[Chem. Pharm. Bull. 34(1) 188—193 (1986)]

Different Sensitivities of Rat Ascites Hepatoma AH13 and Mouse Lymphoid Leukemia L1210 Cells to Typical *N*-Nitrosoureas

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(Received March 14, 1985)

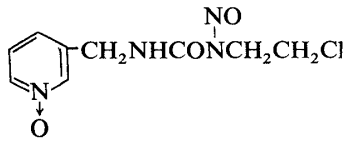
Three structurally typical *N*-nitrosoureas showed different life-prolonging activities on animals bearing ascites hepatoma AH13 and lymphoid leukemia L1210 cells. 1-(2-Chloroethyl)-1-nitroso-3-(3-pyridylmethyl)urea *N*-oxide (CNPyUO) was highly effective against both AH13 and L1210, 1,3-diphenyl-1-nitrosourea (DPhNU) was effective against AH13 but not against L1210, and 1-methyl-1-nitrosourea (MNU) was effective against L1210 but not against AH13.

The cytostatic and deoxyribonucleic acid (DNA)-damaging activities of these *N*-nitrosoureas on both tumor cells *in vitro* or *in vivo* were studied. In the case of CNPyUO treatment *in vivo*, the formation of cross-linked DNA caused cell death of both tumor cells at 72 h after treatment. DPhNU treatment rapidly caused scission of single-stranded DNA of the tumor cells, leading to cell death. MNU treatment caused scission of single-stranded DNA of both cells but showed cytostatic action only on L1210 cells.

Keywords——*N*-nitrosourea; AH13; L1210; 1-(2-chloroethyl)-1-nitroso-3-(3-pyridylmethyl)-urea *N*-oxide; 1,3-diphenyl-1-nitrosourea; 1-methyl-1-nitrosourea; cytostatic action; trypan blue inclusion; DNA injury; alkaline elution

Various types of *N*-nitrosoureas have been synthesized to obtain more efficient and stable antitumor *N*-nitrosoureas in this laboratory.[1] In these *N*-nitrosoureas, we found that there are three structurally typical groups of compounds in the mode of their sensitivities against rat ascites hepatoma AH13 and mouse lymphoid leukemia L1210 cells. As shown in Table I, 1-(2-

TABLE I. Antitumor Activity of CNPyUO, DPhNU and MNU



1-(2-chloroethyl)-1-nitroso-3-(3-pyridylmethyl)urea *N*-oxide

(CNPyUO)

1,3-diphenyl-1-nitrosourea

(DPhNU)

1-methyl-1-nitrosourea

(MNU)

	Donryu-AH13					CDF_1-L1210		
	MTD (mg/kg)	MED (mg/kg)	Days 3—7 dose (mg/kg) × 5	60 d survivors	max. *T/C* % at 60th day	Toxic dose days 2, 6	Days 2, 6 dose (mg/kg) × 2	max. *T/C* % at 30th day
CNPyUO	25	1	5	3/5	>585	20 × 2	10	>417
DPhNU	100	25	10	2/6	>390	30 × 2	15	119
MNU	100	100	20	0/6	142	Not tested	80	236

MTD, maximum tolerance dose. MED, minimum effective dose.

chloroethyl)-1-nitroso-3-(3-pyridylmethyl)urea *N*-oxide (CNPyUO) is highly effective against both AH13 and L1210, 1,3-diphenyl-1-nitrosourea (DPhNU) is effective against AH13 but not against L1210, and 1-methyl-1-nitrosourea (MNU) is effective against L1210 but not against AH13.

