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Assessment of DNA interstrand crosslinks in NIH/3T3 cells induced by Chloroethylnitrosoureas

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Abstract: Aim: To investigated the mechanism of Chloroethylnitrosoureas (CENUs) leading to secondary tumors after therapy, three kinds of CENUs, namely, Nimustine (ACNU), Carmustine (BCNU) and Semustine (MeCCNU) were used to investigated drug-induced DNA interstrand crosslinks(ICLs). **Method:** The alkaline comet assay was adopted to compare DNA damages induced by CENUs under different concentrations. **Results:** With the increase of drug's concentration, DNA migration exhibited a positive concentration-dependent relationship in all three drugs. ACNU was shown to cause significant crosslinking. The other two drugs also induced crosslinking, but this could not be analyzed at high concentrations due to the high cytotoxicity of the drugs which caused cells death.

1 Introduction

Recently, cancer has become a major health issue in the world because of its high mortality and incidence. Although many pathogenic mechanism of cancer have been proposed, its real mechanism remains unclear. Epidemiological studies have shown that almost 80%-90% of human cancers are caused by the environment[1], being chemical pollutants the most important cancer-causing factors. Nitrosoureas are a class of DNA alkylating-agent anticancer drugs that include Chloroethylnitrosoureas (CENUs), such as 1-((4-amino-2-methyl -5-pyrimidinyl) methyl)-3-(2-chloroethyl)-3-nitrosourea (Nimustine, ACNU), 1,3-bis(2-chloroethyl)-1-nitrosourea (Carmustine, BCNU), 1-(2-chloroethyl)-3- cyclohexyl-L-nitrosourea (Lomustine, CCNU), 1-(2-chloroethyl)-3- (4-methlycyclohexyl) -1-nitrosourea (Semustine, MeCCNU) and 1-[N-(2-chloroethyl)-N-nitrosourea] ethylphosphonic acid diethyl ester (Fotemustine, FUNU)[2]. Previous studies have indicated that the cytotoxicity of CENUs is related to the formation of DNA interstrand crosslinks (ICLs)[3]. ICLs are extremely toxic to cells since they prevent the separation of the two strands of a DNA double helix for

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cellular processes, such as replication and transcription, by coordinating chemical reactions with bases on opposing strands[4]. The formation of ICLs between complementary base pair is supposed to be the critical step for the anticancer activity of CENUs[5]. Zhao's group found that the ICLs between the N^1 atom of guanine and the N^3 atom of the complementary cytosine through an ethylene is theoretically determined to be the main crosslinking damage in the reactions of DNA and CENUs[6]. According to the clinical observation, CENUs can also lead to the generation of secondary tumors after the treatment. However, the carcinogenic mechanisms of CENUs are still poorly understood[7]. Reports showed that the pathogenetic mechanism of secondary tumors during therapy due to formation of ICLs[8]. Therefore, studies of drug-induced ICLs to reveal their anticancer mechanism and carcinogenic role are of great significance. To assess genotoxicity of drugs, there are different genetic markers and tests such as chromosomal aberrations, micronucleus test and alkaline comet assay[9]. Alkaline comet assay, also known as single cell gel electrophoresis (SCGE) assay, has been increasingly used in the areas of genotoxicity studies on account of its rapid, simple, sensitive and versatile superiority[10].

In this study, normal NIH/3T3 cells were used for a preliminary study of ICLs induced by three kinds of CENUs: ACNU, BCNU and Me-CCNU. The alkaline comet assay was performed to detect DNA breaks and interstrand cross-links, in order to further study the relationship between CENUs and ICLs.

2 Materials and Methods

2.1 Materials and cell culture

ACNU, BCNU and Me-CCNU, low melting agarose (LMA), and dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. *Tert*-butyl hydroperoxide (tBHP) was obtained from Sinopharm Chemical Reagent Co. Ltd. Propidium iodide was supplied by MERCK and normal melting agarose (NMA) was purchased from Shanghai yito bio-instrument company limited, China. Dulbecco's modified eagle's medium and fetal bovine serum were purchased from Hyclone.

