Nimustine Induces DNA Breaks and Crosslinks in NIH/3T3 Cells

Lin-Na Zhao, Xue-Chai Chen, Yan-Yan Zhong, Qin-Xia Hou, and Ru-Gang Zhong

***Abstract*—The relationship between carcinogenicity and DNA interstrand cross-links of nitrosoureas is poorly defined. 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3- (2-chloroethyl)-3- nitrosourea (ACNU, nimustine) is one of nitrosoureas used in the treatment of high-grade gliomas. It has the capability of causing DNA interstrand cross-links (ICLs) to kill cancer cells. But it can also cause the generation of secondary tumors with carcinogenic side effects. In present study, we investigated DNA interstrand cross-links, DNA double-strand breaks and cell cycle phase in NIH/3T3 cells from the primary mouse embryonic fibroblast cells induced by ACNU. This result indicated that the concentration of 60 and 75μg/ml of ACNU could be detected significantly ICLs, and the γ-H2AX has the ability to be a biomarker for DNA damage associated with ICLs induced by ACNU.**

***Index Terms*—Comet assay, interstrand cross-links, nimustine, nitrosourea, γ-H2AX.**

I. Introduction

The subgroup of nitrosoureas (CNUs) including 1-(4-amino -2-methyl-5- pyrimidinyl) methyl-3 -(2-chloroethyl) -3- nitrosourea (ACNU, nimustine), 1,3- Bis(2-chloroethyl)- 1- nitrosourea (BCNU, carmustine), 1-(2-chloroethyl)- 3- cyclohexyl- nitrosourea (CCNU, lomustine), and methyl-CCNU (Me-CCNU) and 1-[N-(2-chloroethyl)-N-nitrosoureido] ethylphos-phonic acid diethyl ester] (fotemustine) are widely used for malignant brain tumor as second-line chemotherapeutic agent in cancer therapy because of their high potency to induce DNA interstrand cross-links (ICLs) [1], [2]. ICLs are extremely toxic to cells because they prevent the separation of DNA replication and transcription, by coordinated chemical reactions with bases on opposing strands. The resulting covalent linkage is usually irreversible [3]. To maintain the process of cell cycle integrity, cells use multiple DNA repair mechanisms to repair or remove the damaged DNA [4]-[6]. However, it is not only beneficial for the DNA repair mechanisms worked, it can also detriment the integrity of the genome itself. Tumor cells can be resistant for the nitrosoureas and the normal cells can be increase mutation frequency by the genome mutants [7]. Previously, studies showed that nitrosourea's role in cancer or cancer-causing side effects are associated with the formation of DNA interstrand cross-links [8]-[10], and it is significant to further study of drug-induced DNA cross-linking to reveal the difference between the nitrosourea anticancer mechanism and carcinogenic role.

ACNU was discovered in 1974 as the first water-soluble nitrosourea compound [11], and mainly used in the clinic of glioblastoma patients before the introduction of 8-carbamoyl-3-methylimidazo [5, 1-d]-1, 2, 3, 5-tetrazin-4(3H) -one (temozolomide, TMZ) because of its high permeability across blood-brain barrier (BBB) and good cytotoxic activity for gliomas [12]-[13]. BCNU always induces two major types of genotoxic damage to the cell: DNA mono adducts and DNA interstrand cross-links. To understand the mechanism of ACNU induced the second cancer, it is important to investigate the ICLs effect of ACNU and the cell cycle in the process of repairing ICLs.

In this study, the primary mouse embryonic fibroblast cells (NIH/3T3 cells) were induced by ACNU to quantitatively describe the DNA damages. ICLs were detected by a modified version of the comet assay that can observe ICLs block the migration through an agarose gel of DNA by adding DNA breaking agents [14]-[15]. In addition, phosphorylated histone H2AX (γ-H2AX) were examined to demonstrate DNA double-strand breaks and the dependence of ICLs on replication and cell cycle was also assessed.

