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Quantification of DNA interstrand crosslinks induced by ACNU in NIH/3T3 and L1210 cells using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

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RATIONALE: Chloroethylnitrosoureas (CENUs) are important alkylating agents employed for the clinical treatment of cancer. The cellular toxicity of CENUs is primarily due to induction of DNA interstrand crosslinks (ICLs), which has been characterized as l-(3-deoxycytidyl), 2-(l-deoxyguanosinyl)ethane (dG-dC). However, the formation of dG-dC crosslinks can be prevented by O^6-alkylguanine-DNA alkyltransferase (AGT), which removes the O^6-chloroethyl group from O^6-chloroethylguanine (O^6-ClEt-Gua), and ultimately its increased expression can result in drug resistance. Differing levels of AGT expression can lead to varying amounts of dG-dC crosslinking, which influences the sensitivity of cells to CENUs.

METHODS: In this work, a sensitive method for the quantitation of dG-dC crosslinks in cellular DNA has been established using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS).

RESULTS: The limit of detection (LOD) and limit of quantitation (LOQ) of the method were determined to be 2 fmol and 8 fmol on-column, respectively, and the recovery ranged from 96% to 105% with the relative standard deviation (RSD) below 5%. Using this method, the levels of dG-dC crosslink induced by 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) were determined in NIH/3T3 fibroblasts cells (high level of expression of AGT) and L1210 leukemia cells (low level of expression of AGT). The time-course profile indicated that the levels of dG-dC crosslink uniformly increased in the early incubation period and reached the maximum at 12 h. Subsequently, the amount of dG-dC crosslinking decreased to very low levels presumably owing to the repair of O^6-ClEt-Gua by AGT. The crosslinking levels in L1210 cells were significantly higher than those in NIH/3T3 cells at each time point. This provides strong evidence that high express of AGT in CENU-resistant cells inhibits the formation of dG-dC crosslinks.

CONCLUSIONS: This work will contribute to the further understanding of the drug resistance of CENUs, and will provide a means to evaluate the anticancer activity of new bifunctional anticancer agents.

Chloroethylnitrosoureas (CENUs) are an important family of alkylating agents widely used in the clinical treatment of various cancers, including lymphomas, melanomas and brain tumors.[1–4] As shown in Fig. 1, carmustine (BCNU), lomustine (CCNU), semustine (MeCCNU), nimustine (ACNU) and fotemustine (FTMS) represent the typical CENU chemotherapeutics. CENUs are unstable under physiological conditions and spontaneously undergo decomposition to chloroethyldiazohydroxides followed by further degradation to diazonium ions.[5–7] Diazonium ions are very active electrophilic agents, which can directly alkylate DNA and induce various types of DNA damage, including double/single strand breaks and interstrand/intrastrand crosslinks. The formation of DNA interstrand crosslinks (ICLs) is a crucial damage mechanism involved in the anticancer effect of CENUs.[1,3,8–10] Previous investigations indicated that the ICLs induced by CENUs mainly formed between guanine and the complimentary cytosine.[11] As shown in Fig. 2, the mechanism for the formation of dG-dC crosslinks was proposed whereby the chloroethyl diazonium ion originating from CENUs reacted with guanine at the O^6 site to form O^6-chloroethylguanine (O^6-ClEt-Gua) followed by further alkylation of the complimentary cytosine on the N3 site via a cationic intermediate, N1,O^6-ethanoguanine.[10,12] As a result, this covalent crosslink interferes with normal DNA replication by preventing the separation of the double strands, which finally leads to the cytotoxicity of CENUs.

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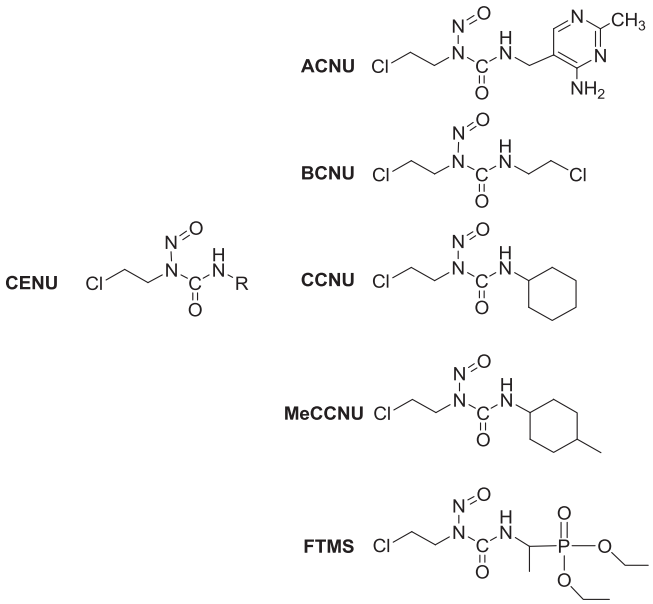

Figure 1. Typical CENU chemotherapeutics used in the clinical treatments of cancer.

To understand the anticancer mechanism of CENUs and develop more efficient chemotherapeutics, *in vitro* and *in vivo* studies have been performed to investigate the CENUs-induced DNA ICLs. Fischhaber *et al.*[12] identified dG-dC crosslinking induced by *N,N'*-bis(2-chloroethyl) nitrosourea (BCNU) using synthetic oligonucleotides and provided the first direct evidence that BCNU had no strong sequence preference for interstrand crosslinking. Subsequently, Bodell and colleagues[13] demonstrated that there was a significant correlation between LD_{10} of CENUs and the levels of dG-dC crosslinks, which suggested that the level of the dG-dC crosslink could be used as a molecular dosimeter for the therapeutic response after treatment with CENUs. Ueda-Kawamitsu *et al.*[14] measured the time course of DNA ICLs in L1210 cells treated with BCNU. The results showed that the percentage of crosslinks reached the maximum after 6 h exposure and subsequently decreased presumably because of DNA repair. Cloretazine, which is a relatively new prodrug with a similar molecular structure to CENUs, was designed and synthesized by Sartorelli's group.[15] They treated leukemia cells with cloretazine and found that its therapeutic action was largely due to the production of dG-dC crosslinks. Due to the high sensitivity and specificity, mass spectrometry has been frequently employed for the determination of DNA adducts in biological samples. Wang's group[16–18] reported the application of liquid chromatography/tandem mass spectrometry (LC/MS/MS) in assessing the levels of ICLs and monoadducts in human cells treated by psoralen and various derivatives. Malayappan *et al.*[19] investigated the formation and repair of the *N,N*-bis [2-(N7-guanyl)ethyl]amine crosslink in human blood induced by cyclophosphamide using LC/MS/MS. Paz *et al.*[20] used LC/MS/MS to map the DNA adducts, including interstrand and intrastrand crosslinks, in different cell lines exposed to mitomycin C and derivatives. In our previous work,[21,22] dG-dC crosslinks induced by MeCCNU in calf thymus DNA and in synthetic oligonucleotides were determined by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS). The results indicated that the dG-dC crosslink level stayed relatively low during the first 2 h of treatment and then underwent an obvious increase, which suggested that the reaction was initiated by the formation of the monoadduct followed by the second alkylation on the complementary strand of DNA to form the crosslink. This provided convincing evidence for the crosslinking mechanism proposed in our theoretical studies.[23,24]