CNPyUO, having a 1-(2-chloroethyl)-1-nitrosoureido group, has both an alkylating activity (including the formation of deoxyribonucleic acid (DNA)–DNA inter- or intra-strand cross-links) and a carbamoylating activity towards protein,[2] like 1,3-bis(2-chloroethyl)urea (BCNU).[3] DPhNU reacts with adenine at the N^6 amino group and guanine at the C^8 carbon to give the corresponding diazo compounds. It also carbamoylates the terminal amino group of lysine and nitrosates the SH group of *N*-acetylpenicillamine.[1f] On the other hand, MNU methylates and carbamoylates the nucleophilic groups of various cells and biological model compounds.[4]

Thus, we started to study the cytostatic and DNA-damaging activities of these three typical *N*-nitrosoureas on AH13 and L1210 cells in order to understand the observed differences in sensitivity of the tumor cells to these compounds.

Results

Cell viability was estimated on the basis of exclusion of trypan blue dye (dead cells are stained with trypan blue). Table II shows the effect of the three *N*-nitrosoureas after a 2 h incubation at 37 °C. The order of cytostatic action against AH13 cells *in vitro* was DPhNU≫CNPyUO>MNU, while that against L1210 cells was DPhNU≫MNU> CNPyUO. The order of effectiveness against each cell line *in vitro* did not correlate with the life-prolonging activity *in vivo*.

The effect of intraperitoneal (*i.p.*) administration of the compounds on survival of AH13 cells is shown in Table III. In the case of CNPyUO, no increase of dead cell ratio was observed until 72 h. The maximum increase was observed at 5 h after DPhNU treatment, and the dead cell ratio slowly recovered to the normal level at 48 h after the treatment. Though the dose of MNU was about five times higher per molecular weight as compared with those of CNPyUO and DPhNU, the dead cell ratio of AH13 cells was scarcely increased by MNU treatment. Table III also shows the effect of *i.p.* administration of the compounds on L1210 cells. In the

Table II. Cytostatic Activity of Compounds *in Vitro*

Treatment		Dead cell ratio (%)[a] at 2 h incubation	
Compd.	Conc. (μg/5 ml)	AH13	L1210
CNPyUO	50	13	2
DPhNU	50	70	88
MNU	100	5	13

a) Dead cell ratio of treated cells − dead cell ratio of control cells.

Table III. Cytostatic Activity of Compounds *in Vivo*

Treatment			Dead cell ratio (%)	
Compd.	Dose (mg/kg)	Time (h) after injection	AH13	L1210
CNPyUO	30	2	3	5
		24	4	22
		48	4	3
		72	11	11
DPhNU	30	2	58	51
		5	75	17
		24	8	3
		48	4	1
MNU	60	2	4	6
		24	3	2
		48	2	6

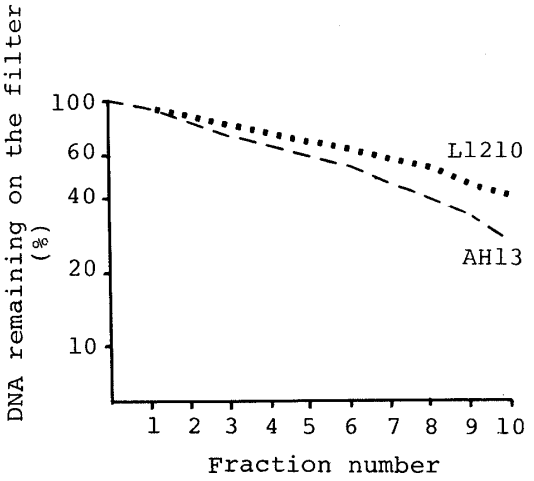


Fig. 1. Elution Profile of DNA from AH13 and L1210 Cells in Ascites Fluid of Animals Inoculated 4 d before

