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Characterization of Nitrogen Mustard Formamidopyrimidine Adduct Formation of bis-(2-Chloroethyl)ethylamine with Calf Thymus DNA and a Human Mammary Cancer Cell Line

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Abstract

A robust, quantitative ultraperformance liquid chromatography ion trap multistage scanning mass spectrometric (UPLC/MS³) method was established to characterize and measure five deoxyguanosine (dG) adducts formed by reaction of the chemotherapeutic nitrogen mustard (NM) bis-(2-chloroethyl)ethylamine with calf thymus (CT) DNA. In addition to the known N7-guanine (NM-G) adduct and its crosslink (G-NM-G), the ring-opened formamidopyrimidine (FapyG) mono-adduct (NM-FapyG) and cross-links in which one (FapyG-NM-G) or both (FapyG-NM-FapyG) guanines underwent ring-opening to FapyG units were identified. Authentic standards of all adducts were synthesized and characterized by NMR and mass spectrometry. These adducts were quantified in CT DNA treated with NM (1 μ M) as their deglycosylated bases. A two-stage neutral thermal hydrolysis was developed to mitigate the artifactual formation of ring-opened FapyG adducts involving hydrolysis of the cationic adduct at 37 °C, followed by hydrolysis of the FapyG adducts at 95 °C. The limit of quantification values ranged between 0.3 and 1.6 adducts per 10⁷ DNA bases, when the equivalent of 5 μ g DNA hydrolysate was assayed on column. The principal adduct formed was the G-NM-G cross-link, followed by the NM-G mono-adduct; the FapyG-NM-FapyG adduct was at the limit of detection. The NM-FapyG adducts formed in CT DNA at a level of ~20% that of the NM-G adduct. NM-FapyG has not been previously quantified and the FapyG-NM-G and FapyG-NM-FapyG adducts have not been previously characterized. Our validated analytical method was then applied to measure DNA adduct formation in the MDA-MB-231 mammary tumor cell line exposed to NM (100 μ M) for 24 h. The major adduct formed was NM-G (970 adducts per 10⁷ bases), followed by G-NM-G (240 adducts per 10⁷ bases) and

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Author Contributions

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Supporting Information.

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NM-FapyG (180 adducts per 10^7 bases), and lastly the FapyG-NM-G cross-link adduct (6.0 adducts per 10^7 bases). These lesions are expected to contribute to the NM-mediated toxicity and genotoxicity in vivo.

Keywords

Nitrogen mustards; DNA adducts; mass spectrometry; cross-links; formamidopyrimidines

Introduction

Nitrogen mustards are a class of bifunctional chemotherapeutic agents that were among the first agents to show effectiveness against non-Hodgkin lymphoma in mice and early human clinical trials.¹⁻³ Mechlorethamine (1949), chlorambucil (1954), cyclophosphamide (1959), uracil mustard (1962) and melphalan (1964) were among the first drugs approved by the FDA for clinical use (Figure 1). They are still used as part of combination drug therapies for the treatment of cancer and autoimmune disease. The mechanism of action of nitrogen mustards is through alkylation of DNA bases. Secondary malignancies, in particular acute myeloid leukemia, are often observed in patients treated with nitrogen mustards, and this side effect is a limiting factor in their clinical use.^{4, 5}

The chemical mechanism of DNA alkylation involves an initial intramolecular S_N2 reaction to form an aziridinium ion intermediate, which is the DNA alkylating species.^{2, 6} As a bis-electrophile, the initial adduct can form a second aziridinium ion intermediate, which can undergo solvolysis to the corresponding alcohol affording a mono-adduct, react with nucleophilic sidechains of proteins (e.g., cysteine) to form DNA-protein cross-links,⁷ or react with another DNA base to generate DNA inter- and intrastrand cross-links.⁸⁻¹⁰ This is shown in Scheme 1 for the alkylation at the N7-position of deoxyguanosine (dG).

Interstrand DNA cross-links are regarded as highly cytotoxic lesions and although they generally represent only a small percentage of the total adduct burden, they are believed to play a disproportionately high role in the mechanism of action of nitrogen mustards and other DNA cross-linking agents.¹¹ Interestingly, nitrogen mustards were shown to form interstrand cross-links in a 5'-GNC-3' rather than 5'-GC-3' sequence.^{12, 13}

Numerous studies have examined the reaction of nitrogen mustards with nucleosides and single and double stranded DNA. The major adducts are from reaction at N7-dG and N3-dA with minor reaction at N3-dC, N1-dA and *O*⁶-dG.^{8, 14-21} In addition, cross-links have been characterized between N7-positions of dG, N3-positions of dA, and between the N7-position dG and N3-position of dA.^{9, 10} Cationic N7-dG adducts can also undergo a secondary reaction involving the addition of hydroxide to C8 followed by imidazole ring-opening to form an *N*⁵-substituted formamidopyrimidine adduct (Fapy-dG) in which the *N*⁵-substituent is derived from the alkylating agent. High pH is usually required to induce Fapy-dG formation for most alkylating agents. Mehta reported that alkylation of dG with a phosphoramidate mustard ($R = -PO_2NH_2$) produced an unusually unstable adduct.²² The decomposition products, which formed at pH 7.4, were ascribed to imidazole ring opening based on their UV spectra. Chetsanga subsequently showed that reacting the phosphoramidate

mustard with DNA produced an adduct that was a substrate for *Escherichia coli* formamidopyrimidine glycosylase (FPG),²³ suggesting the formation of a Fapy-dG adduct from the phosphoramidate mustard. Hemminki observed FapyG adducts from the reaction of nor-nitrogen mustard (R= -H), mechlorethamine, chlorambucil and aziridine.^{15, 24-26} Interestingly, cultured human cells that overexpressed FPG were up to 100-fold less sensitive to thioTEPA or aziridine,²⁷⁻²⁹ 10-fold less sensitive to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and 2-fold less sensitive to the nitrogen mustard mafosfamide.^{30, 31} These results support a role for the *N*⁵-substituted Fapy-dG adducts in the cytotoxic mechanism of these agents.