II. Materials and Methods

A. Materials

3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]- 1-(2-chloroethyl)-1-nitrosourea hydrochloride (nimustine, ACNU), dimethyl sulfoxide (DMSO), and low melting agarose (LMA) was obtained from Sigma-Aldrich. Propidium iodide was supplied by MERCK and Tert-butyl hydroperoxide (tBHP) was purchased from Sinopharm Chemical Reagent Co.,Ltd. Cell Counting Assay Kit-8 and GSH quantification kit was supplied by Dojindo Molecular Technologies (Gaithersburg, MD), Dulbecco's modified eagle's medium and fetal bovine serum were from Hyclone. ACNU was dissolved in ddH2O to prepare the stock solution at 20mg/ml before experiment.

B. Cell Culture

The NIH/3T3 mouse fibroblast cell line was obtained from Cell Resource Center (CRC). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cells were cultured a in a CO_2 incubator at 37℃. Passage them with 0.25% trypsin and 0.02% EDTA, when the cell grew to ~80% confluence.

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C. Cytotoxicity Assay

For determination of ACNU toxicity, NIH/3T3 cells were seeded (1×10^4 cells per well) into a 96-well microtitre plates and cultured at 37 ℃. At 24h intervals, the cells were treated with 100μl various concentration of ACNU in serum-free medium for 24h. For evaluating the cytotoxicity of ACNU, add 10μl the Cell Counting Assay Kit-8 solution to each well [16], and then incubation the plates at 37 ℃ in the incubator for 1h. The absorbance at 450 nm was read on a standard plate reader.

D. The Single Cell Gel Electrophoresis (Comet) Assay

DNA interstrand crosslinking and DNA breaking was analysed using the single cell gel electrophoresis assay described by Günter Speit [17]. Typically, 5×10^4 cells per well were cultured 24h in a 12-well plate. The next day, cells are exposed to 15, 30, 45, 60 or 75 μg/ml ACNU at 37°C for 2h. After treatment, 5×10^4 cells in 0.5% low melting point agarose were spread on microscope slides precoated with 1% nomal melting point agarose. The slides were immersed the slides in cold lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris, pH 10; 1% Triton-X and 10% DMSO were added fresh) at 4 ℃ over night. After that, placed the slides in electrophoresis buffer (300mM NaOH and 1mM EDTA, pH >13) to unwind DNA at 4 ℃ for 30min. The slides were electrophorsed for 30 min at 25 V and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl buffer (pH 7.5) for three times, stained with Gel-Red (10,000×, Biotium) and dried with 75% ethanol. Analyzed the results using a fluorescence microscope (Leica DM3000) and image analysis (Delta Sistemi). 100 cells per sample were evaluated the DNA percentage in the tail.

E. γ-H2AX Immunocytochemistry

NIH/3T3 cells were harvested and cultured 1×10^5cells per well in 6 well plates. After incubated 24h, cells were treated with 15, 30 μg/ml ACNU in serum free medium at 37°C for 2h. Thereafter cells were fixed using ice cold 4% paraformaldehyde, washed with PBS. The γ-H2AX immunocytochemistry assay was described previously [18]. Permeabilized cells with 0.2% Triton X-100 in PBS for 15 min, washed in PBS, blocked with normal goat serum blocking buffer and incubated with anti-phospho-Histone H2A.X Ser139 (Millipore) at 1:1000 dilution over night at 4 ℃. The next day, cells were washed in PBS and incubated with FITC secondary antibody at 37 ℃ for 1h in the dark. Cells were next treated with 0.1 mg/ml RNase A for 30 min and then stained with propidium iodide (2μg/ml). Removed cover slips to microscope slides and captured images using a Leica TCS-SP5confocal laser scanning microscope (100×oil immersion objective).

F. Flow Cytometry Assay

NIH/3T3 cells (1×10^5/well) were seeded in 6-well plates and incubated at 37 ℃ over night. The next day, after the treatment of cells with various concentrations of BCNU for 2h in serum free medium at 37 ℃, cells were cultured in full medium at 37 ℃. Harvested the cells by typsin at the required time point, centrifuged for 5 min at 800×g, washed with PBS, and fixed with chilled 70% ice-cold ethanol at -20 ℃. Prior to analysis, cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min, treated with 0.5 mg/ml RNase A at 37 ℃ for 30 min, stained with 50 μg/ml propidium iodide in PBS. Fluorescence of the PI was measured by COULTER ELITE ESP flow cytometer and the Expo32 software was used to analyze the percentage of cells in each phase of the cell cycle.