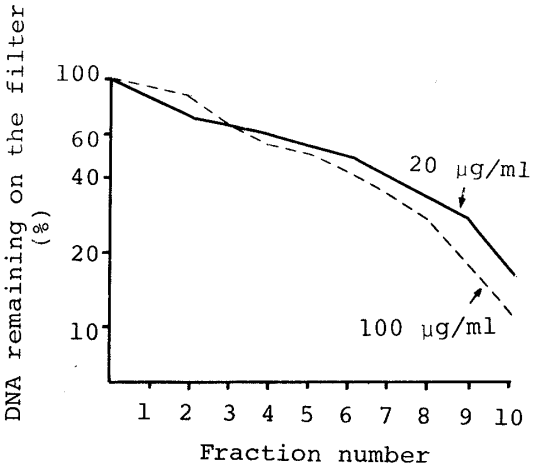


Fig. 2. Elution Profile of DNA from L1210 Cells Treated with MNU *in Vitro*

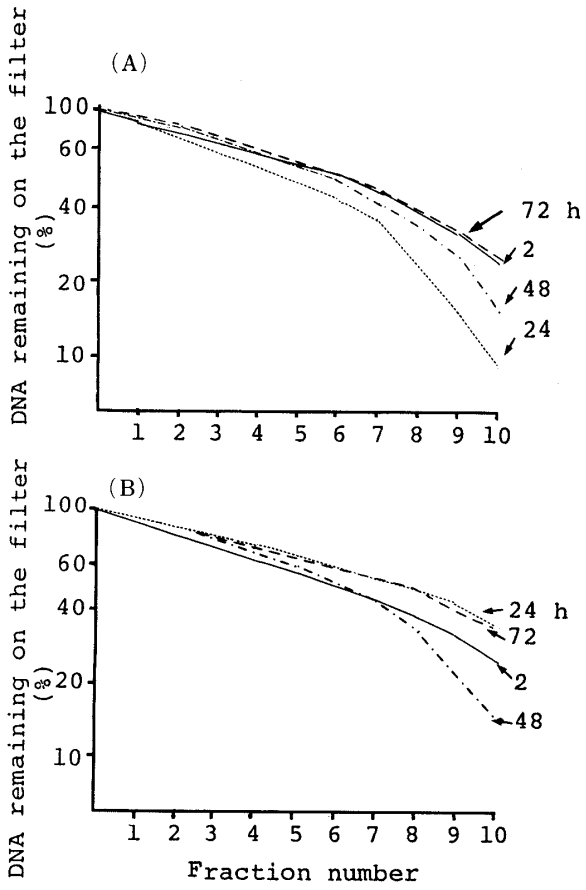


Fig. 3. Elution Profile of DNA from AH13 (A) and L1210 (B) Cells Treated with CNPyUO *in Vivo* (*i.p.*, 30 mg/kg)

case of CNPyUO, the dead cell ratio of L1210 increased biphasically after 24 and 72 h. With DPhNU, the ratio reached a maximum after 2 h and then decreased. With MNU, a slight increase of the dead cell ratio of L1210 cells was observed after 48 h. In the *in vivo* study, the cytostatic effect of DPhNU was the highest against both cells. Moreover, AH13 cells were more sensitive to the cytostatic action of DPhNU than L1210 cells.

The alkaline elution profile is indicative of single-strand scission, and the alteration of DNA length can be estimated by means of the alkaline elution assay.[5] The effect of the *N*-nitrosoureas on DNA of tumor cells was examined *in vivo*. Figure 1 shows the alkaline elution profiles of AH13 and L1210 cells from the ascites fluid of rat or mouse inoculated 4 d previously. Namely, 26 and 40% of DNA of AH13 and L1210 cells, respectively, remained on the filter after passing 20 ml of eluate. After a 2 h incubation with MNU (concentration 100 μg/5 ml) *in vitro*, the alkaline elution of L1210 cells was facilitated. As shown in Fig. 2, at higher concentrations of MNU, more single-strand scission was observed in L1210 cells.