The Fapy-dG adduct in which the formyl nitrogen is unsubstituted is a product of oxidative DNA damage and has been shown to be mutagenic in simian kidney (COS-7) and human embryonic kidney cell culture.^{32, 33} *N*⁵-Substituted Fapy-dG adducts derived from aflatoxin B₁ epoxide (AFB₁-Fapy-dG) and methylating agents (MeFapy-dG) have been reported to be persistent lesions in rodents.^{34, 35} The AFB₁-Fapy-dG and MeFapy-dG adducts have also been shown to be mutagenic in COS-7 cells.^{36, 37} Given the importance of nitrogen mustards in cancer chemotherapy, it is surprising that *N*⁵-nitrogen mustard Fapy-dG adducts have received little attention. Recently, the Fapy-dG adduct derived from bis-(2-chloroethyl)ethylamine has been site-specifically incorporated into oligonucleotides by solid-phase methods and shown to be a substrate for FPG and *E. coli* Endonuclease IV.³⁸

We report here the development of an analytical method to measure nitrogen mustard Fapy-dG adducts (NM-FapyG) from the reaction of bis-(2-chloroethyl)-ethylamine (NM) with calf thymus (CT) DNA by ion-trap multistage scan mass spectrometry. Further, we hypothesized the previously uncharacterized nitrogen mustard cross-links in which one (FapyG-NM-G) or both (FapyG-NM-FapyG) guanines undergo imidazole ring-opening to FapyG units should also be reaction products. The three Fapy adducts along with the N7-G mono-adduct (NM-G) and N7-cross-link (G-NM-G) were detected as their deglycosylated bases (Figure 2) and quantitated by the stable isotope dilution method,³⁹ following neutral thermal hydrolysis and solid phase extraction (SPE).

Experimental Procedures

Synthesis of standards

Authentic standards of NM-G, G-NM-G, NM-FapyG, FapyG-NM-G and FapyG-NM-FapyG were synthesized from dG and NM and described in the Supporting Information. The standards were characterized by NMR and MS and their UV extinction coefficients determined. Isotopically labeled NM-G, G-NM-G, FapyG-NM-G and FapyG-NM-FapyG were synthesized from dG and [²H₅]-ethyl-NM; labeled NM-FapyG was synthesized from [¹⁵N₅]-dG and NM.

Reaction of CT DNA with NM

CT DNA (50 µg) in 0.5 mL of 50 mM potassium phosphate buffer (pH 7.4) was reacted with NM (1 µM) at 37 °C for 14 h. The unreacted NM and decomposition products were removed by solvent extraction with ethyl acetate (3 × 500 µL). A solution of NaCl (5 M, 60 µL) was

added, and the DNA was precipitated by addition of chilled ethanol (1 mL), followed by centrifugation. The DNA pellets were washed with 70%, followed by 100% ethanol, then dried under vacuum for 10 min.

Neutral thermal hydrolysis of CT DNA and recovery of the NM-adducts

CT DNA (50 $\mu\text{g/mL}$) was dissolved in potassium phosphate (50 mM, pH 7.0,) and subjected to neutral thermal hydrolysis at 37 °C, 70 °C, or 95 °C for variable incubation times. Stable, isotopically labeled internal standards were added prior to hydrolysis at a level of 350 pg per 50 μg DNA; this level of spiking with the internal standards corresponds to 53 – 86 adducts per 10^7 bases. The hydrolysis was terminated by placing the solutions on ice and the residual DNA was removed by size exclusion filtration employing Pall 10 kDa molecular weight cut off filter and centrifugation at 5000 g for 20 min. The filters were washed with 2% HCO_2H in H_2O (300 μL), and the combined filtrates containing the adducts were evaporated to dryness by vacuum centrifugation at 65 °C.

The NM adducts were enriched by SPE using Sola SCX cartridges (Thermo Scientific, Bellafonte, PA). The SPE cartridge was pre-conditioned with 5% NH_3 in CH_3OH (1 mL) followed by 0.1 N HCl (1 mL). DNA hydrolysates were resuspended in 0.1 N HCl (1 mL) and loaded on to the SCX resin. The cartridges were sequentially washed with 0.1 N HCl ; 2% HCO_2H ; 2% HCO_2H in CH_3OH ; and H_2O (1 mL each). The NM adducts were eluted from the SPE with a solvent mixture containing 25% H_2O / 70% CH_3OH / 5% NH_3 (1 mL). Samples were evaporated to dryness by vacuum centrifugation at 65 °C. The extracts were dissolved in 2 mM ammonium formate containing 5% CH_3CN (50 μL) immediately prior to UPLC/MS³.

Method validation of NM-DNA adducts

The within-day and between-day reproducibility were assessed by measuring NM adducts from CT DNA treated with NM (1 μM) for 14 h. The DNA was subjected to neutral thermal hydrolysis for 4 h at 95 °C as described above. Calibration curves were constructed in a DNA matrix (50 μg), which underwent neutral thermal hydrolysis and SPE prior to addition of the adduct calibrants. The calibration sample set was comprised of triplicates at nine concentrations: 0, 0.2 to 6 $\text{pg } \mu\text{L}^{-1}$ of unlabeled adduct standards for NM-G, NM-FapyG, FapyG-NM-G, FapyG-NM-FapyG, and 0, 0.2 to 30 $\text{pg } \mu\text{L}^{-1}$ for G-NM-G, with 7 $\text{pg } \mu\text{L}^{-1}$ isotopically labeled internal standards. A 5 μL sample volume out of 50 μL was injected on the column.