As an important family of alkylating agents, CENUs have been widely employed as chemotherapeutics against gliomas, lymphoma and solid tumors since *N*-methyl-*N*-nitrosourea was found to have anticancer activity in 1960s. However, the clinical efficacy of CENUs is greatly limited by drug resistance. Previous studies revealed that O^6-alkylguanine-DNA alkyltransferase (AGT), which acts as a single agent to directly remove the alkyl groups located on the O^6-position of guanine from DNA, provides a powerful resistance mechanism to cancer chemotherapy.[25–30] AGT prevents the formation of CENUs-induced DNA ICL by transferring the chloroethyl groups from O^6-ClEt-Gua to an active-site cysteine residue.[25,31,32] In addition, AGT reacts with the cyclic intermediate $N1,O^6$-ethanoguanine[33,34] to prevent crosslink formation. For understanding the physiological role

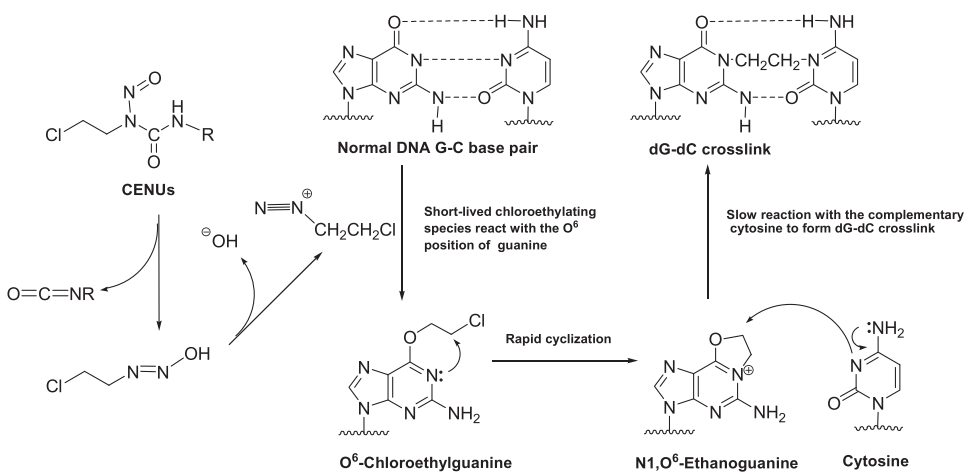

Figure 2. Supposed mechanism for the formation of dG-dC crosslinks induced by CENUs.

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of AGT in drug resistance, a number of cellular experiments were performed by treating various cell lines with CENUs. Melanoma-transfected cells with high AGT level were found to be significantly less sensitive to carmustine and fotemustine.[35] Erickson *et al*.[36] measured the levels of DNA ICL and DNA-protein crosslinks in CENU-treated human cell strains from malignant tumors using alkaline elution assays. They found that the strains deficient in AGT (Mer^- phenotype) produced consistently higher levels of DNA ICL than did the Mer^+ strains. Also using alkaline elution, Bodell *et al*.[37] determined the number of DNA ICLs formed in BCNU-treated cells. The number of DNA ICLs formed in BCNU-resistant cells 9L-2, HU-188, and HU-252-2 are approximately 50% that of the corresponding sensitive cell lines 9L and HU-126. All these studies demonstrated that the formation of DNA ICL induced by CENUs was closely related to the AGT levels in different cell lines. In this work, the levels of dG-dC crosslinks were determined in L1210 leukemia cells (Mer^-) and NIH/3T3 fibroblasts cells (Mer^+) treated with ACNU using HPLC/ESI-MS/MS. The purpose of this study is to establish a highly sensitive quantitation method for DNA ICLs in cells, which can be used to evaluate the anticancer activity and drug resistance of CENUs. We expect this work will contribute to the development of novel bifunctional alkylating anticancer agents with high efficiency and low toxicity.

EXPERIMENTAL

Chemicals and materials

ACNU, acetonitrile (HPLC grade), 2′-deoxyguanosine, 2′-deoxycytidine and phosphodiesterase I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nuclease S1, alkaline phosphatase (CIAP) and deoxyribonuclease I were obtained from TaKaRa Biotechnology (Tokyo, Japan). $^{15}N_3$-2′-Deoxycytidine was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals, reagents and solvents were purchased from Sigma-Aldrich. Microcon YM-10 centrifugal columns were purchased from Millipore (Billerica, MA, USA). Deionized water was purified by a PALL deionizer.

Mouse lymphoid leukemia L1210 cells were purchased from Cell Center of Peking Union Medical College. The NIH/3T3 fibroblasts cells were a gift from the Chinese National Institute for Viral Disease Control and Prevention. All cell culture media and reagents were obtained from Peking Union Medical College.

