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Comparative investigation of the DNA inter-strand crosslinks induced by ACNU, BCNU, CCNU and FTMS using high-performance liquid chromatography–electrospray ionization tandem mass spectrometry

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ABSTRACT

Chloroethylnitrosoureas (CENUs) are an important family of alkylating agents employed in the clinical treatments of cancer. They exert cytotoxicity by inducing DNA interstrand crosslinks (ICLs) between guanine and the complimentary cytosine, namely dG–dC crosslink. Many investigations have been performed on the DNA ICLs involved in the anticancer efficacy of CENUs, but no conclusive comparisons between these agents have been published. In this work, the levels of dG–dC crosslink in calf thymus DNA induced by four CENUs, including nimustine (ACNU), carmustine (BCNU), lomustine (CCNU) and fotemustine (FTMS), were quantitatively determined using HPLC–ESI-MS/MS. The obtained time-courses for the dG–dC crosslinking levels indicated that there is an induction period with very low crosslinking activity at the initial stage of the treatment by BCNU and CCNU. The induction period provides a convincing evidence for the presumed mechanism that the formation of dG–dC crosslinks was initiated by the monoalkylation of guanine followed by the second alkylation of the complimentary cytosine. The crosslinking activity of ACNU is remarkably higher than those of BCNU, CCNU and FTMS at all time points. The crosslinking activities of CENUs were found to be related to their stability in aqueous solution. ACNU has the shortest half-life among the four CENUs, but has highest crosslinking levels; on the contrary, CCNU has the lowest crosslinking activity with the longest half-life. Moreover, a correlation was found between the crosslinking activity and the anticancer efficiency. ACNU with the highest crosslinking activity showed the better survival gain for high-grade glioma than BCNU, CCNU and FTMS as reported in an epidemiological study. This suggests that dG–dC crosslink can possibly be employed as a potential biomarker for evaluating the anticancer efficiency of novel CENU drugs.

1. Introduction

Chloroethylnitrosoureas (CENUs) are an important family of alkylating agents widely employed in the clinical treatment of cancer, including Hodgkin's disease, malignant melanoma, and various solid tumors. Especially, owing to the ability of crossing the blood–brain barrier, CENUs are efficient chemotherapeutics for brain tumor and other central nervous system neoplasms [1–4]. As listed in Table 1, nimustine (ACNU), carmustine (BCNU), lomustine (CCNU) and semustine (MeCCNU) represent the typical CENUs chemotherapeutics used in the clinical treatment of cancer [5–7]. In recent years, several novel CENUs chemotherapies were developed by modifying the structure of the moiety on the N3 atom to achieve higher antineoplastic activity or lower toxicity. Fotemustine (FTMS) is an active nitrosourea in metastatic melanoma, and it was the first drug to show significant efficacy in brain metastases [8,9]. The phosphoalanine group grafted on the N3 atom is highly lipophilic and increase its ability of crossing the blood–brain barrier [10]. 1-(2-Chloroethyl)-3-sarcosinamide-1-nitrosourea (SarCNU) was selected for formulation by the National Cancer Institute because of its therapeutic advantage in the treatment of malignant glioma [11]. Ranimustine (MCNU), a derivative of CENU developed and clinically used in Japan, showed excellent responses against chronic myelogenous leukemia, polycythemia vera and thrombocythemia [12,13].

CENUs are bifunctional alkylating agents, which exert cytotoxicity by inducing DNA interstrand crosslinks (ICLs) within the complimentary guanine and cytosine base pair. This covalent crosslink interferes with the normal DNA replication by preventing the separation of the double strands, and finally leads to the