III. Results

A. Evaluation of the Cytotoxic of the ACNU in NIH/3T3 Cell

To characterized drug sensitivity, NIH/3T3 cells were incubated with various concentration of ACNU for 24h, and growth determined by colormetric assays. Growth curves for NIH/3T3 cells were shown in Fig. 1. After 24h treated of drug, ACNU induced cytotoxicity in NIH/3T3 cells with the IC50 about 600 μg/ml. At lower ACNU concentrations (＜ 100μg/ml), less growth inhibition was observed, but it can produce markedly increased cytotoxicity between the concentration of 100~900μg/ml. Based on this finding, the subsequent experiments involved exposing cells to 15, 30, 45, 60, 75 μg/ml of BCNU for a fixed period of 2 h.

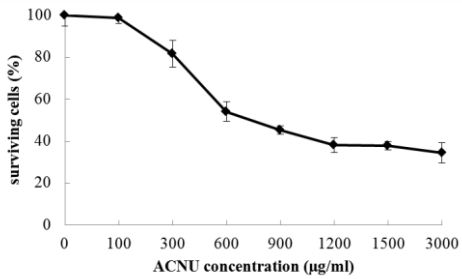

Fig. 1. ACNU induces concentration-dependent cell death in NIH/3T3 cells. Cultures were exposed to increasing concentrations of ACNU for 24h.

B. Induction of DNA Breaking and Cross-Linking in the NIH/3T3 Cells

Five concentration (15, 30, 45, 60, 75 μg/ml) were chosen to investigate the DNA damage and interstrand cross-links capability of ACNU using a modified comet assay. The result was present in Fig. 2, ACNU caused a concentration-related decrease in DNA migration. At all of the five concentration of ACNU, DNA damage was significant observed. Although the cytotoxicity of ACNU is weak, the induced DNA breaks also can be observed, indicated that alkaline comet assay is one kind of a sensitive and superior technique for DNA damages detection.

To observe the DNA migration-retarding effect of cross-links, a co-treatment experiment using a second genotoxic agent was performed. The second genotoxic agent causes DNA breaks, and if a substance induces cross-linking, one will expect a decrease in DNA migration caused by the second genotoxic agent when cells are treated with this substance [19]. In this study, NIH/3T3 cells were exposed to 400 μM tBHP for 1h, and then different concentration of ACNU were treated for 2h. The results in Fig. 3 confirm that all concentration of ACNU caused decreases in DNA migration compared to the control (cells were cultured with tBHP only), and DNA migration was

markedly decreased at 75 μg/ml. This result demonstrated that ACNU (15 to 75 μg/ml) induced a concentration-related decrease in DNA migration.

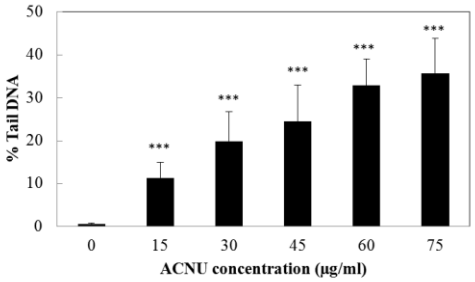

Fig. 2. DNA migration caused by different concentraions of ACNU on NIH/3T3 as determined by alkaline comet assay. Cells were exposed to ACNU at 37℃ for 2h. Results were shown as mean±S.D. of three independent samples (*p<0.05; **p<0.01; ***p<0.001).

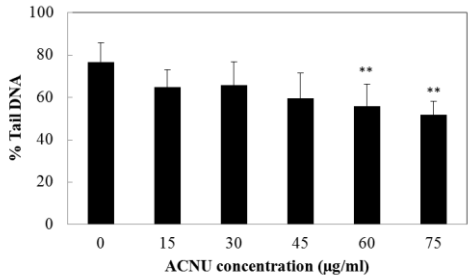

Fig. 3. Confirmation of the presence of ACNU-induced cross-links. NIH/3T3 cells were first treated with 400μM tBHP at 37℃ for 1h and then with different concentrations of BCNU at 37℃ for 2h. Results were shown as mean ±S.D. of three independent samples.