The NIH/3T3 mouse fibroblast cell line was obtained from Cell Resource Center (CRC). The cells were maintained as monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. Cells were grown at 37°C in an incubator with a humidified atmosphere of 5% CO_2.

2.3 Cytotoxicity assay

Cytotoxicity of NIH/3T3 cells against CENUs was performed according to *K. Ishitsuka et al* [11]. NIH/3T3 cells were harvested with 0.25% trypsin and 0.02% EDTA, then were seeded (1×10^4 cells per well) on a 96-well plate. After incubated for 24 h, cells were treated with various concentrations of CENUs in serum-free medium for 24 h. To evaluate the cytotoxicity, 10 μl of Cell Counting Assay Kit-8 (Dojindo molecular Technologies, Japan) solution was added to each well, afterwards the microplates were incubated at 37°C for 1-2 h. Absorbance was measured at 450 nm using a microplate reader.

2.4 The alkaline comet assay

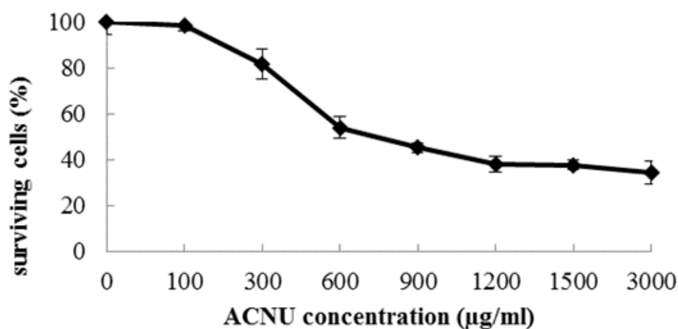
The alkaline comet assay described by Günter Speit[12] was used to assess CENUs -induced DNA damage and DNA interstrand cross-links in NIH/3T3 cells. Cells were cultured at 5×10^4 cells /well into a 12-well plate. The next day, cells were exposed to concentrations of 15, 30, 45, 60 or 75 μg/ml CENUs at 37°C for 2 h. After treatment, approximately 2500 NIH/3T3 cells in 50 μl DMEM were mixed with 5 μl 1% low melting point agarose. The mixture was added to microscope slides precoated with 1% nomal melting point agarose. The slides were immersed overnight in lysis solution (2.5 M

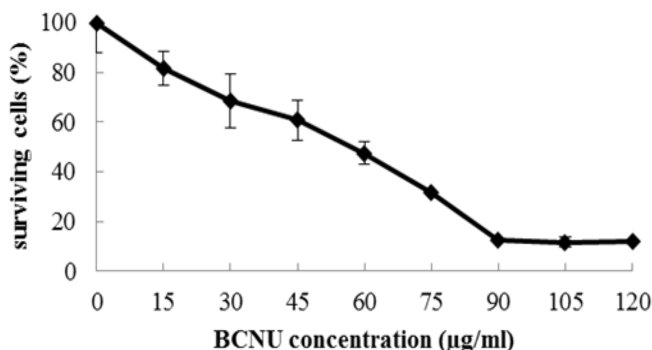
NaCl, 100 mM EDTA, 10mM Tris, pH10, 1% Triton-X and 10% DMSO were added fresh) at 4°C, and then placed in electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH >13) at 4°C for 30 min to unwind DNA. Electrophoresis was performed at 25 V and 300 mA for 30 min. The slides were then washed in neutralizing solution (0.4 M Tris-HCl, pH7.5) three times, stained with Gel-Red (10,000×, Biotium) and dried with 75% ethanol. The results were analyzed using a fluorescence microscope (Leica DM3000) and image analysis software I.A.S (Delta Sistemi), and the comet parameter (mean percentage of DNA in the tail) was used to demonstrate the damage of DNA.