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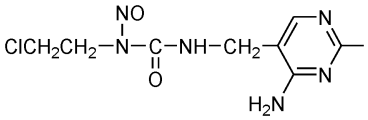
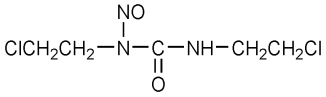
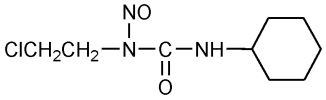
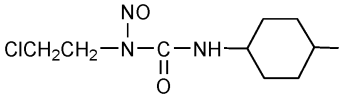
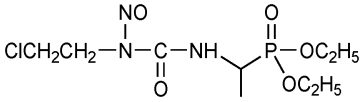
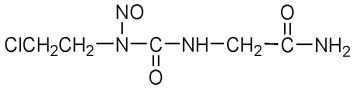
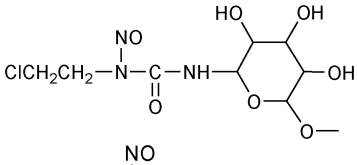
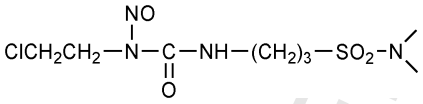
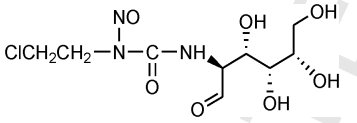
Table 1
Chloroethylnitrosoureas used in clinical treatment or developed as preclinical therapeutics for cancer.

Proprietary name	Chemical structures	Indications
Nimustine (ACNU)		Brain tumors, small cell lung cancer and Hodgkin's disease
Carmustine (BCNU)		Brain tumors and Hodgkin's disease
Lomustine (CCNU)		Brain tumors
Semustine (MeCCNU)		Brain tumors
Fotemustine (FTMS)		Brain tumors and melanoma
SarCNU		Malignant glioma
Ranimustine (MCNU)		Brain tumors, myeloma, malignant lymphoma, and leukemia
Tauromustine (TCNU)		Myeloma, malignant glioma and lymphoma
Chlorozotocin (DCNU)		Pancreatic tumors

apoptosis of cancer cells [14–16]. The CENUs-induced DNA ICLs were demonstrated to occur between the N1 site of guanine and the N3 site of the complimentary cytosine. The supposed mechanism of the formation of dG–dC crosslink is shown in Fig. 1. The chloroethyl diazonium ion produced by the decomposition of CENUs alkylates guanine on the O^6 site to form O^6-chloroethylguanine (O^6-ClEt-Gua) followed by further alkylation of the complimentary cytidine on the N3 site via a cationic intermediate, N1,O^6-ethanoguanine [17–19]. The levels of dG–dC crosslink in cells are much lower than the monoadducts induced by CENUs, such as N7-(2-hydroxyethyl)-guanine and N7-(2-chloroethyl)-guanine [20–22]. However, there is considerable correlation between the crosslinking activity and the cytotoxicity of CENUs. Therefore, dG–dC crosslink is possibly be used as a biomarker for evaluating the chemotherapeutic effect of CENUs.

To understand the anticancer mechanism of CENUs and develop more efficient chemotherapeutics, significant efforts have been devoted to investigate the CENUs-induced DNA ICLs by in vitro or

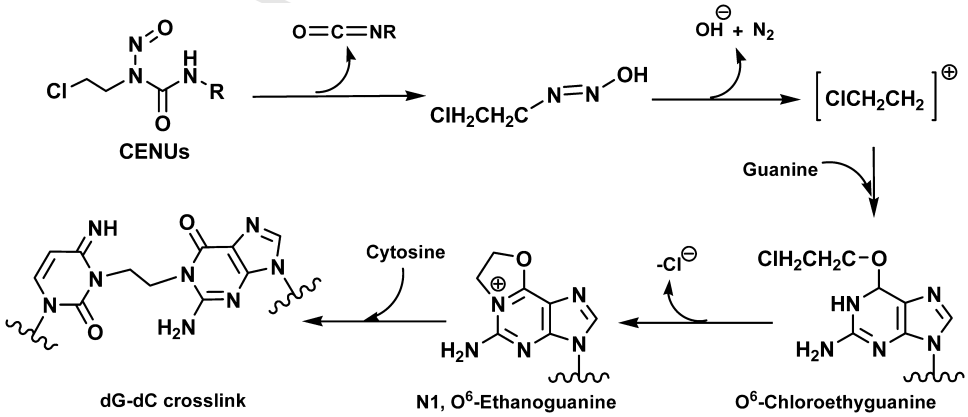


Fig. 1. Supposed mechanism for the formation of dG–dC crosslinks induced by CENUs.