C. Immunofluorescence Microscope Detection of γ-H2AX Foci

γ-H2AX formation has been used as a marker for DNA damage associated with ICLs [20], [21].The histone H2AX is rapidly phosphorylated in large chromatin domains that flank the DNA DSBs; these domains can be observed by immunofluorescence microscopy and are termed γ-H2AX foci [22]. In this experiment, NIH/3T3 cells were treated with ACNU for 2h and stained for γ-H2AX post-treatment. As shown in Fig. 4, compare to the control, after treatment of 15μg/ml ACNU, the number of γ-H2AX foci did not exhibit a significant increase. When the cells were treated with 30 and 45μg/ml, the number of γ-H2AX foci increased slightly. However, with ACNU of 60 and 75μg/ml, the foci presented notable increase. The data, therefore, supported the conclusion that high concentration of ACNU caused noticeable DNA interstrand crosslinks.

D. Cell Cycle Dependence of ACNU-Induced Toxicity

It is known that ICLs can lead to a stalled replication fork in S-phase and the cells damaged by ICLs activate cell-cycle checkpoint and arrest at late S to G2 [23]. Therefore G2-phase arrest is an important mechanism to provide time for repairing DNA damage before the cells enter mitosis and propagate. To examine the effect on cell cycle induced by ACNU, cells were treated with different concentration of ACNU for 2h and cultured with full medium for 6 days. From the result (Fig. 5), it was observed the cell cycle distribution of control NIH/3T3 cells was 65±5, 20±3, and 15±6% in the G1, S and G2 phases, respectively. After the following 24h of incubation, there was markedly accumulated in G2 phases at all of concentration and reached a maximum at 15, 30 and 45μg/ml (30±6, 32±7 and 38±10). However, the G2 fraction gradually declined to at 2d, 3d and 4d at the concentration of 15, 30 and 45μg/ml (7±5, 9±7 and 6±4). At 60 and 75μg/ml, the percentage of G2 cells reached maximum after incubated for 48h (49±6 and 57±7). Subsequently, restore to normal cells at 6d and7d (13±8 and 9±8). All this results showed that DNA damages will induce cells arrest in G2/M phase, and come to normal after DNA damages are repaired.

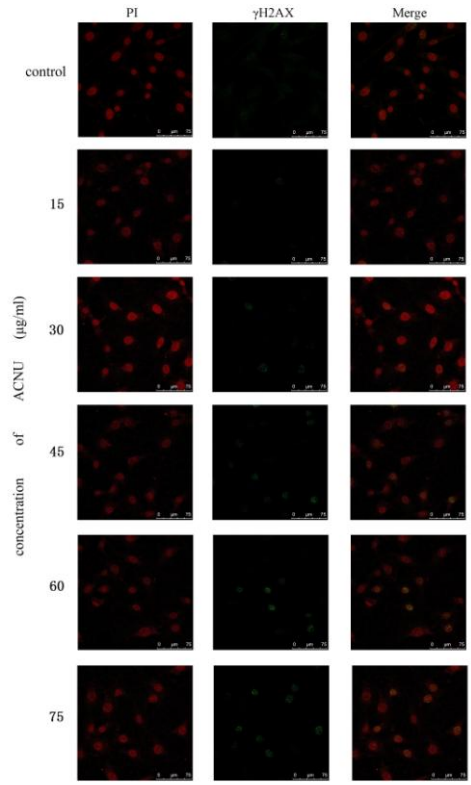

Fig. 4. Immunocytochemistry staining for γ-H2AX phosphorylation (green) in NIH/3T3 cells after 2h treated with 15 to 75μg/ml of ACNU. Control is untreated by ACNU, and DNA counterstaining is with PI.