3 Results

3.1 Concentration-dependent death of NIH/3T3 cell caused by CENUs

To investigate the effect of CENUs on normal cell death, the NIH/3T3 were exposed to increasing concentrations of CENUs for 24 h, and cell viability was measured using the CCK-8 assay. As shown in Figure 1, all of the three kinds of CENUs induced a concentration-dependent decrease in cell viability, and half maximal inhibitory concentration (IC50) of ACNU, BCNU, Me-CCNU were 750, 60 and 65 μg/ml respectively. These results indicated that the cytotoxicity for normal cells of both BCNU and Me-CCNU is more than 10 fold higher than ACNU. Based on this, several concentrations nearby the IC50 of BCNU or Me-CCNU were further analyzed, and cells were treated with concentrations of 15, 30, 45, 60 and 75 μg/ml for all three drugs.





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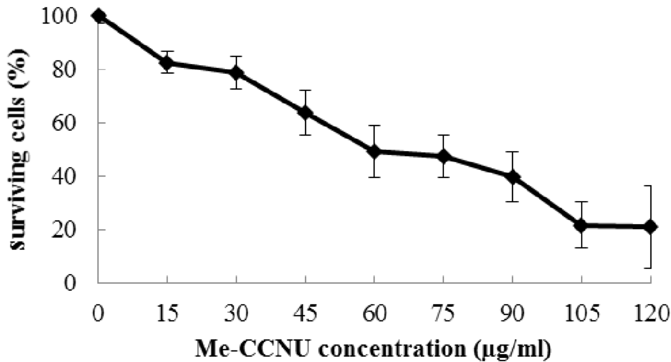
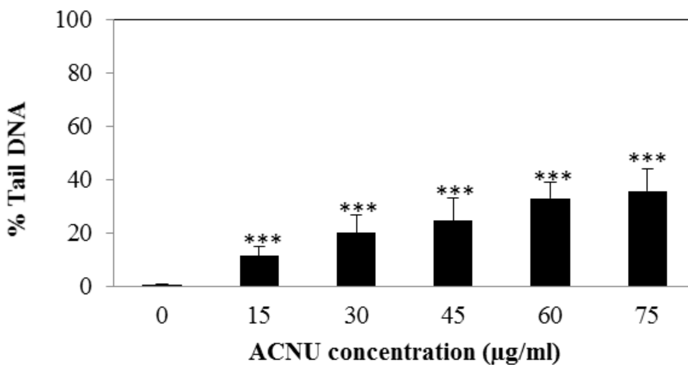
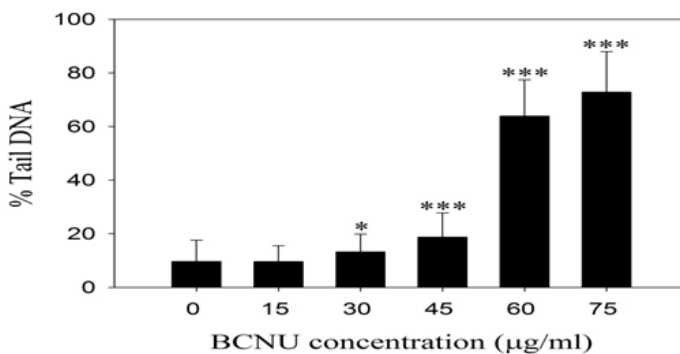


Figure 1. Cytotoxicity of CENUs in different concentrations in NIH/3T3 cells.

3.2 CENUs -induced DNA breaks

The concentration-dependent DNA damage in NIH/3T3 cells treated with CENUs (15, 30, 45, 60, 75 μg/ml) was measured using a modification of the comet assay. As shown in Figure 2, all of the three drugs showed a positive concentration-dependent relationship, DNA migration is directly proportional to the concentration of drugs. In the given concentrations, DNA breaks caused by ACNU were in a lesser degree than the other two drugs, the rate of tail to total being lower than 50%. The result indicates that at lower concentration of BCNU, DNA breaks are in lower level, however, at higher concentration, BCNU induced DNA breaks are predominant.