Synthesis of the standards

The synthesis of [l-(3-deoxycytidyl),2-(l-deoxyguanosinyl) ethane (dG-dC) was carried out according to a previously published method, with some modifications.[11] Briefly, N^2,3′,5′-triacetyl-2′-deoxyguanosine was produced from the reaction of 650 mg (2.3 mmol) of 2′-deoxyguanosine with 2.4 mL (26 mmol) of acetic anhydride, 30 mg (0.25 mmol) of 4-dimethylaminopyridine, and 3.8 mL (27 mmol) triethylamine in 100 mL of dry pyridine at 50°C for 20 h. After crystallization and filtration, the obtained residue was dissolved in 6 mL of dry dioxane followed by incubation with 1260 mg (4.8 mmol) of triphenylphosphine, 750 μL (4.8 mmol) of diethylazodicarboxylate, and 312 μL (4.8 mmol) of 2-fluoroethanol dioxane at room temperature for 1 h. Then 30 mL of 5% $NaHCO_3$ solution were added. The mixture was stirred at room temperature for 15 min. The product was extracted three times with 50 mL of methylene chloride. The oily residue was dissolved in 10 mL of methanol after evaporation of CH_2Cl_2. After the addition of 60 mL of concentrated ammonium hydroxide, the mixture was kept at 60°C for 3 h. After evaporation of methanolic NH_4OH, the product, O^6-(2-fluoroethyl)-2′-deoxyguanosine (O^6-FEt-dGuo), was dissolved in methanol and purified by silica gel column chromatography (200–300 mesh) using ethyl acetate and petroleum ether as the solvent. The obtained O^6-FEt-dGuo was used as the material for the following synthesis of dG-dC crosslinks. O^6-FEt-dGuo (20 mg, 64 μmol) was mixed with 2′-deoxycytidine (5 mg, 22 μmol). The mixture was suspended in DMSO (100 μL) at 55°C for 20 days. HPLC purification with fraction collection of dG-dC was performed using a 4.6× 250 mm ZORBAX SB-C18 column (5 μm particle size) and a 10 mM ammonium acetate buffer (0.1% acetic acid, pH 6.8) (A) and acetonitrile (B) at a flow rate of 1 mL/min. A gradient of 5 to 10% B in 20 min was employed with a linear gradient to 30% B over 10 min with a 3 min isocratic wash at 30% B. The fraction during 20 to 22 min was collected. UV detection was performed at 258 nm. The synthesis of the internal standard, $^{15}N_3$-dG-dC, was carried out using the same procedure as the unlabeled dG-dC except $^{15}N_3$-2′-deoxycytidine was used in the final step. The final product was characterized by NMR, MS, IR and UV spectroscopy. The data were consistent with the results obtained previously.[11]

Cell culture

Murine lymphoid leukemia L1210 cells were grown, as suspension cultures, in Dulbecco's modified Eagle's medium (DMEM) high glucose medium supplemented with 10% (v/v) horse serum (HS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C under a 5% CO_2 atmosphere. The mouse embryonic fibroblast cell line NIH/3T3 was routinely sub-cloned and then cultivated in DMEM high glucose medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in an air atmosphere containing 5% CO_2. Cells were passaged as required every 2–3 days. The culture medium was discarded when cells achieved a saturation density of 1–2× 10^6 cells/mL. Afterwards, the HS-free and FCS-free medium containing ACNU was supplemented.

Treatment of NIH/3T3 and L1210 cells with ACNU

ACNU was dissolved in deionized water immediately prior to use and directly added to fresh culture medium to achieve final concentrations of 0.2, 0.6 and 1 mM, respectively. Cells were incubated in freshly prepared culture solutions containing ACNU at 37°C for various times of treatment (3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h and 24 h). NIH/3T3 cells were trypsinized after the treatment. Cells were collected by centrifugation followed by washing with phosphate-buffered saline (PBS). The obtained cell pellets were kept at –20°C for

DNA extraction. For each time point, the control cells were cultured under the same conditions as the treated cells without addition of ACNU to the culture medium.

DNA extraction from cell samples

DNA extraction from cell pellets was performed following the previously reported procedures with modifications.[38–40] Typically, L1210 and NIH/3T3 cells were homogenized in 10 mL of lysis buffer (10 mM Tris-HCl, 150 mM NaCl, and 10 mM EDTA, pH 8.0) with addition of 60 μL of proteinase K solution (20 mg/mL), shaking at 37°C for 12 h. The mixture was extracted with phenol/chloroform/isoamyl alcohol solution (25:24:1, v/v/v) twice. Then the supernatant containing DNA was collected and the DNA was precipitated by addition of 20 mL of ice-cold ethanol followed by centrifugation at 12000 rpm for 5 min. The DNA pellets were washed with 70% ethanol and then 100% ethanol. All the DNA samples were dried with a stream of nitrogen and stored at –20°C until enzymatic hydrolysis.

Enzymatic hydrolysis of DNA

The purity and concentration of the DNA samples were determined before enzymatic hydrolysis. The obtained DNA pellets were redissolved in 1 mL of Tris-HCl buffer (10 mM, pH 7.0). The purity of DNA was confirmed by measuring UV absorption at 230, 260 and 280 with the ratio of 260/230 and 260/280 at 2.4 and 1.8, respectively. The concentration of the DNA solution was determined by UV absorbance at 260 nm. One OD_{260nm} corresponded to approximately 50 μg/mL for double-stranded DNA.

Following the previously reported protocols,[21,22] DNA samples were digested by four enzymes, including DNase I, nuclease S1, alkaline phosphatase and snake venom phosphodiesterase. Briefly, the solutions were heated at 98°C for 5 min and promptly chilled in an ice-bath for 10 min. Each solution (200 μL) was hydrolyzed using 150 units of DNase I (30 μL, buffered in CH_3COONa 20 mM, NaCl 150 mM, pH 5.0) and 500 units of nuclease S1 (20 μL, buffered in CH_3COONa 10 mM, NaCl 150 mM, $ZnSO_4$ 0.05 mM, pH 4.6). After incubation at 37°C for 6 h, the mixture was further incubated overnight at 37°C with the addition of 40 units (40 μL) of alkaline phosphatase and 1 milliunit of phosphodiesterase I (10 μL) buffered in Tris-HCl 500 mM, $MgCl_2$ 10 mM (pH 9.0). Finally, the DNA samples were filtered with molecular weight centrifugal filters (Microcon YM-10) for HPLC/ESI-MS/MS analysis. A buffer control without DNA was prepared for each set of samples and processed as negative controls following the same procedure.

Quantitation of dG-dC crosslinks by HPLC/ESI-MS/MS

HPLC/ESI-MS/MS analysis was carried out on a Thermo TSQ Quantum Discovery MAX triple quadrupole tandem mass spectrometer interfaced with a HPLC system (Thermo Finnigan, San Jose, CA, USA). The electrospray ionization (ESI) was performed in positive mode. Chromatography was performed with a 2.1×150 mm (5 μm particle size) Zorbax SB-C18 column (Agilent Technologies, Palo Alto, CA, USA) and a flow rate of 0.1 mL/min. The injection volume was 25 μL. The mobile phase consisted of deionized water with 0.01% acetic acid (solvent A) and acetonitrile (solvent B). The initial elution step was 2% B for the first 5 min. The gradient was linearly increased to 80% B in 25 min and held at that composition for 3 min. Then the solvent composition was brought back to the initial composition of 2% B in the next 2 min and equilibrated for 30 min. The instrumental parameters of the mass spectrometer were set as follows: spray voltage 4000 V; sheath gas (nitrogen) pressure 50 psi; auxillary gas (nitrogen) pressure 15 psi; capillary temperature 300°C; and tube lens offset 89 V. Collision energy was 20 eV with an argon pressure of 1.0 mTorr. The source collision-induced dissociation (CID) was set to 8 V. The fragmentation patterns of dG-dC and $^{15}N_3$-dG-dC are shown in Fig. 3. The amounts of dG-dC crosslink were quantified by selecting reaction monitoring (SRM) with the transition of *m/z* 521 → 289 for dG-dC and 524 → 292 for $^{15}N_3$-dG-dC.