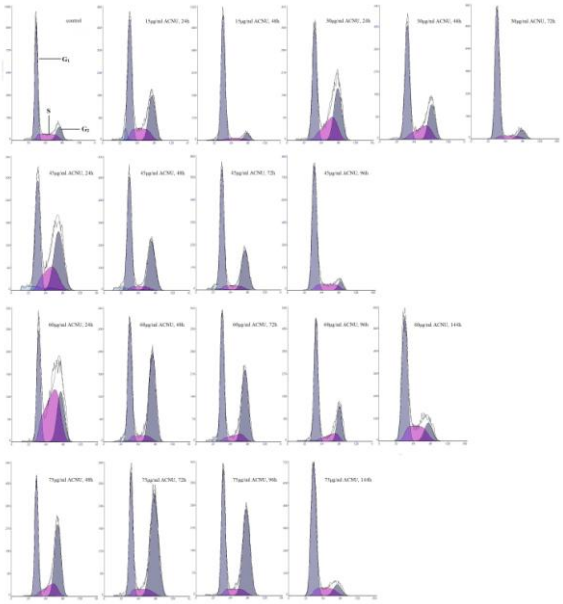

Fig. 5. Flow cytometric analysis of NIH/3T3 cells after treated with ACNU for 2h.The cells were seeded at 1×105/well in in 6-well plates and harvested at the indicated time points. The cell cycle profiles shown are representative of at least three independent experiments.

IV. Discussion

As bifunctional alkylating agents, nitrosoureas were widely used to be anti-cancer drugs because their ability of inducing DNA interstrand crosslinks. However, they are limited due to the resistant of cancer cells and the potential threat in inducing acute myeloid leukaemia (AML). It is not cleardr. It is not clear that the mechanism of nitrosoureas caused the cancer. Therefore, it is very important to study the relationship between nitrosoureas and DNA cross-links in normal cells, and further to know the correlation of DNA cross-links and cancer. In this work, we study the damage, specially ICLs induced by ACNU which is one of nitrosoureas in the primary mouse embryonic fibroblast cells (NIH/3T3 cells) and the impact on cell cycle. As a bifunctional electrophile, ACNU is able to form DNA monoalkylated adducts and cross-links. Monoalkylated adducts prone to spontaneous depurination to form single-strand breaks (SSB) or alkali-labile sites (ALS), that presented DNA migration-increase in comet assay. On the contrary, DNA cross-links presented DNA migration-reducing in comet assay. Therefore, we used alkaline comet assay to demonstrate the DNA damage induced by ACNU. From the result, a positive concentration-dependent relationship was exhibited for DNA breaking damage (Fig. 3). The migration increased with the enhancing concentration of ACNU. At 15μg/ml, the DNA interstrand cross-links were not detected (Fig. 4). At 30 and 45μg/ml, the ICLs slightly increased were observed and the damage become significantly at 60 and 75μg/ml. It demonstrated that the DNA breaking damage predominated at low concentration of ACNU (⩽45μg/ml); however, the ICLs damage appeared at high concentration (⩾45μg/ml).

There has been reported when the DNA replication fork is encountered or during its repair, DNA interstrand crosslinks can be formed to be DSBs through the repair system of cells [24]. The appearance of phosphorylated γ-H2AX foci is considered to be a marker for recruiting DSBs repair proteins. Consequently, γ-H2AX may also be a potential and useful biomarker for predicting chemosensitivity during cancer therapy involving ICL drugs [25]. We investigated the number of γ-H2AX foci increased significantly after incubated with 60 and 75μg/ml of ACNU for 2h. These coincide with the trend of ICLs detected using comet assay. It indicated that DNA DSBs could be detected immediately after exposed to ACNU by forming γ-H2AX foci. γ-H2AX has the potential as a biomarker for detecting ICLs damage induced by ACNU.

DNA damage induced by ICLs is most critical in the S-phase of proliferating cells that rapidly slows down S-phase progression and ongoing DNA synthesis [26]. To repair the DNA damage, cells activate repair mechanisms and arrest at late S to G2. In agreement, our results demonstrated cells are arrested in the G2 phase of cell cycle to repair the DNA ICLs after treated with ACNU and the cells enter into next mitosis when finished repairing.

In conclusion, the present study describes the DNA interstrand crosslinks induced by ACNU to normal cells and the effect on cell cycle. To understand the mechanism of how nitrosoureas caused the second cancer in clinical cases, it necessary to choose normal cells as the study model. In our research, we used NIH/3T3 cell as study model and found that the concentration of 60 and 75μg/ml of ACNU could be detected significantly ICLs. The γ-H2AX has the ability to be a biomarker for DNA damage associated with ICLs induced by ACNU.

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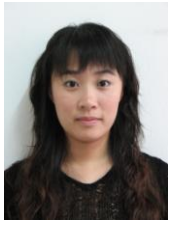

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