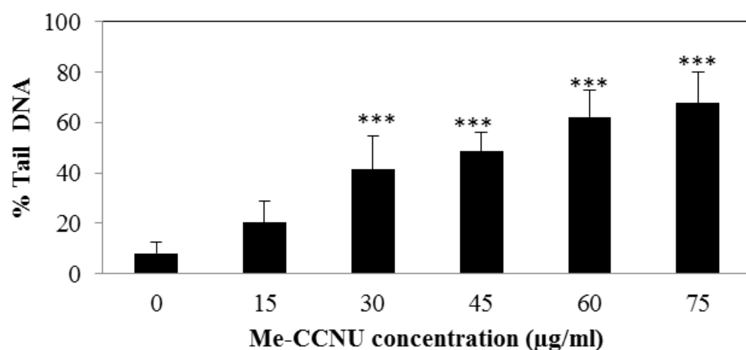
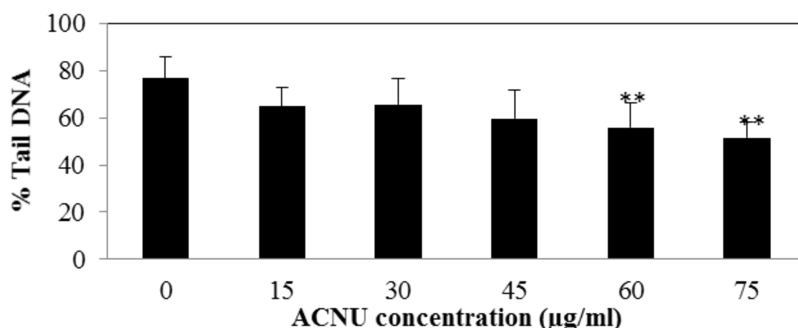


Figure 2. DNA migration caused by different concentrations of CENUs as determined by alkaline comet assay. Results were shown as mean±S.D. of three independent samples(n=3). (*p<0.05; **p<0.01; ***p<0.001)

3.3 CENUs -induced DNA interstrand crosslinks

To observe the DNA migration-retarding effect of cross-links, a co-treatment experiment using a second genotoxic agent was performed. In the present research, *tert*-butyl hydroperoxide (tBHP) was used as the second genotoxic agent[13]. This genotoxic agent can cause DNA breaks, and if a substance induces cross-linking, one will expect a decrease in DNA migration caused by the second genotoxic agent. In this experiment, NIH/3T3 cells were exposed to 400 μM tBHP for 1h, and then different concentrations of CENUs were added. As shown in Figure 3, with the increase concentration of ACNU, DNA migration decreased, which indicated that ACNU could cause the DNA ICLs. Because of the low cytotoxicity of ACNU, higher concentrations around IC50 were also selected for further analysis. The result showed that a significant rate of ICLs were observed especially at 150 and 300 μg/ml. However, the DNA breaks increased at 450 and 600 μg/ml, which indicated that at 150 and 300 μg/ml of ACNU the rate of interstrand cross-link is higher than the rate of DNA breaks. For BCNU, a similar result was observed at concentrations of 15 and 30μg/ml. BCNU led to statistically significant decreases in DNA migration. However, the decreases were not statistically significant at 45 μg/ml, and the DNA migration showed a small increase at 60 and 75 μg/ml. This result indicates that the effect is very strong, confirming the presence of cross-links at concentrations of 15 and 30 μg/ml. Nevertheless, ICLs weren't observed under the given concentration for Me-CCNU. Its high cytotoxicity causes cell death at higher concentrations, making it difficult to conclude whether Me-CCNU could cause ICLs.