Treatment of NM-modified CT DNA with FPG

NM-modified CT DNA (17 μg) was diluted in sodium phosphate buffer (50 mM, pH 7.0, 200 μL) containing MgCl_2 (10 mM) and DTT (1 mM).^{40, 41} Stable, isotopically labeled internal standards (117 pg, a level of internal standard corresponding between 53 to 86 adducts per 10^7 bases) were added, followed by the addition of FPG (40 units). The incubation was conducted at 37 °C and the released adducts were measured at time intervals of 7, 21, 30 and 44 h. The reaction was terminated by adding 2 volumes of chilled ethanol followed by sample storage at -20 °C for 1 h. The supernatant containing the released NM

adducts was vacuum centrifuged to dryness and processed by SPE as described above. Additional FPG (40 units) was added to the remainder of the reaction at each time interval.

Treatment of MDA-MB-231 Cells with NM

The human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in Leibovitz's L-15 medium (SIGMA, St Louis, MO) with a seeding density of 2 million in a T75 TPP® flask (SIGMA, St Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (50 U/mL, 50 µg/mL) (SIGMA, St Louis, MO) at 37 °C without CO₂. Cells were grown for 24 h and then treated with 100 µM NM (Mittenwald, Germany) in PBS. The culture media was 12 mL and the carcinogen volume was kept at less than 0.1% of the total media volume. The NM treatment was performed in duplicate along with a PBS (Corning Life Sciences, Corning, NY) serving as the negative control. The NM treatment was performed for 24 h. Two independent experiments were performed.

DNA isolation

After 24 h of treatment, the media was aspirated and the dead cells were removed and the adhered cells were washed twice with PBS and collected by incubating with 0.5% Trypsin-EDTA (GIBCO, Grand Island, NY) at 37 °C for 5 minutes. The trypsin was neutralized with equal volume of the culture media and centrifuged to pellet the cells. The cells were then resuspended in PBS (10 mL) and pelleted again. This washing was done twice more. Cell toxicity of the NM-treated cells versus PBS negative control cells was assessed by the Trypan blue exclusion assay using a Countess Automated Cell Counter (Invitrogen, Life Technologies, Carlsbad, CA). Cells were frozen at -80 °C before processed for DNA extraction.

The cells were homogenized and treated with RNase A (13 µL of 10 mg/mL) and RNase T1 (1.5 µL of stock 200KU/mL) (SIGMA, St Louis, MO) for 30 min at 37 °C, followed by addition of proteinase K (20 µL of 20 mg/mL) (Novagen- Merck, Darmstadt, Germany) and 30 µL of 10% SDS (Alfa Aesar, Ward Hill, MA) for 1 h at 37 °C. DNA was then extracted using DNeasy Blood & Tissue kit (Qiagen, Redwood City, CA) following manufacturer's instructions with minor modifications. Neutral thermal hydrolysis of the NM-modified MDA-MB-231 Cell DNA was done for 4 h at 95 °C, or 72 h at 37 °C, followed by 4 h hydrolysis at 95 °C. The amount of DNA assayed ranged between 3.7 and 6.0 µg, with internal standards added at a level ranging between 240 and 360 adducts per 10⁷ bases.

UPLC/MS³ measurements

UPLC/MS³ measurements were performed on a LTQ Velos Pro MS (Thermo Scientific, San Jose, CA), operating in the positive ESI mode. The MS was interfaced with an Advance CaptiveSpray™ source from Michrom Bioresource Inc. (Auburn, CA) and a NanoAcquity UPLC system (Waters Corp., Milford, MA). All DNA adducts were analyzed in a single scan event at the MS³ scan stage employing the MS parameters described in Table 1. Typical MS tuning parameters were as follows: capillary temperature, 270 °C; source spray voltage, 1.3 kV; S-lens RF level, 69%; no source fragmentation. Helium, set at 1 millitorr, was used as the collision and damping gas in the ion trap. There was no sheath or auxiliary

gas. The reconstructed ion chromatograms were obtained using the m/z ions reported at the MS³ scan stage and employed for quantitative measurements.

The DNA adducts were separated on an Inertsil C18 ODS-4 column (0.3 × 250 mm, 5 μm particle size) GL Sciences, Torrance, CA). Mobile phase A contained 2 mM ammonium formate and 5% CH₃CN in H₂O, and mobile phase B contained 2 mM ammonium formate and 5% H₂O in CH₃CN. A linear gradient was employed for separation of the DNA adducts. The solvent was held at 95:5 A/B for 1 min, and then increased to 5:95 A/B at 11 min, and held at this solvent composition for 2 min. The solvent composition was re-equilibrated to starting conditions over 2 min, followed by a 7 min equilibration, at a flow rate of 5 μL/min. The column was heated at 65 °C, to optimize the peak shape and chromatography. All connecting tubing employed was PEEKsil™ (50 μm inner diameter) because the NM G adducts strongly interacted with PEEK tubing, resulting in very broad peaks.

Statistical methods

The *t*-test or non-linear regression comparisons of curve fits were performed using GraphPad Prism (v. 6) for Windows, GraphPad Prism Software (La Jolla, CA). A *P* value <0.05 was considered statistically significant.