According to previous work,[23,24] the level of dG-dC crosslink in DNA from cells were reported as the number of crosslinked dG and dC in every 10^9 base pairs calculated by Eqn. (1). In Eqn. (1), C refers to the determined molar concentrations of the dG-dC crosslink; V refers to the volume of the enzymatic digestion solution, which is 300 μL; C_0 is the concentration of the DNA duplexes; V_0 is the volume of the DNA sample before enzymatic digestion, which is 200 μL; and M_0 represents the average molecular weight of the four deoxynucleotides (325 g/mol):

$$\text{dG-dC crosslinks}/10^9 \text{ base pairs} = (C \times V \times 10^9)/(C_0 \times V_0/2M_0) \quad (1)$$

RESULTS AND DISCUSSION

Method validation

The synthesized dG-dC and isotope-labeled $^{15}N_3$-dG-dC were purified by HPLC and identified by MS employing ESI in positive mode. As shown in Fig. 3, the $[M+H]^+$ ion of dG-dC (*m/z* 521) yields two fragment ions at *m/z* 405 and 289 after losing one and two deoxyribose moieties, respectively. A transition of *m/z* 521 → 289 was selected for SRM quantitative analysis of dG-dC crosslinks because *m/z* 289 is the dominant fragment ion. Similarly, neutral loss of deoxyribose leads to two fragment ions of *m/z* 408 and 292 from the $[M+H]^+$ ion of $^{15}N_3$-dG-dC (*m/z* 524), and the transition *m/z* 524 → 292 was monitored for the internal standard $^{15}N_3$-dG-dC. Under the HPLC conditions used here, dG-dC and $^{15}N_3$-dG-dC coeluted at 22.6 min (see Fig. S1, Supporting Information).

The standards were prepared by mixing the synthesized dG-dC (0.32, 1.6, 8, 16, 32 and 60 nM) with a fixed amount of $^{15}N_3$-dG-dC (9.6 nM) in deionized water. They were subjected to HPLC/ESI-MS/MS analysis as described above for quantitative analysis. The calibration curve was constructed by plotting the corresponding SRM peak area ratios of dG-dC/$^{15}N_3$-dG-dC versus the corresponding concentration ratios. As shown in Fig. 4, the calibration curve was linear over the range from 0.32 to 60 nM with a correlation coefficient (R^2) of 0.9999.

The sensitivity, accuracy and specificity of the method were validated by determining the limit of detection (LOD), limit of quantification (LOQ), recovery and analysis of control

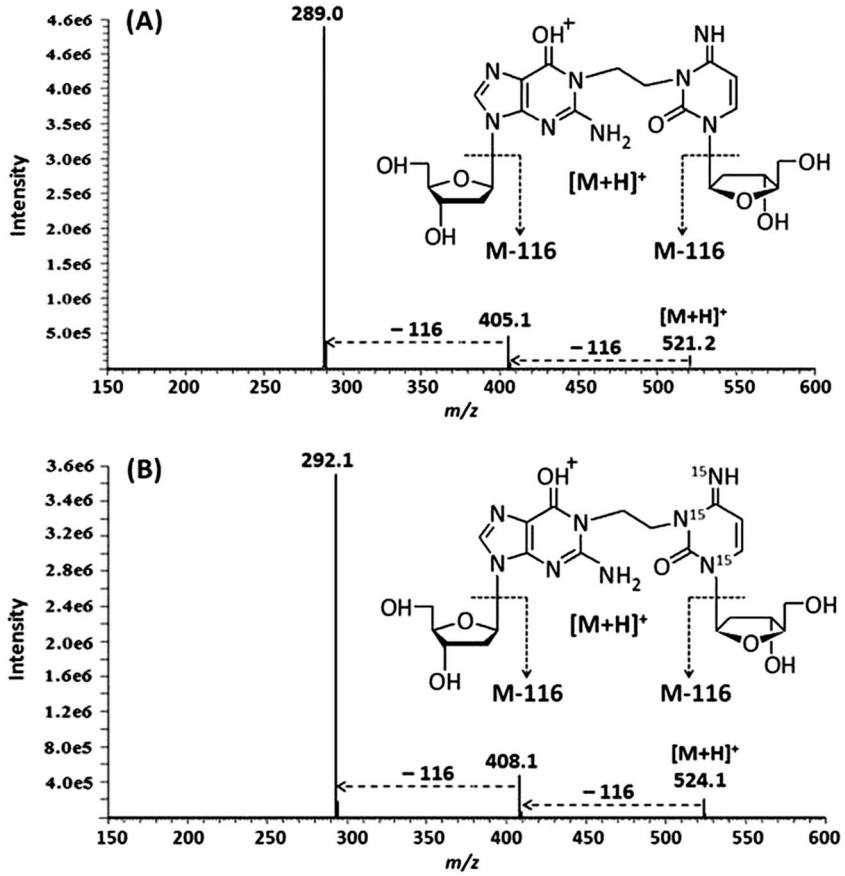

Figure 3. Positive ESI product ion spectra of the $[M+H]^+$ ions of dG-dC (A) and $^{15}N_3$-dG-dC (B).