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in vivo studies. Hayes et al. [23] determined the DNA ICLs induced by FTMS and MeCCNU in linear pBR322 plasmid using agarose gel electrophoresis, and observed that the coincubation of ellagic acid reduced ICLs considerably. Penketh et al. [24] compared the levels of ICL induced by CENUs and 1,2-bis(sulfonyl)hydrazine derivatives (BSHs) in T7 DNA using fluorescent assay with Hochest 33258 as a probe specifically recognizing the crosslinked double strand DNA. Also using fluorescent assay, Ueda-Kawamitsu et al. [25] measured the time course of DNA ICLs in L1210 cells treated with BCNU, and observed that the percentage of crosslinks reached the maximum after 6 h exposure and subsequently decreased presumably because of DNA repair. Tong et al. [26] first isolated 1-[*N*3-deoxycytidyl],2-[*N*1-deoxyguanosinyl]ethane (dG–dC crosslink) from DNA exposed to BCNU. Bodell et al. [22] measured the levels of dG–dC crosslink using HPLC, and observed a significant correlation between LD_{10} of CENUs and the dG–dC crosslinking level. High performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) has been frequently used for the quantitative analysis of DNA adducts because of its high sensitivity, specificity and accuracy. Fischhaber et al. [27] determined the levels of BCNU-induced dG–dC crosslink using HPLC–MS/MS and provided the first direct evidence that BCNU had no strong sequence preference for interstrand crosslinking. In our previous work [28–30], dG–dC crosslinks induced by MeCCNU in calf thymus DNA and in synthetic oligonucleotides were determined by HPLC–MS/MS. The results indicated that the dG–dC crosslink stayed at a relatively low level during the first 2 h of the treatment and then underwent obvious increase. This provided convincing evidence for the crosslinking mechanism proposed in our theoretical studies [31,32], 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 crosslinks. Even though many previous studies were focused on the DNA ICLs involved in the anticancer efficiency of CENUs, no conclusive comparisons between these agents have been published. In this work, the levels of CENU-induced dG–dC crosslink were determined using HPLC–ESI-MS/MS. Comparison was performed between the levels of dG–dC crosslink in calf thymus DNA treated by ACNU, BCNU, CCNU and FTMS, respectively.

2. Experimental

2.1. Chemicals and materials

ACNU, BCNU, CCNU, FTMS, acetonitrile (HPLC grade), 2′-deoxyguanosine, 2′-deoxycytidine, calf thymus DNA and snake venom 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.

2.2. Synthesis of the standards

The synthesis of [1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)] ethane (dG–dC) and the internal standard $^{15}N_3$-dG-dC were carried out according to a previously published method [29,33], with some modifications. Briefly, 325 mg of 2′-deoxyguanosine was incubated with 1.8 mL of acetic anhydride in a mixture containing 18 mg of 4-dimethylamiopyridine, 2.0 mL of triethylamine and 50 mL of dry pyridine at 50 °C for 20 h. After crystallization and filtration, the obtained N^2,3′,5′-triacetyl-2′-deoxyguanosine was dissolved in 4 mL of dry dioxane followed by incubation with 160 μL of 2-fluoroethanol dioxane, 650 mg of triphenylphosphine and 400 μL of diethylazodicarboxylate at room temperature for 1 h. Then 15 mL of 5% $NaHCO_3$ solution was added. The mixture was stirred at room temperature for 15 min. The product was extracted with 30 mL of methylene chloride for three times. The oily residue was dissolved in 6 mL of methanol followed by addition of 30 mL of concentrated ammonium hydroxide. The mixture was kept at 60 °C for 3 h to produce O^6-(2-fluoroethyl)-2′-deoxyguanosine (O^6-FEt-dGuo), which 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 starting material for the following synthesis of dG–dC crosslink. Twenty milligram of O^6-FEt-dGuo were incubated with 5 mg of 2′-deoxycytidine in 100 μL of DMSO at 55 °C for 20 days. The final product was purified by HPLC with a 4.6 mm × 250 mm Zorbax SB-C18 column (5 μm in particle size) to collect the fractions containing dG–dC. The mobile phase consists of ammonium acetate solution at the concentration of 10 mM (0.1% acetic acid, pH 6.8) (A) and acetonitrile (B) with a flow rate of 1 mL/min. A gradient of 5–10% buffer B in 20 min was employed with a linear gradient to 30% buffer B over 10 min. Then an isocratic wash of 30% buffer B was used for 3 min followed by a gradient down to 5% buffer B over 2 min. The fraction between 20 and 22 min was collected. UV detector was set 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 [29,33].