Results

Synthesis of Standards

Standards for the five adducts of interest (Figure 2) were synthesized from the reaction of dG with NM in trifluoroethanol as described in the Supporting Information. Isotopically labeled standards were synthesized using [²H₅]-ethyl-NM. The isotopic label was lost at the MS² scan stage for NM-FapyG; thus the labeled NM-FapyG was prepared from [¹⁵N₅]-dG. The product ion spectra of the NM-G, G-NM-G, NM-FapyG, FapyG-NM-G, and FapyG-NM-FapyG adducts and isotopically labeled internal standards at the MS² and MS³ scan stages with the proposed principal product ions are shown in Figures S16-S20 of the Supporting Information.

Calibration Curves and Quantitation of Monomer and Dimer Adducts of NM adducts

The linear regressions of the calibration curves were based on the ratio of the peak area of the unlabeled adduct to that of labeled internal standard plotted against the amount of unlabeled adduct versus the internal standard. Data were fitted to a straight line using ordinary least-squares with equal weightings (Figure S21 of the Supporting Information). For all five adducts, the linearity of the calibration curve was shown by the slope, the goodness-of-fit linear regression values, $r^2 > 0.99$, over the range 0.1 to 30 pg or 150 pg of adduct.⁴² Based upon recommended guidelines for data acquisition and quality evaluation in environmental chemistry, the values of the limit of detection (LOD) and limit of quantitation (LOQ) for analytes were set at 3σ and 10σ standard deviation units above the background level signal of an uncontaminated matrix.⁴³ The LOQ values ranged from 0.3 to 1.6 adducts per 10⁷ DNA bases, when the equivalent of 5 μg DNA hydrolysate was assayed on column.

Development of an Adduct Enrichment Protocol

All measurements were performed with CT DNA (50 $\mu\text{g/mL}$) in potassium phosphate buffer (100 mM, pH 7.0) modified with NM (1 μM). Cationic N7-dG adducts are thermally labile. The FapyG lesion has been removed from DNA by hot piperidine treatment.⁴⁴ We therefore decided to liberate the adducts of interest through a two-step process in which the NM-G and G-NM-G adducts would be removed by neutral thermal hydrolysis (pH 7.0, 95 °C) followed by hot piperidine treatment (0.2 M, 90°C, 1 h) to liberate the Fapy adducts. This protocol removed the DNA adducts of interest as their modified nucleobases while leaving the bulk unmodified DNA intact, which was removed by size exclusion filtration with a 10 kDa molecular weight cut off filter. Treatment of the NM-modified DNA with just hot piperidine induced artifactual NM-FapyG formation. In addition, the residual piperidine recovered following SPE, adversely affected the chromatography and signal of response for all adducts. We subsequently determined that prolonged hydrolysis of DNA at 95 °C in neutral phosphate buffer also caused hydrolysis of the Fapy adducts, thus obviating the need for the piperidine treatment.

The employment of SPE resins without polyethylene frits was essential for isolation of the modified nucleobases and optimal sensitivity by ion trap MS. We observed that solid phase resins containing polyethylene frits resulted in very strong ion suppression effects for all NM-G adducts, similar to the ion suppression effects previously seen with DNA adducts of heterocyclic aromatic amines enriched by SPE.⁴⁵ A representative chromatogram of the adducts recovered from NM-modified CT DNA following neutral thermal hydrolysis (pH 7.0, 95 °C, 4 h) is shown in Figure 3.

Method Validation

The within-day and between-day reproducibility were determined for the reaction of CT DNA with NM, following neutral thermal hydrolysis at 95 °C for 4 h. Four independent replicate measurements were determined for the five adducts. The studies were repeated on 3 different days, and the data are summarized in Table 2. The within-day and between-day data precision, reported as the % coefficient of variation (% CV), are 10 % for the NM-G, NM-FapyG, FapyG-NM-G and G-NM-G adducts; formation of the FapyG-NM-FapyG adduct was below the LOQ value.

Time Course of recovery of NM adducts from CT DNA as a function of temperature and time, and artifactual formation of NM-FapyG adducts

The hydrolysis and recovery of NM CT DNA adducts was conducted at 70 and 95 °C as a function of time (Figure 4). The hydrolysis of the cationic NM-G and G-NM-G adducts was rapid at 95 °C, and the recovery of adducts reached a maximum level after ~0.5 h, whereas the NM-FapyG and FapyG-NM-G adducts were hydrolyzed more slowly, reaching their maximum level between 2 and 4 h. The FapyG-NM-FapyG adduct was observed at low levels, approaching the limit of detection (LOD). The cationic NM-G and G-NM-G adducts were released more slowly at 70 °C, reaching their maximum levels between 2 and 4 h. The levels of NM-FapyG and FapyG-NM-G continued to slowly increase, reaching levels of 2.5 and 1.2 adducts per 10^7 bases, respectively, over this 8 h time course.

Once again, the FapyG-NM-FapyG level approached the LOD. The level of NM-FapyG was ~9-fold greater when the hydrolysis was conducted at 95 °C compared to 70 °C (23.1 ± 1.8 vs 2.5 ± 0.3 adducts per 10^7 DNA bases, t-test, $p < 0.001$, Mean $\pm$ SEM); the level of FapyG-NM-G was ~3-fold greater at 95 °C (3.6 ± 0.3 vs 1.2 ± 0.1 adducts per 10^7 DNA bases, t-test, $p < 0.001$); and the amount of NM-G decreased by ~0.3 fold when hydrolysis was done at 95 °C compared to hydrolysis at 70 °C (24.0 ± 1.6 vs 34.1 ± 1.9 , $p < 0.002$). There are significant differences between the levels of NM-G, NM-FapyG, FapyG-NM-G adducts obtained by the two-step hydrolysis conditions (Figure 4). These kinetic data signify that a portion of the NM-G adducts undergo ring-opening to form NM-FapyG at 95 °C.