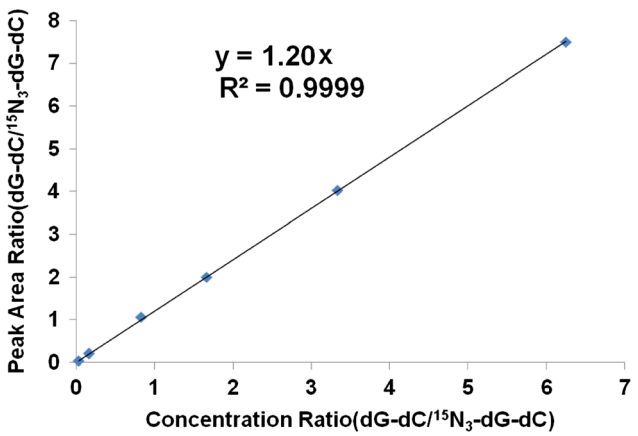

Figure 4. Calibration curve of dG-dC crosslinks by plotting SRM peak area ratios versus the concentration ratio between dG-dC and $^{15}N_3$-dG-dC.

samples to measure any contamination or artifactual formation of dG-dC. The LOD and LOQ were estimated at the levels giving signal-to-noise ratios (S/N) of 5 and 15, respectively. The LOD and LOQ for a dG-dC crosslink standard spiked in calf thymus DNA were determined to be 2 and 8 fmol on-column, respectively. In order to evaluate the influence of sample preparation and matrix effects on the measured results, the recovery of dG-dC in the hydrolysates was studied using calf thymus DNA spiked with dG-dC and $^{15}N_3$-dG-dC. Appropriate amounts of dG-dC were prepared in the calf thymus DNA solution at the concentration of 2, 4, 8 and 16 nM with a fixed $^{15}N_3$-dG-dC concentration of 9.6 nM to obtain the quality control (QC) samples. Enzyme hydrolysis was conducted for the QC samples. The resulting mixtures were subjected to HPLC/ESI-MS/MS analysis. As summarized in Table 1, the mean recovery was demonstrated to fall within the range of 96 to 105%. The precision of the assay was evaluated by determining the relative standard deviation (RSD). Each QC sample was analyzed in triplicate. The measured RSD levels were below 5% for all samples. The measured recovery and precision indicated that the influence of sample preparation and matrix effects is negligible on the obtained results of the dG-dC concentration. Figure 5 shows the SRM chromatograms of dG-dC and $^{15}N_3$-dG-dC in the DNA enzymatic hydrolysates

Table 1. Recovery of the dG-dC quantitation method

Added concentration (nmol/L)	Determined concentration (nmol/L) (n = 3)	Recovery (%)	RSD (%)
2	1.9	96	3.7
4	4.0	100	4.3
8	8.4	105	2.9
16	15.9	99	3.9

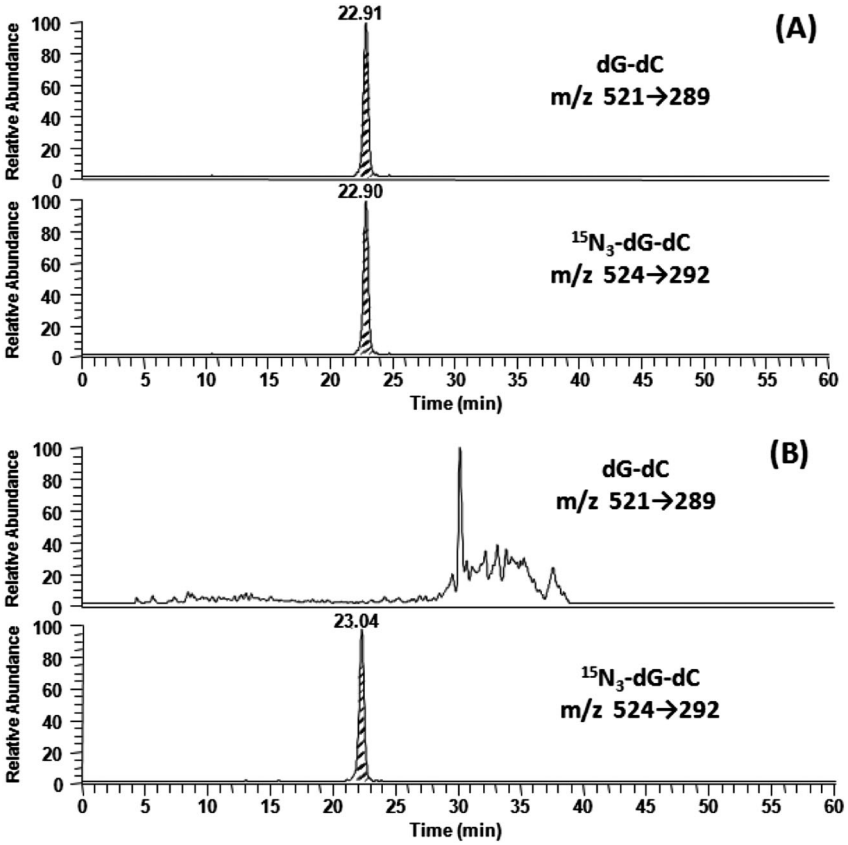

Figure 5. SRM chromatograms of dG-dC crosslinks in the DNA digestion mixtures from cells treated with 0.6 mM ACNU for 12 h (A) and from control cells (B).

from the cells treated with 0.6 mM ACNU for 12 h (Fig. 5(A)) and from the control cells (Fig. 5(B)). In Fig. 5(A), the retention time for dG-dC in the digestion mixture is 22.9 min, and its corresponding isotope-labeled standard, $^{15}N_3$-dG-dC, has the same retention time. Figure 5(B) indicates that there is no signal detected with the SRM transition for dG-dC in the DNA hydrolysates from the control cells. This indicates that there is no significant matrix interference or contamination in the analyte channels from internal standards or hydrolytic enzymes, so the specificity of the method was acceptable.

Quantitation of dG-dC crosslinks in the hydrolysates of ACNU-treated NIH/3T3 and L1210 cells

To provide insight into the relationship between crosslinking level and cellular sensitivity to CENUs, the established quantitation method was employed to determine the dG-dC crosslink levels in DNA extracted from mouse leukemia L1210 cells and fibroblast NIH/3T3 cells treated with ACNU. The determined time courses for the levels of dG-dC crosslink are indicated in Fig. 6, and the corresponding values of the crosslinking level at each time point were summarized in Supplementary Table S1 (Supporting Information). In both L1210 and NIH/3T3 cells, the levels of dG-dC crosslink displayed dose-dependence with increasing ACNU concentrations from 0.2 to 1 mM. For the treatments at three different ACNU concentrations, the crosslinking levels showed a common increasing trend from 0 to 12 h, and reached a maximum at 12 h with 359, 594, and 895 dG-dC crosslinks/10^9 base pairs in NIH/3T3 cells at the ACNU concentrations of 0.2, 0.6 and 1 mM, respectively. In L1210 cells, the crosslinking levels also reached the maximum at 12 h with 986 and 1306 dG-dC crosslinks/10^9 base pairs at the ACNU concentrations of 0.6 and 1 mM, while at 0.2 mM ACNU the maximum was reached at 9 h with 490 dG-dC crosslinks/10^9 base pairs. Subsequently, the crosslinking levels decreased to very low levels in both the cell lines. The crosslinking levels at 24 h fell back to levels lower than those at 3 h. Most notably, in NIH/3T3 cells the crosslinking level was below the detection limit at 24 h and an ACNU concentration of 0.2 mM. The decrease in the dG-dC crosslinking levels reflected the repair of alkylated guanine by AGT. The decrease in the crosslinking level is also possibly due to death of the cells containing high levels of DNA damage. Moreover, besides the AGT-mediated repair mechanism, there are other pathways repairing the crosslinked base pair, such as the base excision repair (BER) processing, in which the BER proteins are capable of recognizing and removing the crosslinked base pairs induced by chemotherapeutic agents.[41]