Figure 3 shows the elution profiles of DNA from AH13 and L1210 cells treated with CNPyUO *in vivo*. Thus, CNPyUO apparently affected DNA scission at 24 and 48 h after treatment *in vivo*. After the administration of DPhNU, single-strand scission was clearly observed at 5 and 2 h after treatment of AH13 and L1210 cells, respectively (Fig. 4). These

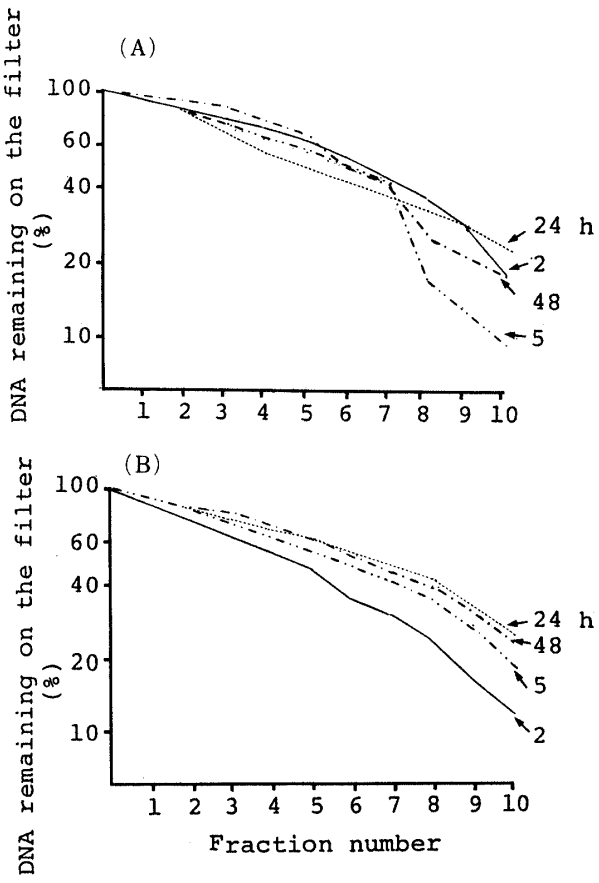


Fig. 4. Elution Profile of DNA from AH13 (A) and L1210 (B) Cells Treated with DPhNU *in Vivo* (*i.p.*, 30 mg/kg)

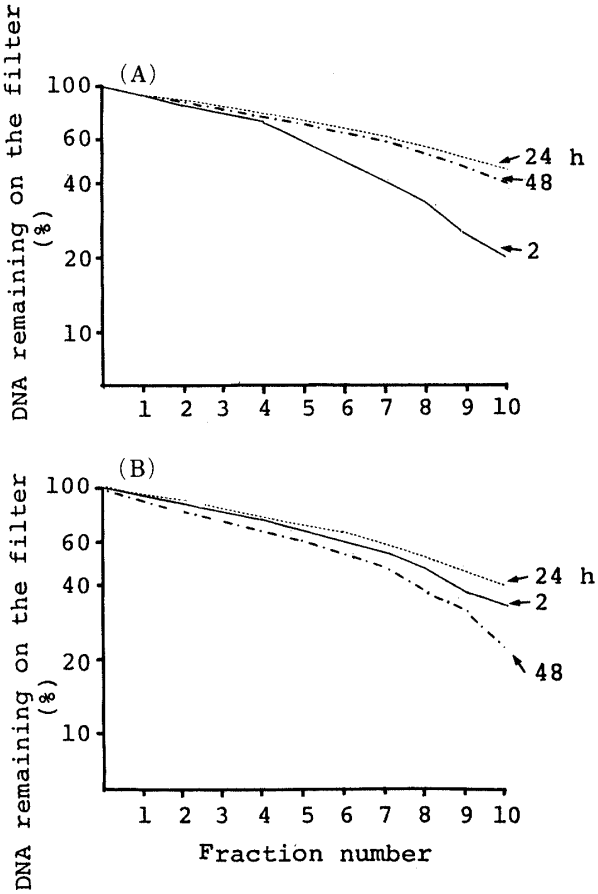


Fig. 5. Elution Profile of DNA from AH13 (A) and L1210 (B) Cells Treated with MNU *in Vivo* (*i.p.*, 60 mg/kg)

data show that recovery of L1210 cells from DNA damage was rapid. MNU treatment (Fig. 5) scissored DNA of AH13 and L1210 cells at 2 and 48 h, respectively.