Since the cationic NM-G and NM-G-NM adducts undergo hydrolysis at elevated temperatures more rapidly than the ring-opened NM-FapyG adducts, we explored the selective depurination of the cationic adducts from DNA under mild hydrolysis at 37 °C while retaining the more stable NM-FapyG adducts in the DNA backbone.⁴⁶ The time course of neutral thermal hydrolysis of NM-modified CT DNA was conducted over time up to 72 h, followed by precipitation of DNA and recovery of depurinated NM-G and G-NM-G adducts in the supernatant. The DNA was subjected to further hydrolysis at 95 °C to recover the three NM FapyG adducts.

The time course of hydrolysis is summarized in Figure 5A and 5B. The level of NM-G reached a plateau at ~32 h with greater than 95% of the NM-G released from the CT DNA. The hydrolysis of the G-NM-G adduct was less rapid than the mono-adduct, and ~15% of the adduct still remained bound to the DNA after 72 h. The ensuing hydrolysis of the partially depurinated DNA at 95 °C for 4 h showed that the remainder of G-NM-G was recovered at the elevated temperature (Figure 5B). The ring-opened NM Fapy adducts were relatively stable towards hydrolysis of DNA at 37 °C with <10% of the adducts recovered in the supernatant during the 72 h time course. The remaining ~ 90% of the NM-FapyG and FapyG-NM-G fractions were recovered from the DNA by the subsequent hydrolysis at 95 °C. The total amount of NM-FapyG and FapyG-NM-G recovered by sequential hydrolysis of NM-modified DNA at 37 °C followed by 95 °C were 6.9 ± 0.7 and 3.4 ± 0.6 adducts per 10^7 DNA bases, respectively. We conclude that neutral thermal hydrolysis at 37 °C for 72 h to remove cationic adducts, followed by hydrolysis of thermally stable ring-opened NM FapyG adducts at 95 °C greatly minimizes artifactual formation the NM FapyG adducts.

Enrichment of NM-FapyG adducts by FPG excision of NM CT-DNA

The NM-FapyG adduct is a substrate for FPG when placed in a 24-mer duplex.³⁸ We examined the use of FPG to analyze the NM-FapyG in NM-modified CT DNA (Figure 6). The level of NM-FapyG and FapyG-NM-G based on sequential neutral thermal hydrolysis were, respectively, 6.9 ± 0.7 and 3.4 ± 0.6 adduct per adducts per 10^7 DNA bases versus 1 NM-FapyG adduct per 48 DNA bases in the 24-mer duplex.³⁸ Despite the >50,000-fold dilution of NM-FapyG in CT DNA, FPG excised NM-FapyG from the CT DNA, albeit much less efficiently.

The amount of NM-FapyG recovered by FPG from the treated CT DNA after 72 h was ~4-fold greater than the level of NM-FapyG due to background hydrolysis without FPG. The recovery of the NM G lesions, with and without FPG, versus time was assessed by non-

linear regression analysis of adduct and showed significant differences for NM-FapyG and FapyG-NM-G adducts ($p < 0.003$). The levels of NM-FapyG and FapyG-NM-G recovered by FPG after 72 h were, respectively 8.6 ± 1.0 and 1.5 ± 0.2 adducts per 10^7 DNA bases. These adducts levels are similar to those recovered by sequential neutral thermal hydrolysis at 37 °C for 72 h followed by at 95 °C for 4 h (Figure 5A and B). In contrast, the recovery of the NM-G 72 h with FPG was nearly identical to that of the control without FPG ($p = 0.78$). Interestingly, the recovery of the G-NM-G adduct modestly increased in the presence of FPG over control (–FPG) ($p = 0.01$).

The recoveries of NM CT DNA adducts as a function of the different hydrolysis conditions are summarized in Table 3. The recoveries of cationic adducts, and NM-FapyG by the two stage neutral thermal hydrolysis at 37 °C followed by 95 °C are in excellent agreement to the level of adducts recovered by the FPG. In contrast, the amount of FapyG-NM-G recovered by treatment with FPG is about half the level recovered by two-stage neutral thermal hydrolysis. The FapyG-NM-G appears to be a poorer substrate for FPG than NM-FapyG, and the recovery of FapyG-NM-G did not go to completion at 44 h.

NM-DNA Adduct Formation in MB-231 Cells

Formation of the NM adduct was measured in MDA-MB-231 cells treated with NM (100 μ M) for 24 h. DNA was hydrolyzed by neutral thermal hydrolysis (95 °C for 4 h, or 37 °C for 72 h, followed by hydrolysis of remaining NM adducts bound to DNA at 95 °C for 4 h). The results of NM DNA adduct formation are summarized in Figure 7, and chromatograms of DNA adducts of the untreated cells and NM treated cells as a function of hydrolysis conditions are shown in Supporting Figures S22-S23.

Similar to the data observed with CT DNA modified with NM, greater than 95% of the cationic NM-G adduct was depurinated from DNA over 72 h at 37 °C with little adduct retained to the DNA backbone. In contrast, only a trace of NM-FapyG was recovered in the supernatant following hydrolysis at 37 °C for 72 h with greater than 95% of this adduct being retained in the DNA backbone; NM-FapyG was recovered in the ensuing hydrolysis of DNA at 95 °C. The levels of NM-FapyG are about 20 – 25% of the level of NM-G formation, which is similar to the relative amounts observed in CT DNA treated with NM (1 μ M). The G-NM-G cross-link was the major adduct formed with CT DNA, but the adduct levels were ~25% of the level of NM-G at this single time point in cellular DNA. The FapyG-NM-G cross-link was detected at a level of 6 adducts per 10^7 bases, or < 1% of the level of NM-G. Further studies are required to investigate the levels of NM DNA adducts formed as a function of dose and rate of adduct removal to understand relative levels of NM adduct formation and their persistence in cells and in vivo. These results demonstrate that both NM cross-links and FapyG adducts are formed at appreciable levels in nuclear DNA of MB-231 cells.