2.3. Treatment of calf thymus DNA with CENUs

Calf thymus DNA was dissolved in phosphate-buffered saline (10 mmol/L Tris–HCl, 50 mmol/L NaCl, 50 mmol/L NaH_2PO_4, pH = 7.4) to a concentration of 0.5 mg/mL. ACNU was dissolved in deionized water immediately prior to use and directly added to 8 mL of DNA solution to achieve the final concentrations of 1, 2, 4 and 8 mM, respectively. The reaction mixtures were incubated at 37 °C for 12 h in the dark. Aliquots of 400 μL solution were removed from the reaction mixture at 1, 2, 3, 4, 6, 8, 10 and 12 h, respectively. For each sample, DNA was precipitated by adding 800 μL of ice-cold ethanol followed by centrifuge at 12,000 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. The treatments of calf thymus DNA with BCNU, CCNU and FTMS, respectively, were carried out using the same protocol as ACNU except that the drugs were dissolved in ethanol, but not deionized water. For each time point, the control DNA samples were incubated at the same conditions as the treated DNA samples.

2.4. Enzymatic hydrolysis of DNA

The concentration of DNA samples was determined before the enzymatic hydrolysis. The obtained DNA pellets were redissolved in 200 μL of Tris–HCl buffer (10 mM, pH 7.0). The concentration of the DNA solution was determined by the UV absorbance at 260 nm. One OD260 corresponds to approximately 50 μg/mL for double-strand DNA.

Each DNA sample was spiked with 15 μL of internal standard $^{15}N_3$-dG-dC (1 μM) followed by enzymatic hydrolysis according to the previously reported protocols [29,30]. DNA samples were digested by four enzymes, including DNase I, nuclease S1, alkaline phosphatase and snake venom phosphodiesterase I. Briefly, the DNA solutions were first heated at 98 °C for 5 min and promptly

chilled in an ice-bath for 10 min. Each DNA solution (200 μL) was hydrolyzed by adding 180 units of DNase I (60 μL, buffered in CH_3COONa 20 mM, NaCl 150 mM, pH 5.0) and 300 units of nuclease S1 (60 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 30 units (60 μL) of alkaline phosphatase and 10 mill units of phosphodiesterase I (5 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.

2.5. Quantitation of dG–dC crosslink 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 Thermo Finnigan HPLC system (Thermo Finnigan, San Jose, CA). The electrospray ionization (ESI) was performed in the positive mode. The fractions of dG–dC crosslink were separated with a 2.1 mm × 150 mm (5 μm in particle size) Zorbax SB-C18 column (Agilent Technologies, Palo Alto, CA) and eluted at 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 gradient was started from an isocratic elution with 2% B for the first 5 min followed by a linear increase to 80% B in 25 min. After being held at 80% B for 3 min, the solvent composition was brought back to the initial composition of 2% B in the next 2 min and equilibrated at those conditions for 30 min. The instrumental parameters of the mass spectrometer were set as follows: spray voltage 4000 V; sheath gas (nitrogen) pressure 50 psi; aux gas (nitrogen) pressure 15 psi; capillary temperature 300 °C; and tube lens offset 89 V. Collision energy was set to 20 eV using argon at 1.0 mTorr. The source CID was set to 8 V. The fragmentation pattern of dG–dC and $^{15}N_3$-dG-dC was shown in Fig. S1 in the Supporting Information. 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. Under the proposed HPLC conditions, dG–dC standard and $^{15}N_3$-dG-dC internal standard coeluted at 22 min (see Fig. S2 in the Supporting Information).