Discussion

As reported in our preceding paper,[2] CNPyUO reacted with piperidine as a model compound for nucleic acid bases to form firstly 2-chloroethylated piperidine, and this further reacted with piperidine to form a cross-linked piperidine, 1,2-bispiperidinoethane, in good yield. These data show that CNPyUO will react with DNA to form alkylated DNA or cross-linked DNA, as occurs with BCNU.[6] *In vivo*, the CNPyUO treatment of AH13 cells increased the alkali-eluted DNA without any increase of the dead cell ratio at 24 h. In the case of L1210 cells, CNPyUO treatment increased the dead cell ratio at 24 h after treatment but did not increase the alkali-eluted DNA at that time. This indicates that the formation of mono-substituted DNA causes cell death of L1210 cells at 24 h, and cross-link intercepted alkaline elution of break-DNA. In both cells, cross-linked DNA should be a cause of cell death at 72 h after treatment. It causes a marked life-prolonging effect in treated animals bearing both tumors, respectively.

As reported in our previous paper[1f] DPhNU permeated quickly into the cytoplasm or nucleus when administered, and decompose to give active chemical species (phenyl diazonium ion, phenyl isocyanate and nitrosonium ion). The phenyl diazonium ion reacts with adenine and guanine to form diazophenylated nucleic acid bases. Phenyl isocyanate reacts with amino

groups of protein to form *N*-carbamoylated protein. The nitrosonium ion reacts with SH groups to form *S*-nitrosated protein. Thus, *N*-carbamoylation and *S*-nitrosation of enzyme protein should inhibit DNA repair enzyme activity. In combination therapy, administration of phenyl diazonium salts and phenyl isocyanate increased the life-span of AH13-bearing rats synergistically. We found that DPhNU treatment increased both alkali-eluted DNA and cell death in both cell systems. These results suggested that DNA single-strand excision causes cell death in both cells. Fig. 4 shows that regrowth of L1210 cells started more quickly than that of AH13 cells. Since L1210 cells have a shorter cell cycle time (12.8 h)[7] than AH13 cells (22 h)[8] and have an effective repair system, they recovered quickly from DNA damage caused by the treatment with DPhNU. In our data, daily administration of DPhNU to mice bearing L1210 cells did not increase the life span ($T/C\% = 107$). This suggests that L1210 readily acquired resistance to DPhNU.

MNU was reported to be a methylating[4a] and carbamoylating[4b] agent. MNU treatment caused DNA scission of AH13 but did not cause AH13 cell death. The repair system worked quickly, and AH13 growth continued, leading to the death of the tumor-bearing rats. On the other hand, DNA scission in L1210 was clearly observed at 48 h after administration of MNU. In AH13 cells, the excision repair system caused DNA scission at 2 h, whereas in L1210 cells, other repair systems work and after that, excision repair starts. According to Pegg and Hui,[9] the ability of cells to remove the O^6-methyl substituent from DNA is owing to O^6-alkylguanine-DNA alkyltransferase in animals, and this starts to work within a few minutes after methylation of DNA.

A possible action mechanism of these three *N*-nitrosoureas is proposed in Chart 1. These *N*-nitrosoureas react with DNA and the DNA causes DNA scission or DNA repair. The modified groups in the reacted DNA and enzymes are different with the three agents, and consequently the antitumor spectra against AH13 and L1210 cells differ.

