N*⁶-CMdA.

DISCUSSION

Owing to its sensitivity, accuracy and specificity, LC-MS/MS or MS³ combined with the stable isotope-dilution technique constitutes a rigorous analytical method for the quantitation

of DNA lesions in complex biological matrices.^{24,25} This technique is advantageous over other conventional methods for DNA adduct measurement (e.g. ³²P-postlabeling and immunoassay) as it allows for unequivocal identification and reliable quantitation of chemically modified nucleosides by providing structural information. In this method, the stable isotope-labeled standards of the lesions were added to the nucleoside mixture prior to enzymatic digestion. The determination of the levels of the lesions depends on the measurement of the molar ratios of the analytes over their corresponding internal standards, which is not affected by the variation in experimental conditions during enzymatic digestion, HPLC enrichment, and LC-MS analysis.

Compared to the previously reported LC/MS-based quantification of *O*⁶-CMdG and *O*⁶-MedG in selected-reaction monitoring (SRM) mode on a triple quadrupole mass spectrometer,^{17,26} our MS³ detection mode in the LTQ XL linear ion trap mass spectrometer provided better specificity (see Figures S7–S12 for representative selected-ion chromatograms and tandem mass spectra acquired from MS² and MS³ modes of detection). Additionally, in contrast to the solid-phase extraction method previously used for analyte enrichment,^{17,26} the off-line HPLC enrichment method reported herein offered better sample cleanup by removing excess amount of unmodified nucleosides and buffer salts added during the enzymatic digestion,³⁰ which further improved the sensitivity of our method. Moreover, to our knowledge, our technique is the first application of the nLC-nESI-MS³ for the simultaneous quantification of multiple carboxymethylated and methylated DNA lesions in cellular DNA, which increased the sensitivity of the analysis by improving ionization efficiency and transmission of ions from the ionization source into the mass analyzer.

We found a dose-dependent induction of *O*⁶-CMdG and *O*⁶-MedG in genomic DNA of both XPA-deficient GM04429 cells and HCT-116 cells treated with azaserine, which validated our analytical method. The detection of *N*⁶-CMdA in both cell lines provided evidence to support that this lesion could be induced in cells. In contrast to *O*⁶-CMdG and *O*⁶-MedG, statistically significant increase in the level of *N*⁶-CMdA was only observed when XPA-deficient GM04429 cells, but not HCT-116 cells, were treated with the highest dose (i.e. 450 μM) of azaserine, indicating the existence of potential pathway for the facile repair of this lesion *in vivo*. Along this line, Li *et al.*³¹ observed that *N*⁶-methyl-2'-deoxyadenosine (*N*⁶-MedA) could be repaired by *Escherichia coli* AlkB through a direct reversal mechanism. Recent studies also revealed the presence of demethylase for *N*⁶-MedA in *Drosophila melanogaster* and *Caenorhabditis elegans*.^{32,33} It is possible that these enzymes may also be involved in the repair of *N*⁶-CMdA, owing to its structural similarity with *N*⁶-MedA. In this connection, it is of note that *O*⁶-MedG can be repaired readily by *O*⁶-methylguanine-DNA methyltransferase (MGMT),³⁴ and *O*⁶-CMdG was also found to be a substrate for MGMT *in vitro*.³⁵

Taken together, we reported, for the first time, the simultaneous quantifications of *O*⁶-CMdG, *O*⁶-MedG and *N*⁶-CMdA in cultured human skin fibroblasts and human colorectal carcinoma cells treated with azaserine using nLC-nESI-MS³ with the stable isotope-dilution method. This method may serve as a useful tool for the future exploration of the repair of these lesions and for the investigation about the involvement of DNA carboxymethylation and methylation in the etiology of human gastrointestinal tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative selected-ion chromatograms (SICs) for monitoring the m/z 326→210→152 (A) and 328→212→152 (B) transitions for the $[M+H]^+$ ions of the unlabeled O^6 -CMdG and $[D_2]$ - O^6 -CMdG, respectively, in the nucleoside mixture of genomic DNA extracted from the XPA-deficient human skin fibroblast cells treated with azaserine. Shown in the insets are the MS^3 spectra for the unlabeled and labeled O^6 -CMdG.

Figure 2. Representative SICs for monitoring the m/z 282 $\rightarrow$ 166 $\rightarrow$ 149 (A) and m/z 285 $\rightarrow$ 169 $\rightarrow$ 152 (B) transitions for the $[M+H]^+$ ions of unlabeled O^6 -MedG and $[D_3]$ - O^6 -MedG, respectively, in the nucleoside mixture of genomic DNA extracted from the XPA-deficient human skin fibroblast cells treated with azaserine. Shown in the insets are the positive-ion MS³ spectra for the unlabeled and labeled O^6 -MedG.

Figure 3. Representative SICs for monitoring the *m/z* 310→194→148 (A) and *m/z* 313→197→150 transitions (B) for the [M+H]⁺ ions of the unlabeled *N*⁶-CMdA and [¹³C₂,¹⁵N]-*N*⁶-CMdA, respectively, in the nucleoside mixture of genomic DNA extracted from the XPA-deficient human skin fibroblast cells treated with azaserine. Shown in the insets are the positive-ion MS³ spectra for the unlabeled and labeled *N*⁶-CMdA.

Figure 4. LC-MS³ quantification results for *O*⁶-CMdG (A), *O*⁶-MedG (B) and *N*⁶-CMdA (C) in DNA samples isolated from XPA-deficient human skin fibroblast cells and human colorectal carcinoma HCT-116 cells treated with different concentrations of azaserine. The data represent the means and standard deviations of results obtained from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The p values were calculated by using an unpaired two-tailed t -test.

Scheme 1. Formation of diazoacetate and its induction of *O*⁶-CMdG, *O*⁶-MedG and *N*⁶-CMdA.

Table 1

Intra-day and inter-day precision and accuracy for the measurements of O^6 -CMdG, N^6 -CMdA, and O^6 -MedG, reported as Relative Standard Deviation (RSD) and Percent Recovery, respectively.

	Intra-day		Inter-day	
O^6 -CMdG (nM)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
0.063	5.4	92.3	16.6	86.9
0.197	20.5	75.6	7.9	82.7
O^6 -MedG (nM)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
0.009	20.4	84.6	6.9	77.2
0.014	8.5	76.0	18.5	80.9
N^6 -CMdA (nM)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)
0.020	10.9	83.3	9.6	70.0
0.028	11.6	85.7	7.5	88.1