According to previous work [29,30], the level of dG–dC crosslink in DNA from cells were reported by the number of crosslinked dG and dC in every 10^7 base pairs calculated by Eq. (1). In Eq. (1), C refers to the determined concentrations of dG–dC crosslink; V refers to the volume of the enzymatic digestion solution, which is 400 μL; C_0 is the concentration of the calf thymus DNA measure by UV; V_0 is the volume of the DNA sample before enzymatic digestion,

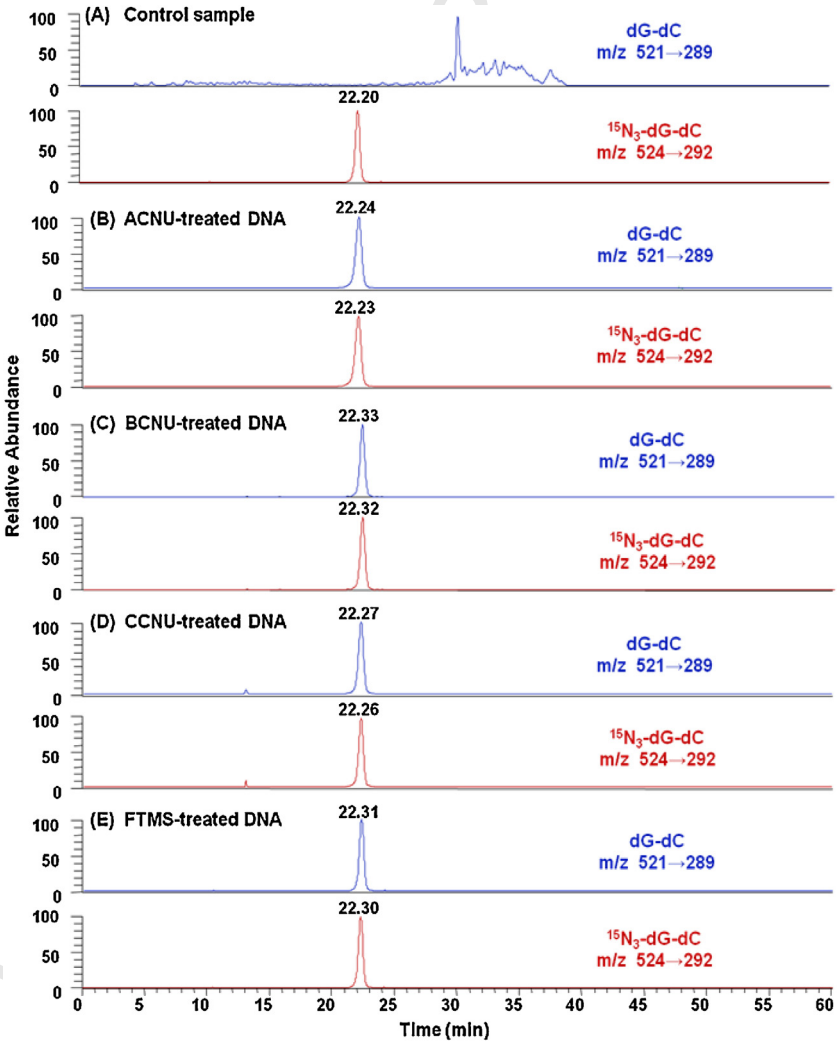

Fig. 2. SRM chromatograms of dG–dC crosslinks in the DNA digestion mixtures from (A) control samples, (B) ACNU-treated DNA, (C) BCNU-treated DNA, (D) CCNU-treated DNA and (E) FTMS-treated DNA.

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which is 200 μL; and M_0 represents the average molecular weight of the four deoxynucleotides (325 g/mol).

$$\frac{\text{dG – dC crosslinks}}{10^7 \text{ base pairs}} = \frac{C \times V \times 10^7}{C_0 \times V_0/2M_0} \quad (1)$$

3. Results and discussion

Fig. 2 shows the SRM chromatograms of dG–dC and $^{15}N_3$-dG-dC in the DNA enzymatic hydrolysates from the control sample (Fig. 2A), and DNA treated with ACNU (Fig. 2B), BCNU (Fig. 2C), CCNU (Fig. 2D) and FTMS (Fig. 2E). In Fig. 2B–D, the retention times for dG–dC in the digestion mixtures are about 22 min, and their corresponding isotope labeled standard, $^{15}N_3$-dG-dC, has the same retention time. Fig. 2A indicates that there is no signal detected with the SRM transition for dG–dC in the DNA hydrolysates from the control samples. 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.