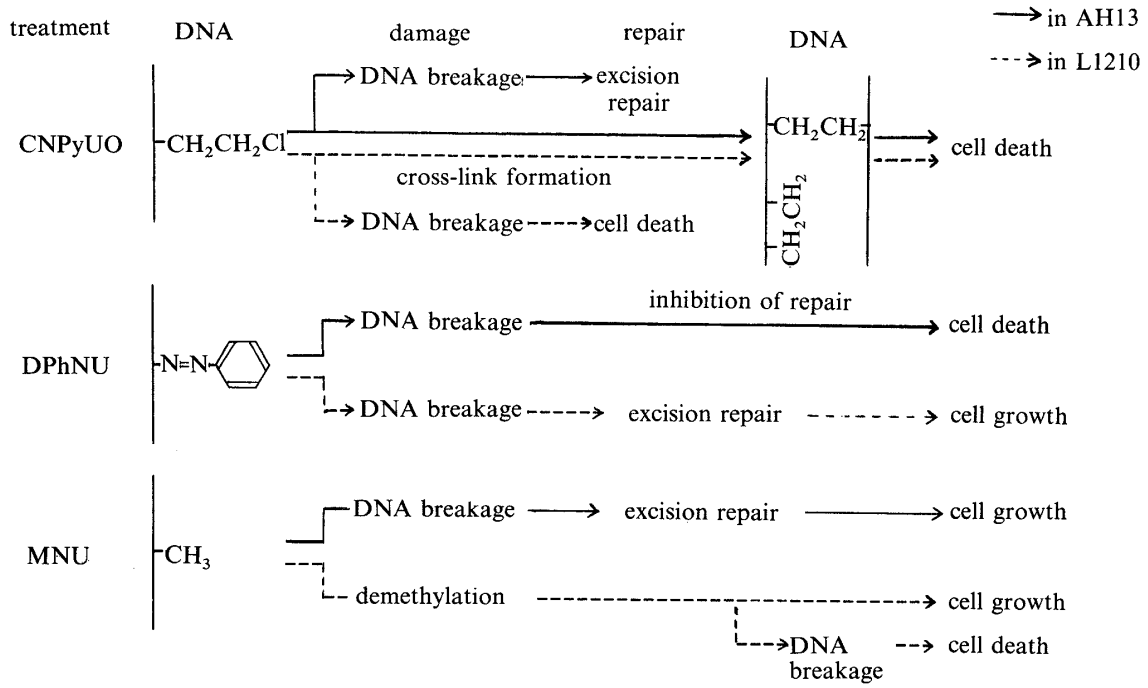


Chart 1. A Possible Action Mechanism of Three Typical *N*-Nitrosoureas

Materials and Methods

Cells——AH13 cells were kindly donated by the Sasaki Institute, Tokyo, and were propagated by intraperitoneal inoculation in Donryu rats (Nihon Rat Co., Tokyo) every 6 d. L1210 cells were kindly donated by the

Cancer Chemotherapy Center, Tokyo, and were propagated by intraperitoneal inoculation in DBA/2 mice every 6 d; CDF_1 mice (Shizuoka Agricultural Association for Laboratory Animals) were used for test.

Assay of Dead Cell Ratio——A 0.1 ml aliquot of 0.4% trypan blue solution in phosphate-buffered saline was added to the 0.5 ml cell suspension (10^6 cells/ml). Five min after the addition, total cells and stained cells were counted in a hemocytometer (dead cells were stained by trypan blue). The dead cell ratio was calculated as follows. Dead cell ratio (%) = (stained cells/total cells) × 100.

Alkaline Elution Assay——Alkaline elution was performed by the method reported by Cavanna *et al.*[5b,c] at 0.2 ml/min. Each fraction was 2 ml, and 10 fractions were collected. After the alkaline elution, the contents of DNA in the collected fractions were determined by the reported method. The fluorescence was read at 489 nm with excitation at 397 nm in a Hitachi MPF-4 fluorescence spectrophotometer. The blank readings were made with tubes containing 2 ml of eluting solution subjected to the same procedure.

Cell Preparation——*In Vitro* Assay: Cells were collected from the ascites fluid of tumor-bearing animals. Approximately 10^6 cells were suspended in Hank's solution.

In Vivo Assay: Tumor cells were intraperitoneally inoculated 4 d before treatment. Materials[10] were dissolved or suspended in physiological saline solution containing Tween 80 (0.5%). Animals were given a single intraperitoneal injection.

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