Ueda-Kawamitsu *et al.*[14] performed a quantitative analysis of BCNU-induced dG-dC crosslinks in L1210 leukemia cells by fluorescence assay with BCNU exposure for 24 h. The results indicated that the maximum level of DNA ICL was observed at 6 h followed by a decline, which was attributed to the repair mechanism. Both of the two studies gave direct evidence for the AGT-mediated repair of DNA ICLs by observing obvious decreases in the crosslinking levels, although different peak times of the crosslinking levels

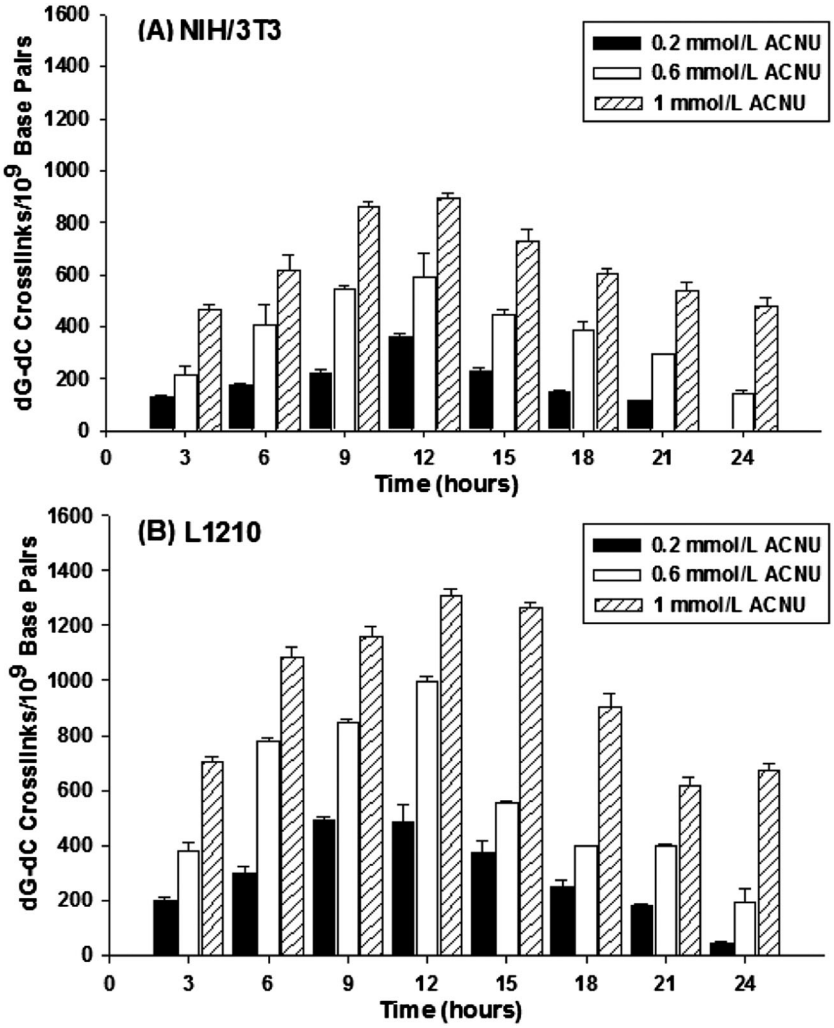

Figure 6. Profiles of dG-dC crosslinks in NIH/3T3 cells (A) and in L1210 cells (B) treated with ACNU for 0–24 h.

were observed. This difference may be due to a different CENU being used in treatment of the cells. Further studies of dG-dC crosslink levels in cells induced by ACNU, BCNU and other CENUs, which have different crosslinking activities and half-lives, are being performed in our laboratory.

As shown in Fig. 6, there were significant differences ($P < 0.05$) between the dG-dC levels in the DNA from L1210 cells and NIH/3T3 cells. At each time point, the levels of dG-dC crosslink were higher in L1210 cells than in NIH/3T3 cells. The levels of dG-dC crosslink in L1210 cells were 1.02 to 2.19 times higher than those in NIH/3T3 cells throughout the experiment. This result suggested that the dG-dC crosslinks induced by ACNU were more efficiently repaired in NIH/3T3 cells than in L1210 cells. NIH/3T3 mouse fibroblasts represent a stable diploid line that expresses stable levels of AGT activity.[42] Dunn *et al.*[43] demonstrated that NIH/3T3 cells were more resistant to ACNU than MGMT-deficient cells because of their relatively high levels of AGT. Previous studies indicated that L1210 cells are deficient in expression of AGT,[44] although they were not considered completely defective in crosslink repair because low levels of AGT activity are present.[45] Bodell *et al.*[46] obtained similar results as our study by comparing the levels of dG-dC crosslinks in the three glioma lines, BTRC-19, 9L-2 and 9L cells. They concluded that the reduction in the levels of dG-dC crosslink in 9L-2 and BRC-19 cells, which were 10-fold more resistant to CENU than 9L cells, was associated with increased levels of AGT activity. These results convincingly demonstrated that the suppression of the formation of CENU-induced crosslinks occurred as a result of chloroethyl transferase action of AGT.

CONCLUSIONS

In summary, a method for quantitation of dG-dC interstrand crosslinks in cells was established using HPLC/ESI-MS/MS providing high sensitivity, specificity and accuracy. Using this method, the levels of dG-dC crosslinks induced by ACNU were determined in NIH/3T3 fibroblasts cells (high level of expression of AGT) and L1210 leukemia cells (low level of express of AGT). The time-course profiles of dG-dC crosslinks in the two cell lines both showed a time-dependent pattern over a 24 h period. The crosslinking levels upon exposure to ACNU at various concentrations uniformly increased in the early incubation period reaching the maximum at 12 h.

Subsequently, the crosslinking levels declined after 12 h, presumably due to AGT-mediated DNA repair. The crosslinking levels in L1210 cells were significantly higher than those in NIH/3T3 cells at each time point, which suggested that NIH/3T3 cells have higher repairing activity than L1210. Moreover, the rate of disappearance of the dG-dC crosslink from 12 to 24 h is more rapid in L1210 than in NIH/3T3 cells, reflecting that the higher levels of the dG-dC crosslink in L1210 cells might be due to the higher efficiency of its formation by better uptake of the drug. The difference in the crosslinking levels of L1210 and NIH/3T3 provides direct evidence for AGT-repair of dG-dC crosslinks.