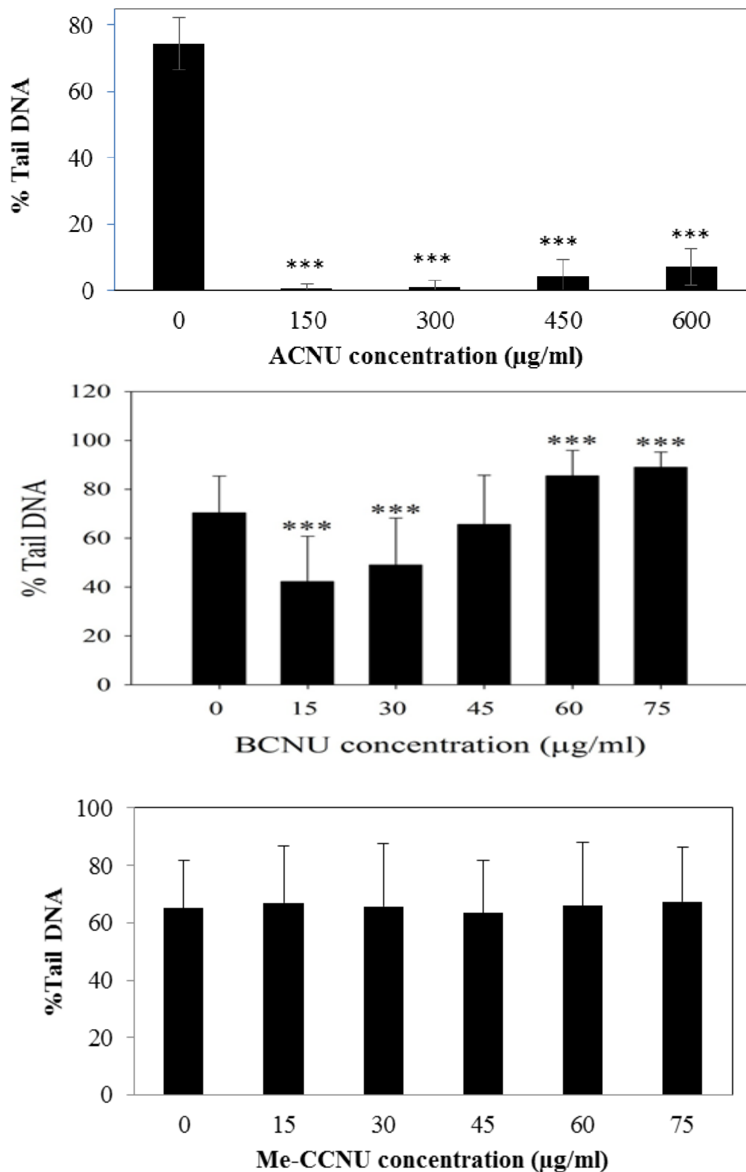


Figure 3. Confirmation of the presence of cross-links. NIH/3T3 cells were first treated with 400 μM tBHP at 37°C for 1h and then with different concentrations of CENUs at 37°C for 2 h. Results were shown as mean ±S.D. of three independent samples(n=3) (*p<0.05; **p<0.01; ***p<0.001)

4 Discussion

CENUs are widely used in the treatment of different cancers. They damage DNA by alkylation of bases, single/double strand breakages and interstrand crosslinks[14-15]. DNA damage caused by different nitrosoureas has been studied via different methods of detection, such as agarose gel electrophoresis assay[16], fluorescence assay[17] and lipid chromatography/mass spectrometry (LC/MS)[18]. Being highly sensitive and specific, the comet assay proved to be a useful technique to evaluate the degree of DNA damage[19].

Our study compared the interstrand crosslinking induced by different kinds of CENUs via single cell gel electrophoresis (SCGE) assay firstly, and it showed that SCGE assay is a convenient method

to detect the DNA breaks and interstrand crosslinks. Clinical observations showed that CENUs may also cause secondary tumors during the treatment of cancer. For this reason, it is important to establish a DNA cross-links cell model for normal cells, therefore NIH/3T3 cells from the primary mouse embryonic fibroblast were used in our research.

From the results of the cytotoxicity test, IC50 for both BCNU and Me-CCNU are similar, and ACNU displayed the lowest toxicity with 10 fold lower than the other two. Furthermore, ACNU is water soluble with Me-CCNU slightly soluble, and BCNU has a higher solubility in organic solvents. In the same series concentrations, all of the three drugs showed a positive concentration-dependent relationship, DNA breakage increased in direct proportion concentration of drugs. The results of interstrand crosslinks indicated that the treatment with BCNU at concentrations lower than 100 μg/ml, in particular at 15 and 30 μg/ml, present the most obvious ICLs. At concentration higher than 100 μg/ml, ACNU could induce a big rate of ICLs, but cells treated with the other two drugs died because of their high cytotoxicity. Based on these results, ACNU is the most suitable CENUs for further study of DNA interstrand crosslinks since its water-solubility, low cytotoxicity and high DNA crosslinking.

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