Discussion

The chemistry and reactivity of nitrogen^{8-10, 18, 20, 24, 47-56} and sulfur^{44, 57-64} mustards with nucleosides and DNA in vitro is well studied. There are also numerous studies that examine the mustard derived DNA adducts from treated cell culture and animals,^{46,48,50,54,56,59,64,65}

including white blood cells of patients on chemotherapeutic regimens that include a nitrogen mustard.^{53, 57} A variety of methods have been used to identify these adducts including ³²P-postlabeling,^{47, 58} co-chromatography by HPLC with authentic standards,^{8, 9, 24, 48, 57} and mass spectrometry.^{18, 20, 49-52, 56, 59, 64} Recently, tandem mass spectrometry with stable isotope dilution has been employed to identify and quantitate some of these DNA adducts.^{53, 54, 61-63} Collectively, the studies indicate that these electrophiles react predominantly at the N7 atom of dG, although N3-dA modification can also be significant.⁶⁶ The toxicity of mustard agents has been attributed to interstrand DNA cross-links. Interstrand cross-links inhibit DNA replication and transcription by preventing strand separation and thus, selectively induce toxicity and cell death of rapidly proliferating tumor cells over quiescent cells.¹¹ The cationic N7-dG mono-adducts are generally believed to be benign since N7 does not participate in Watson-Crick base pairing; N7-adducts can undergo depurination and the cellular consequence of N7-dG alkylation is often attributed to the apurinic (AP) site or less abundant adducts that arise for reaction at other position, most notably O⁶-dG and N3-dA.⁶⁷⁻⁶⁹ However, some cationic N7-dG adducts can undergo ring-opening to produce stable N⁵-substituted Fapy-dG adducts.⁶⁶ These lesions may persist in vivo, and contribute to the long term genotoxicity of alkylating agents.^{34, 35, 70} N⁵-Substituted FapyG adducts have not been extensively studied, unlike the unsubstituted counterpart derived from oxidative damage.^{71, 72}

The scission of 8,9-bond of the purine ring of guanine to form a ring-opened Fapy adduct was first reported by Hems, who identified 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) in which the formyl nitrogen is unsubstituted, as a major product formed after treatment of guanosine with ionizing radiation.⁷³ Fapy-dG has been identified in oxidized DNA⁷⁴ and also in liver of fish exposed to toxic chemicals.⁷⁵ However, reports on the formation of other ring-opened N⁵-substituted FapyG adducts of genotoxic carcinogens in vivo are few. The major adduct of aflatoxin B₁ (AFB₁) is the cationic N7-G adduct formed by reaction of dG with AFB₁ epoxide.³⁴ A portion of N7-AFB₁-G undergoes ring-opening to form the formamidopyrimidine derivative, AFB₁-FapyG. This adduct persists in vivo and over time becomes the predominant AFB₁-lesion in rodents.³⁴ HPLC with radioactive detection was originally used to measure AFB₁-N7-G and AFB₁-FapyG from the DNA of livers of rodents treated with [³H]-AFB₁. Both adducts were recently characterized by LC/MS².⁷⁶ One study reported the occurrence of N⁵-methyl-formamidopyrimidine (MeFapyG) formation in hepatic DNA of rats treated with radiolabeled N,N-dimethylnitrosamine (DMN), 1,2-dimethylhydrazine, or N-methyl-nitrosourea. The identity of the adduct as MeFapyG was based on HPLC co-chromatography of a synthetic standard with the radiolabeled adduct obtained from rat hepatic DNA.^{35, 77} However, a related study could not detect the MeFapyG lesion from the DNA of rodents treated with DMN.⁷⁸ It was also reported that the C8-dG adduct of the carcinogenic aromatic amines 2-naphthylamine (2-NA) gave a related ring-opened product that was found to be a persistent lesion in urothelium but not in the liver of dogs given 2-NA.⁷⁰

Previous studies of nitrogen and sulfur mustard adducts from their reaction with DNA in vitro, in cultured cells, or in vivo have concentrated on characterizing the N7-G and N3-A mono adducts and their cross-links. One study reported the identification of bis-N7-guanine

cross-links in white blood cells of cancer patients receiving cyclophosphamide therapy,⁵³ and sulfur mustard (bis-(2-chloroethyl)-sulfide, SM) also forms bis[2-(guanin-7-yl)ethyl]sulfide cross-links in tissues of rodents, and urine of rabbits exposed to SM.^{62, 63} The potential formation of ring-opened FapyG adducts was not addressed in those studies. Hoes et al identified the products from the reaction of melphalan with CT DNA by mass spectrometry.⁴⁹ After the modification reaction and enzymatic hydrolysis, a product had a mass consistent with a 5'-p-(melphalan-FapyG)-p-C-3' dinucleotide. Masses consistent with a melphalan-Fapy-dA adduct has also been reported.^{65, 79} However, no further characterization of these products was reported.

The labile N7-G and N3-A nitrogen and sulfur mustard adducts and their cross-links are often thermally hydrolyzed from DNA under neutral^{8-10, 51, 57, 61} or acidic^{9, 10, 24, 46, 48, 50, 52, 53, 59} conditions. Alternatively, enzymatic digestion to the nucleosides has also been employed.^{18, 20, 47, 49, 54, 58, 65} and is necessary for DNA modifications that do not increase base lability (e.g., *O*⁶-dG adducts). Fapy nucleosides can exist as a number of slowly interconverting isomeric constituents such as α - and β -anomers, furanose and hexanose forms of the deoxyribose, geometric isomers of the formamide group, and possible atoisomers.⁸⁰ As a result, Fapy nucleoside adducts often exhibit poor chromatographic properties, which diminish the sensitivity of detection. The advantage of neutral thermal hydrolysis is that the labile adducts are selectively hydrolyzed while leaving the unmodified DNA in tact, allowing for its facile removal by precipitation and/or size exclusion filtration.