Fig. 3 shows the time-course of the levels of dG–dC crosslinks induced by the four CENUs. The corresponding values for the determined levels of dG–dC crosslinks at each time point are listed in Table S1 in the Supporting Information. For the four CENUs, the levels of dG–dC crosslink display dose-dependence with the concentration increased from 1 to 8 mM. The crosslinking levels show a common increasing trend during 0–12 h, and reached a maximum at 12 h. For ACNU, the crosslinking levels at 12 h were 527, 1203, 1358 and 1445 dG–dC crosslinks/10^7 base pairs for 1, 2, 4 and 8 mM concentration, respectively. For BCNU, CCNU and FTMS, the maximum crosslinking levels were 505, 263 and 585 dG–dC crosslinks/10^7 base pairs, respectively, observed at 12 h for the highest concentration of 8 mM. However, there is a significant difference between the patterns of the time-course of the four CENUs. In Fig. 3B and C, there is an "induction period" in the initial stage of the treatment by BCNU and CCNU, in which the dG–dC crosslinks stay at relatively low levels. Approximately, the induction period is 2 h for BCNU, and 4 h for CCNU. Especially for CCNU, the induction period is very apparent for all the four concentrations of the drug with the crosslinking levels lower than 20 dG–dC crosslinks/10^7 base pairs. In our previous work [29], a similar induction period was also observed in MeCCNU treated oligonucleotide duplexes. The induction period provides a convincing evidence for the supposed mechanism of the formation of dG–dC crosslinks induced by CENUs, which postulates that the reaction was initiated by the formation of the guanine monoadduct induced by chloroethyl cations arising from the decomposition of CENUs, and then the dG–dC crosslink is formed via the second alkylation of the complimentary cytosine base.

For the time-courses of the crosslinking levels induced by ACNU and FTMS (Fig. 3A and D), no obvious induction period was observed in the initial stage of the treatments. This suggests that the formations of dG–dC crosslinks induced by ACNU and FTMS are faster than those by BCNU and CCNU. CENUs can undergo spontaneous decomposition to yield chloroethyldizonium ions, which alkylate guanine followed by the formation of dG–dC crosslinks. Our theoretical study revealed that the decomposition of CENUs was the rate-limiting step in the whole process of the formation of crosslinks [31,32]. Previous studies reported that the half-lives of ACNU, BCNU, CCNU and FTMS were 34, 49, 53 and 43 min, respectively [34,35]. From these theoretical and experimental evidences, it can be inferred that ACNU and FTMS with shorter half-lives decompose to chloroethyl diazonium ions more quickly and consequently induce the formation of dG–dC crosslinks more quickly, which finally results in the disappearance of the induction period. On the contrary, BCNU and CCNU, which have longer half-lives and decompose slower, slow down the formation of dG–dC crosslinks by yielding less chloroethyl diazonium ions in the initial stage of the treatment. Therefore, there is an induction period in the formation of crosslinks induced by BCNU and CCNU.