This work provided a robust method for quantitation of DNA ICLs in cell lines. This method provides direct quantitation of dG-dC crosslinks allowing for higher specificity and accuracy than previous methods, such as fluorescence and alkaline elution based assays, which measure the crosslinked double strands. This work not only contributes to a further understanding of the drug resistance to CENU-based anticancer drugs induced by AGT, but also will assist in the development of new bifunctional anticancer agents with high specificity and efficiency.

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REFERENCES

[1] C. T. Gnewuch, G. Sosnovsky. A critical appraisal of the evolution of N-nitrosoureas as anticancer drugs. *Chem. Rev.* **1997**, *97*, 829.
[2] A. Silvani, M. Eoli, A. Salmaggi, A. Erbetta, L. Fariselli, A. Boiardi. Intra-arterial ACNU and carboplatin versus intravenous chemotherapy with cisplatin and BCNU in newly diagnosed patients with glioblastoma. *Neurol. Sci.* **2002**, *23*, 219.
[3] J. K. Wiencke, J. Wiemels. Genotoxicity of 1,3-bis(2-chloroethyl)-l-nitrosourea (BCNU). *Mutat. Res. Rev. Gene. Toxicol.* **1995**, *339*, 91.
[4] A. De Rossi, L. Rossi, A. Laudisi, V. Sini, L. Toppo, F. Marchesi, G. Tortorelli, M. Leti, M. Turriziani, A. Aquino, E. Bonmassar, L. De Vecchis, F. Torino. Focus on fotemustine. *J. Exp. Clin. Cancer Res.* **2006**, *25*, 461.
[5] A. Hussein, E. Mioglou-Kalouptsi, A. Papageorgiou, I. Karapidaki, Z. Iakovidou-Kritsi, Th. Lialiaris, E. Xrysogelou, Ch. Camoutsis, D. Mourelatos. Comparison of new nitrosoureas esters with modified steroidal nucleus for cytogenetic and antineoplastic activity. *In Vivo* **2007**, *21*, 389.
[6] Y. J. Lu, H. W. Yang, S. C. Hung. Improving thermal stability and efficacy of BCNU in treating glioma cells using PAA-functionalized graphene oxide. *Int. J. Nanomed.* **2012**, *7*, 1737.
[7] A. M. Zheleva, V. G. Gadjeva. Spin labelled nitrosoureas and triazenes and their non-labelled clinically used analogues - a comparative study on their physicochemical properties and antimelanomic effects. *Int. J. Pharmaceut.* **2001**, *212*, 257.
[8] P. G. Penketh, K. Shyam, A. C. Sartorelli. Comparison of DNA lesions produced by tumor-inhibitory 1,2-bis (sulfonyl)hydrazines and chloroethylnitrosoureas. *Biochem. Pharmacol.* **2000**, *59*, 283.
[9] O. D. Scharer. DNA interstrand crosslinks: Natural and drug-induced DNA adducts that induce unique cellular responses. *Chembiochem* **2005**, *6*, 27.
[10] F. X. Chen, W. J. Bodell, G. N. Liang, B. Gold. Reaction of N-(2-chloroethyl)-N-nitrosoureas with DNA: Effect of buffers on DNA adduction, cross-linking, and cytotoxicity. *Chem. Res. Toxicol.* **1996**, *9*, 208.
[11] W. J. Bodell, K. Pongracz. Chemical synthesis and detection of the cross-link 1-[N3-(2′-deoxycytidyl)]-2-[N1-(2′-deoxyguanosinyl)] ethane in DNA reacted with 1-(2-chloroethyl)-1-nitrosourea. *Chem. Res. Toxicol.* **1993**, *6*, 434.
[12] P. L. Fischhaber, A. S. Gall, J. A. Duncan, P. B. Hopkins. Direct demonstration in synthetic oligonucleotides that N, N′-bis(2-chloroethyl)nitrosourea cross-links N-1 of deoxyguanosine to N-3 of deoxycytidine on opposite strands of duplex DNA. *Cancer Res.* **1999**, *59*, 4363.
[13] W. J. Bodell. DNA alkylation products formed by 1-(2-chloroethyl)-1-nitrosourea as molecular dosimeters of therapeutic response. *J. Neuro-oncol.* **2009**, *91*, 257.
[14] H. Ueda-Kawamitsu, T. A. Lawson, P. R. Gwilt. In vitro pharmacokinetics and pharmacodynamics of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). *Biochem. Pharmacol.* **2002**, *63*, 1209.
[15] P. G. Penketh, R. P. Baumann, K. Ishiguro, K. Shyam, H. A. Seow, A. C. Sartorelli. Lethality to leukemia cell lines of DNA interstrand cross-links generated by Cloretazine derived alkylating species. *Leuk. Res.* **2008**, *32*, 1546.
[16] S. Liu, Y. S. Wang. A quantitative mass spectrometry-based approach for assessing the repair of 8-methoxypsoralen-induced DNA interstrand cross-links and monoadducts in mammalian cells. *Anal. Chem.* **2013**, *85*, 6732.
[17] C. F. Lai, H. C. Cao, J. E. Hearst, L. Corash, H. Luo, Y. S. Wang. Quantitative analysis of DNA interstrand cross-links and monoadducts formed in human cells induced by psoralens and UVA irradiation. *Anal. Chem.* **2008**, *80*, 8790.
[18] H. C. Cao, J. E. Hearst, L. Corash, Y. S. Wang. LC-MS/MS for the detection of DNA interstrand cross-links formed by 8-methoxypsoralen and UVA irradiation in human cells. *Anal. Chem.* **2008**, *80*, 2932.
[19] B. Malayappan, L. Johnson, B. Nie, D. Panchal, B. Matter, P. Jacobson, N. Tretyakova. Quantitative high-performance liquid chromatography-electrospray ionization tandem mass spectrometry analysis of bis-N7-guanine DNA-DNA cross-links in white blood cells of cancer patients receiving cyclophosphamide therapy. *Anal. Chem.* **2010**, *82*, 3650.
[20] M. M. Paz, S. Ladwa, E. Champeil, Y. Liu, S. Rockwell, E. K. Boamah, J. Bargonetti, J. Callahan, J. Roach, M. Tomasz. Mapping DNA adducts of mitomycin C and decarbamoyl mitomycin C in cell lines using liquid chromatography/electrospray tandem mass spectrometry. *Chem. Res. Toxicol.* **2008**, *21*, 2370.
[21] B. Q. Bai, L. J. Zhao, R. G. Zhong. Quantification of meCCNU-induced dG-dC crosslinks in oligonucleotide duplexes by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 2027.
[22] B. Q. Bai, L. J. Zhao, R. G. Zhong. Analysis of deoxyribonucleic acid interstrand cross-links induced by nitrosourea with high performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Chinese J. Anal. Chem.* **2010**, *38*, 532–536.
[23] L. J. Zhao, X. Y. Ma, R. G. Zhong. A density functional theory investigation on the formation mechanisms of DNA interstrand crosslinks induced by chloroethylnitrosoureas. *Int. J. Quantum Chem.* **2013**, *113*, 1299.