The data show the cationic NM-G and G-NM-G adducts can be converted to the NM-FapyG and Fapy-G-NM-G adducts at elevated temperature and neutral pH. The artifactual FapyG formation was mitigated by hydrolysis of cationic NM-G and G-NM-G adducts present in DNA under physiological conditions (pH 7.0, 37 °C, 72 h) prior to high temperature neutral thermal hydrolysis of the ring-opened FapyG adducts.

Our preliminary data show that NM forms high levels of DNA adducts in MDA-MB-231 cells. The relative amount of NM-FapyG to NM-G was comparable to that observed in vitro with CT DNA modified with NM. In contrast, the relative amount of G-NM-G to total NM DNA adducts was considerably lower in cells than in CT DNA. The lower amounts of G-NM-G formed in cells may be attributed to differences in reactivity of the NM in the nuclear genome where the DNA is packaged with histones versus commercial DNA, which has nearly all of protein removed during DNA isolation. Additionally, the cellular packaging of DNA is likely to prevent non-specific and inter-helix cross-linking, which may occur in the reaction of DNA in solution. We have also shown that the FapyG-NM-G cross-link is formed in cells and may contribute to the cytotoxicity of NM along with NM-G-NM.

Previously reported comparative quantitative measurements of cationic NM-G and its cross-linked adducts by MS are limited. Ganesan and Keating reported that the treatment of rat ovarian cells with phosphoramidate mustard (6 μ M) resulted in the formation of N-(2-hydroxyethyl)-N-[2-(N7-guaninyl)ethyl]-amine (NOR-G-OH) and the cross-linked adduct N,N-bis[2-(N7-guaninyl)-ethyl]amine (G-NOR-G) at levels of several adducts per 10⁵ DNA bases.⁵⁶ Wang et al reported that the SM (100 μ M) also forms the structurally related N7-[2-

[(2-hydroxyethyl)thio]ethyl]-guanine (N7-HETEG) and bis[2-(guanin-7-yl)ethyl]-sulfide (Bis-G), among other adducts, as the biomarkers for DNA damage in rodents and human keratinocytes, hepatocytes and lung fibroblasts.⁶⁴ Very high levels N7-HETEG and G-SM-G cross-link (> 4 adducts per 10^5 bases) were formed in all cell lines. The $t_{1/2}$ values of N7-HETEG and its cross-link showed no significant differences among the liver, lungs, and skin-derived cells. The relative amount of cross-link to N7-HETEG was $>20\%$ in all cell lines.

The NM-FapyG adduct was shown to be a good substrate for FPG.³⁸ As expected, the NM-FapyG adduct was released when the NM-modified CT DNA was treated with FPG. The NM-FapyG level increased over the course of 72 h to 8.6 ± 1.0 adducts per 10^7 DNA bases, which compares favorably to the amount of adduct recovered under neutral thermal hydrolysis conditions. An unexpected result was that the level of the FapyG-NM-G cross-link adduct also increased over time to 1.5 ± 0.2 adducts per 10^7 bases after 72 h, indicating that this adduct is a substrate for FPG. The hydrolytic mechanism of glycosylases involves flipping the modified base out of the DNA helix and into the enzyme active site, which is likely to be inhibited by a cross-link. We therefore propose that the substrate for FPG is the Fapy-dG-NM-G mono-adduct where the cationic guanine has depurinated rather than the intact cross-link. In the case of interstrand cross-links, depurination of the cationic G followed by excision of the FapyG portion by a DNA glycosylase (e.g., NEIL1 or OGG1) could lead to highly cytotoxic double strand breaks (Scheme 2). The over-expression of FPG in human cultured cell was shown to have a modest (2-fold) protective effect against mafosfamide.³⁰ While removal of the mafosfamide-FapyG mono-adduct is expected to be protective, repair of the much less abundant FapyG-mafosfamide-G adduct from hemi-depurinated interstrand cross-link would lead to double strand breaks and likely to enhance cytotoxicity. Similarly, depurination of the bis-cationic N7-G cross-link followed by base excision repair of the hemi-depurinated G-NM-G adduct (e.g, N-methylpurine glycosylase, MPG) would also lead to double strand breaks. This is related to the observation that overexpression of MPG sensitized cells towards methylating agents.⁸¹ Defects or inhibition of homologous recombination has been shown to sensitize cells to mustard agents.⁸²⁻⁸⁴ An agent that could catalyze strand scission of an AP site would also be expected to enhance the therapeutic index of mustards and other DNA alkylating agents.⁸⁵

Conclusions

We have developed an analytical protocol that allows simultaneous detection and quantitation of five dG adducts of a nitrogen mustard. The three FapyG adducts have not been fully characterized or previously quantitated, and two cross-links (FapyG-NM-G and FapyG-NM-FapyG) were previously unknown. The level of FapyG-NM-FapyG formed was observed at the LOD and its formation in cells is likely to be well below the LOD. NM-FapyG and FapyG-NM-G were observed in cultured MDA-MB-231 cells treated with NM suggesting that the contribution of these lesions to NM genotoxicity should be considered. With our validated analytical methodology, we will explore the relative contribution of ring-opened FapyG adducts of NM and their persistence in cell lines and experimental animals models. Such studies may provide further understanding about the contribution N^5 -

substituted Fapy-dG lesions in the carcinogenicity of DNA alkylating agents and secondary tumor development from chemotherapeutic alkylating agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

NM	bis-(2-chloroethyl)ethylamine
CT DNA	calf thymus DNA
Fapy	formamidopyrimidine
FPG	formamidopyrimidine DNA glycosylase
LOD	level of detection
LOQ	level of quantitation
CV	coefficient of variation
SM	bis-(2-chloroethyl)sulfide

Figure 1.
Early chemotherapeutic nitrogen mustard.