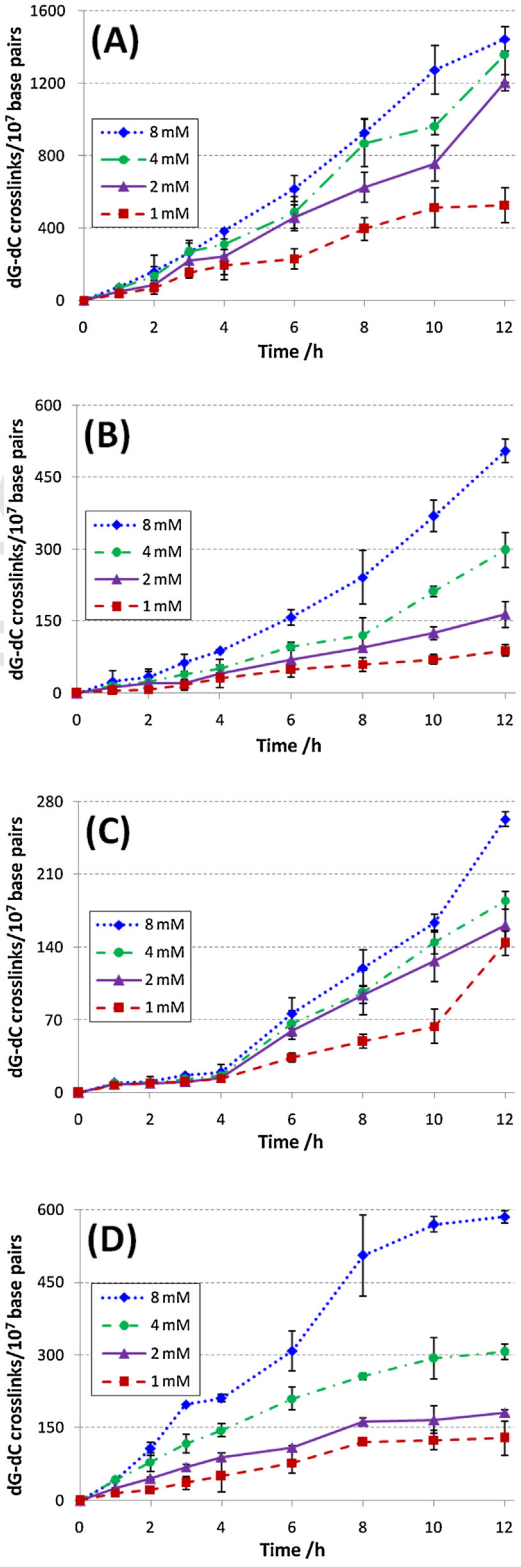

Fig. 3. Plots of dG–dC crosslinking levels (crosslinks/10^7 base pairs) vs time (h) in DNA treated by (A) ACNU, (B) BCNU, (C) CCNU and (D) FTMS with the drug concentrations at 1, 2, 4 and 8 mM ($n = 3$).