[24] L. J. Zhao, X. Y. Ma, R. G. Zhong. Comparative theoretical investigation of the formation of DNA interstrand crosslinks induced by two kinds of N-nitroso compounds: nitrosoureas and nitrosamines. *J. Phys. Org. Chem.* **2012**, *25*, 1153.
[25] S. L. Gerson. MGMT: Its role in cancer aetiology and cancer therapeutics. *Nature Rev. Cancer* **2004**, *4*, 296.
[26] A. E. Pegg. Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools. *Chem. Res. Toxicol.* **2011**, *24*, 618.
[27] J. L. Tubbs, A. E. Pegg, J. A. Tainer. DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by O-6-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy. *DNA Repair* **2007**, *6*, 1100.
[28] R. J. Hansen, R. Nagasubramanian, S. M. Delaney, M. M. Cherian, S. Lin, S. C. Kogan, M. E. Dolan. Role of O6-alkylguanine-DNA alkyltransferase in protecting against 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-induced long-term toxicities. *J. Pharmacol. Exp. Ther.* **2005**, *315*, 1247.
[29] G. P. Margison, M. F. Santibanez-Koref. O-6-alkylguanine-DNA alkyltransferase: role in carcinogenesis and chemotherapy. *Bioessays* **2002**, *24*, 255.
[30] T. P. Brent, P. J. Houghton, J. A. Houghton. O-6-Alkylguanine-DNA alkyltransferase activity correlates with the therapeutic response of human rhabdomyosarcoma xenografts to 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 2985.
[31] T. P. Brent. Suppression of cross-link formation in chloroethylnitrosourea-treated DNA by an activity in extracts of human leukemic lymphoblasts. *Cancer Res.* **1984**, *44*, 1887.
[32] A. E. Pegg. Mammalian O6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res.* **1990**, *50*, 6119.
[33] T. P. Brent, J. S. Remack, D. G. Smith. Characterization of a novel reaction by human O6-alkylguanine-DNA alkyltransferase with 1,3-bis(2-chloroethyl)-1-nitrosourea-treated DNA. *Cancer Res.* **1987**, *47*, 6185.
[34] P. E. Gonzaga, P. M. Potter, T. Niu, D. Yu, D. B. Ludlum, J. A. Raerty, G. P. Margison, T. P. Brent. Identification of the cross-link between human O6-methylguanine-DNA methyltransferase and chloroethylnitrosourea-treated DNA. *Cancer Res.* **1992**, *52*, 6052.
[35] I. Passagne, A. Evrard, P. Depeille, P. Cuq, D. Cupissol, L. Vian. O-6-methylguanine DNA-methyltransferase (MGMT) overexpression in melanoma cells induces resistance to nitrosoureas and temozolomide but sensitizes to mitomycin C. *Toxicol. Appl. Pharmacol.* **2006**, *211*, 97.
[36] L. C. Erickson, G. Laurent, N. A. Sharkey, K. W. Kohn. DNA cross-linking and monoadduct repair in nitrosourea-treated human-tumor cells. *Nature* **1980**, *288*, 727.
[37] W. J. Bodell, T. Aida, M. S. Berger, M. L. Rosenblum. Repair of O6-(2-chloroethyl)guanine mediates the biological effects of chloroethylnitrosoureas. *Environ. Health Persp.* **1985**, *62*, 119.
[38] Y. N. Yang, D. Nikolic, S. M. Swanson, R. B. van Breemen. Quantitative determination of N-7-methyldeoxyguanosine and O-6-methyldeoxyguanosine in DNA by LC-UV-MS-MS. *Anal. Chem.* **2002**, *74*, 5376.
[39] F. G. Zhang, M. J. Bartels, L. H. Pottenger, B. B. Gollapudi, M. R. Schisler. Quantitation of lower levels of the DNA adduct of thymidylyl(3′-5′)thymidine methyl phosphotriester by liquid chromatography/negative atmospheric pressure chemical ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1043.
[40] E. D. Michaelson-Richie, X. Ming, S. G. Codreanu, R. L. Loeber, D. C. Liebler, C. Campbell, N. Y. Tretyakova. Mechlorethamine-induced DNA-protein cross-linking in human fibrosarcoma (HT1080) cells. *J. Proteome Res.* **2011**, *10*, 2785.
[41] A. Kothandapani, S. M. Patrick. Evidence for base excision repair processing of DNA interstrand crosslinks. *Mutat. Res.* **2013**, *743*, 44
[42] L. C. Harris, P. M. Potter, K. Tano, S. Shiota, S. Mitra, T. P. Brent. Characterization of the promoter region of the human O6-methylguanine-DNA methyltransferase gene. *Nucleic Acids Res.* **1991**, *19*, 6163.
[43] W. C. Dunn, K. Tano, G. J. Horesovsky, R. J. Preston, S. Mitra. The role of O6-alkylguanine in cell killing and mutagenesis in Chinese-hamster ovary cells. *Carcinogenesis* **1991**, *12*, 83.
[44] K. Ishiguro, H. A. Seow, P. G. Penketh, K. Shyam, A. C. Sartorelli. Mode of action of the chloroethylating and carbamoylating moieties of the prodrug cloretazine. *Mol. Cancer Ther.* **2006**, *5*, 969.
[45] N. Yajima, S. Hochi, N. Miyata, T. Kishi, G. Kawanishi. DNA breaks and repair in the mouse leukema-L1210 cells exposed to 3 different types of interstrand DNA cross-linkers. *Mutat. Res.* **1990**, *236*, 43.
[46] W. J. Bodell. Repair of DNA alkylation products formed in 9L cell lines treated with 1-(2-chloroethyl)-1-nitrosourea. *Mutat. Res.* **2003**, *522*, 85.

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