Figure 2.
Guanine adducts of a bis-(2-chloroethyl)ethylamine

Figure 3.

Neutral thermal hydrolysis of the NM-modified CT DNA for 4 h at 95 °C. (A) Untreated CT DNA and (B) DNA modified with NM (1 μ M). Internal standards were added at a level ranging between 53 and 87 adducts per 10^7 bases. The retention time (t_R) and area of peak integration (A) are reported.

Figure 4.

Time course of neutral thermal hydrolysis and recovery of NM-G adducts as a function of temperature (70 or 95°C) and time.

Figure 5.

(A) Time course of the neutral thermal hydrolysis and recovery of the NM-G adducts in the supernatant as a function of time at 37 °C, (B) followed by neutral thermal hydrolysis of remaining NM-modified DNA at 95 °C for 4 h. The level of FapyG-NM-FapyG was below the limit of quantification (LOQ).

Figure 6.
FPG Assay with CT DNA Modified with NM (1 μ M)

Figure 7.

NM DNA adducts in MDA-MB-231 cells treated with NM (100 μ M) for 24 h. Isolated DNA was hydrolyzed as described in Fig. 5 (N = 4 and reported as the average and SD of 2 independent experiments performed in duplicate). Levels of FapyG-NM-G are reported with the right Y scale, all other adducts are reported with the left Y scale

Scheme 1.

Scheme 2.

Table 1

ESI/MS³ parameters for NM-G adducts. The activation time for all analyses was 10 ms.

MS ⁿ	Adduct	<i>m/z</i>	Isolation width (<i>m/z</i>)	CE	Activation Q	Ions monitored at MS ³ scan stage
MS ²	NM-G	267.2	3	35	0.25	72.1, 98.1
MS ³		116.1	2	40	0.35	
MS ²	[² H ₅]-NM-G	272.2	3	35	0.25	77.1, 103.1
MS ³		121.1	2	40	0.35	
MS ²	NM-FapyG	285.2	3	35	0.35	168.1
MS ³		196.1	2	35	0.35	
MS ²	[⁵ N ₁₅]-NM-FapyG	290.2	3	35	0.35	173.1
MS ³		201.1	2	35	0.35	
MS ²	G-NM-G	400.2	2	35	0.30	178.1, 234.2
MS ³		249.1	2	32	0.32	
MS ²	[² H ₅]-G-NM-G	405.2	4	35	0.30	178.1, 239.2
MS ³		254.2	2	32	0.32	
MS ²	FapyG-NM-G	418.2	3	32	0.30	196.1
MS ³		267.1	3	30	0.30	
MS ²	[² H ₅]FapyG-NM-G	423.3	4	32	0.30	196.1
MS ³		272.2	2	30	0.30	
MS ²	FapyG-NM-FapyG	436.2	3	35	0.30	164.1
MS ³		223.1	2	40	0.30	

Table 2
Performance of the Method: CT DNA Modified with NM (1 μ M) With-day and Between-day Reproducibility.^{a,b}

Adduct/10 ⁷ bases		Day 1 (pH7)	Day 2 (pH7)	Day 3 (pH7)	CV (%) within-day	CV (%) between-day
NM-G	Mean	20.7	19.4	19.9		
	SD	1.7	0.2	1.3		
	RSD (%)	8.3	1.1	6.7	6.4	6.2
G-NM-G	Mean	132	118	127		
	SD	14.1	1.3	9.7		
	RSD (%)	10.6	1.1	7.5	8.2	9.1
NM-FapyG	Mean	19.9	18.8	19.2		
	SD	1.1	0.2	1.1		
	RSD (%)	5.7	1.0	5.4	4.8	5.2
FapyG-NM-G	Mean	3.8	3.7	3.7		
	SD	0.2	0.1	0.2		
	RSD (%)	6.7	1.3	7.4	5.9	5.5

^a CT DNA (50 μ g/mL in potassium phosphate (50 mM, pH 7.0) was subjected to neutral thermal hydrolysis at 95 °C for 4 h.

^b FapyG-NM-FapyG was below the LOQ value (0.3 adduct per 10⁷ bases) and not reported.

Table 3
Mean Levels of NM Adduct for CT DNA as a Function of Neutral Thermal Hydrolysis Conditions ^a

	95 °C (4 h)	37 °C (72 h) adducts in supernatant	95 °C (4 h) after 37 °C (72 h)	Sum of adducts 37 °C (72 h) and 95 °C (4 h)	FPG
NM-G	24.0 ± 1.6	33.8 ± 2.0	2.2 ± 0.4	36.0 ± 1.9	33.7 ± 3.0
G-NM-G	102 ± 3.0	75.7 ± 8.7	23.0 ± 4.8	98.7 ± 7.7	99.2 ± 12.3
NM-FapyG	23.1 ± 1.8	0.8 ± 0.1	6.1 ± 0.7	6.9 ± 0.7	8.6 ± 1.0
FapyG-NM-G	3.6 ± 0.3	0.3 ± 0.1	3.1 ± 0.6	3.4 ± 0.6	1.5 ± 0.2

^a, 3 independent measurements (Mean ± SD)