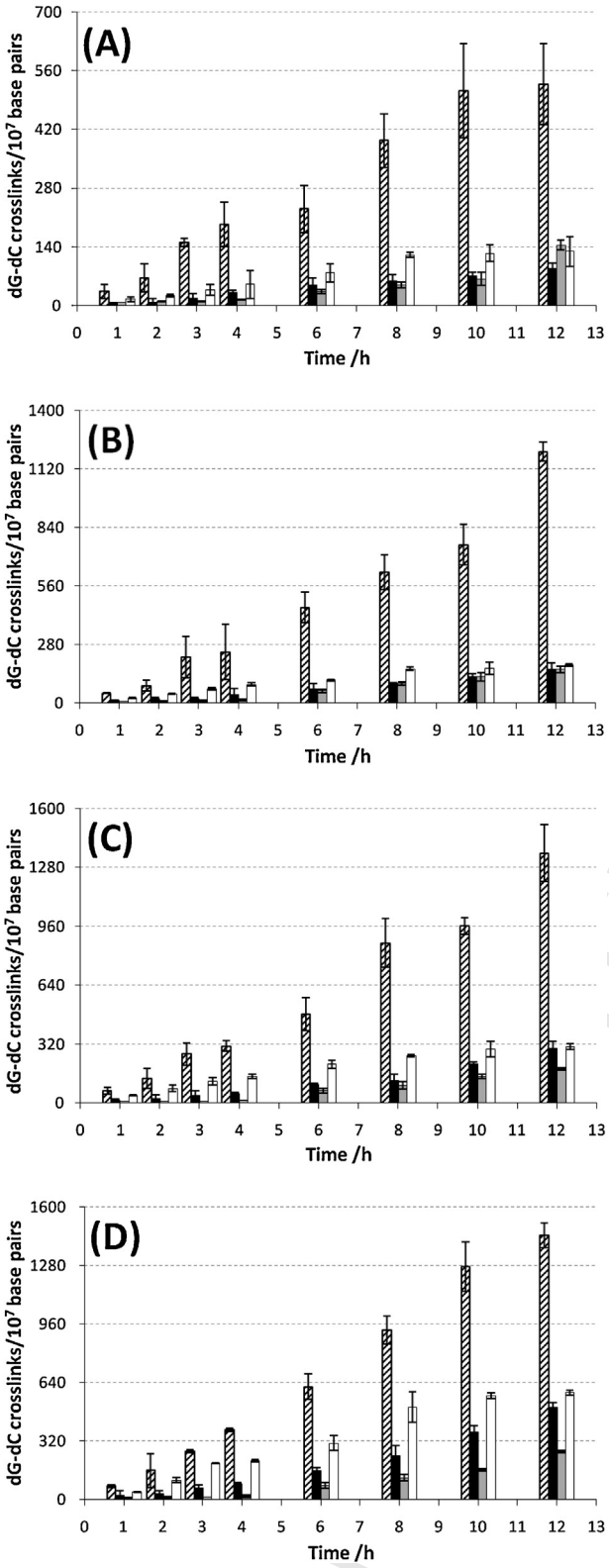

Fig. 4. Levels of dG–dC crosslinks in calf thymus DNA treated by the four CENUs at the concentration of (A) 1 mM, (B) 2 mM, (C) 4 mM and (D) 8 mM. Symbol designations are ▨ for ACNU, ■ for BCNU, ▩ for CCNU and □ for FTMS treatment ($n = 3$).

Fig. 4 shows the comparison of the crosslinking levels induced by the four CENUs at various concentrations. It is predominant that ACNU has the highest crosslinking activity followed by FTMS, BCNU and CCNU. The results indicates that ACNU with the shortest half-life exhibit highest crosslinking activity; while CCNU with the longest half-life lead to the lowest crosslinking level. From the above results, we presumed that the crosslinking activity of CENUs might be related to their stability in aqueous solution. Because the cytotoxicity of CENUs is related to the formation of DNA ICLs, it was presumed that the anticancer activity of the drugs was correlated to the level of dG–dC crosslinks. Wolff et al. [36] compared the anticancer efficiency of ACNU, BCNU, CCNU, FTMS and other CENUs by performing the survival gain analysis of 364 studies describing 24,193 patients with high-grade glioma treated in 504 cohorts. They demonstrated that the highest survival gain was achieved by ACNU (8.9 months) followed by CCNU (5.3 months) and FTMS (2.0 months), while BCNU provided no survival gain. Except for CCNU, the dG–dC crosslinking levels obtained in this work are correlated to the survival gain reported in the epidemiological study, which is ACNU > FTMS > BCNU. CCNU has relatively high survival gain, but lowest crosslinking level, which may be related to its relatively high lipophilicity in the treatment of glioma. Bodell et al. [37,38] treated human glial-derived cells and 9 L rat gliosarcoma cells by ACNU and BCNU, and determined the DNA ICLs using alkaline elution assay. They observed that the crosslinking levels induced by ACNU were higher than those induced by BCNU in all cell lines, which is also consistent with our results.

4. Conclusion

In summary, the levels of dG–dC crosslinks induced by ACNU, BCNU, CCNU and FTMS were quantitatively determined using HPLC–ESI-MS/MS. The obtained time-courses of the crosslinking levels for the four CENUs showed a time-dependent trend at all concentrations of the drugs. At the initial stage of the treatment (approximately the first 2–4 h), an obvious induction period with very low crosslinking levels was observed in the time-courses of BCNU and CCNU. The existence of the induction period corresponded to the dG–dC crosslinks being rarely detectable at the beginning of the treatment. This provides convincing evidence for the presumed mechanism that the first step for the formation of dG–dC crosslinks was the monoalkylation of guanine followed by the second alkylation of the complimentary cytosine. The comparison of the crosslinking levels for the four drugs indicated that ACNU induced remarkably higher levels of dG–dC crosslinks than the other three drugs at all time points. The crosslinking activities of CENUs were found to be related to their stability in aqueous solution, i.e., ACNU with the lowest stability has the highest crosslinking levels; on the contrary, CCNU with the highest stability has the lowest crosslinking levels. Moreover, the low stability may also contribute to the disappear of the induction period in the formation of crosslinks induced by ACNU and FTMS. A correlation was found between the determined crosslinking level and the previously reported anticancer efficiency. ACNU, which has the highest crosslinking activity, showed better survival gain for high-grade glioma than BCNU, CCNU and FTMS in an epidemiological study. This suggests that dC–dG crosslink can possibly be employed as a potential biomarker for evaluating the anticancer efficiency of novel CENU drugs. This work contributes to a further understanding of the anticancer mechanism of CENUs, and will assist in the development of novel bifunctional anticancer agents with high specificity and efficiency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijms.2014.04.018.

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