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Effects of ACNU, a Water-soluble Nitrosourea Derivative, on Survival and Cell Kinetics of Cultured HeLa S3 Cells

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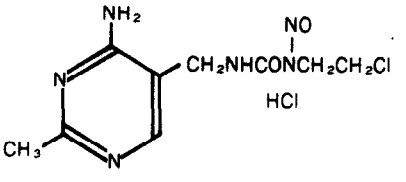
Abstract—*Effects of a water-soluble nitrosourea, 1(4-aminomethylpyrimidine-5yl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) was investigated on cultured HeLa S3 cells with regard to their lethality and cell progression through the cell cycle. The survival curve of exponentially growing cells exposed to increasing concentrations of the drug for* 1 hr *was characterized by a threshold type of response* (D_o = 7.0 μg/ml·1 hr, D_q 3.5 μg/ml·1 hr). *Throughout the cell cycle, ACNU exerted its main killing effect on cells in the* G_1 *and* $G_2 + M$ *phases, whereas cells in S were resistant to the drug. The change in their age-response was due to the* D_o *value of the dose-survival curve rather than the* D_q. *Effects of cell progression were also examined at a low concentration of ACNU* (5.0 μg/ml), *which allowed* 80% *of treated cells to survive. Delayed transit was observed in the S phase, and more markedly in the* $G_2 + M$ *phase. The magnitude of these perturbations depended on the position of the cell cycle at which the drug was administered. Cells treated in the* G_1 *and early S phases showed a much longer duration of S and* $G_2 + M$ *phases than cells treated in mid-S phase. Cells treated in the late S and* G_2 *phases could normally pass through mitosis, but were subsequently blocked in the* G_2 *phase following a prolonged S phase in the next cell cycle. These studies revealed that there seems to be some positive relationship of the effect of ACNU on cell progression and cell killing throughout the cell cycle.*

INTRODUCTION

ACNU (Fig. 1) is a water-soluble nitrosourea derivative synthesized by Nakao *et al.* [1] in 1974. Shimizu and Arakawa [2] found that the compound was highly active against L-1210 leukemia in BDF_1 mice and several other experimental tumors, whereas it has relatively low toxicity in experimental animals. Based on these animal studies, ACNU is now widely used to treat malignant tumors in clinical practice, in particular leukemia, lymphoma, brain tumors and lung tumors [13].

The antitumor activity of this compound is primarily assumed to be caused by alkylation of intracellular DNA, resulting in the inhibition of DNA synthesis [4]. However, there is little information available regarding the effects of ACNU on tumor cell kinetics. In this study the lethality and disturbance of progression through the cell cycle of ACNU were examined by using cultured HeLa S3 cells.



1–(4–amino–2–methylpyrimidin–5yl)
–methyl–3–(2–chloroethyl)–3–
nitrosourea hydrochloride

Fig. 1. Chemical structure of ACNU.

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Our findings are aimed to be an aid to the design of cancer chemotherapy using ACNU.

MATERIALS AND METHODS

Cell culture

HeLa S3 cells were used throughout the experiments. The cells were normally grown in monolayer culture in F-10 HI medium supplemented with 10% calf serum in a humidified atmosphere of 5% CO_2-air mixture at 37°C. The average cell cycle time under these conditions was about 20 hr, with a G_1 period of 6.5 hr, an S period of 9.5 hr and a G_2 + M period of 4 hr. All experiments were performed on cells in the exponential growth phase.

Drug treatment and survival assay

ACNU was supplied by Sankyo Co. Ltd, Tokyo, Japan. This compound was dissolved in Dulbecco's phosphate-buffered medium adjusted to pH 3.0 and stored at 4°C for stability. Just before use, the stock solution was diluted with prewarmed F-10 medium adjusted to pH 7.4 with HEPES buffer (20 mmol) to give the required drug concentrations.

Survival was determined by the ability of drug-treated cells to form colonies. Suspensions of single cells were harvested by trypsinization or mitotic collection, counted with a Coulter Counter (Model Z-B) and plated into plastic dishes (60 × 15 mm). The cell number to dish was adjusted to produce about 100 colonies. The cells could normally attach to the bottom of the dish within 2 hr after plating; they were then exposed to ACNU for 1 hr by replacing the culture medium by medium containing the drug. At the end of treatment, cells were rinsed twice with prewarmed medium and incubated for about 2 weeks.

Cell synchronization

Synchronous cells were obtained by a selection of mitotic cells as previously described [5]. Mitotic frequency of harvested cells throughout experiments was usually about 80%. The degree of synchrony was monitored by pulse labeling the cells with [^{3}H]TdR (1.0 μCi/ml for 20 min: sp. act. 5.0 Ci/mmol, Radiochemical Centre, Amersham, U.K.) and by measuring the cell number with a window counting technique.

Colcemide treatment

In order to analyze the effects of ACNU on cell progression through the G_2 phase and mitosis, a stathmokinetic method was employed: asynchronously growing cells pulse prelabeled with [^{3}H]TdR (5.0 μCi/ml for 4 min) were successively treated with colcemide (0.05 μg/ml; Gibco, Grand Island, NY, U.S.A.) followed by ACNU (5.0 μg/ml). At various intervals of the treatment, cells were removed from dishes with 0.1% trypsin, centrifuged (1000 rpm for 2 min), fixed in Carnoy's solution and spread on glass slides for autoradiography. The percentage of labeled and unlabeled mitotic cells was determined by scoring 1000 cells.

Autoradiography

The slides for autoradiography were covered with nuclear emulsion (NR-M2, Konishiroku Co. Ltd., Tokyo, Japan) and exposed in a light tight box at 4°C for about 2 weeks. The slides were then developed and stained with Giemsa solution. Cells having more than five grains were scored as labeled cells. Five hundred cells were counted to determine the labeling index.

RESULTS

Cell killing effect

The survival response of asynchronous exponentially growing HeLa S3 cells exposed to increasing concentrations of ACNU in the range of 2.5–30 μg/ml for 1 hr is shown in Fig. 2. The curve is characterized by a small shoulder followed by an exponentially decreasing of survival fraction (D_o = 7.0 μg/ml·hr, D_q = 3.5 μg/ml·1 hr). In Fig. 3 synchronous cells obtained by mitotic cell collection were exposed to 10 or 25 μg ACNU/ml for 1 hr at different times after mitosis. Survival response to the drug through the cell cycle revealed a fluctuation in sensitivity: the most sensitive phase of the cell cycle was G_1 and G_2 + M phases, whereas cells in S phase were resistant to the drug. The dose-

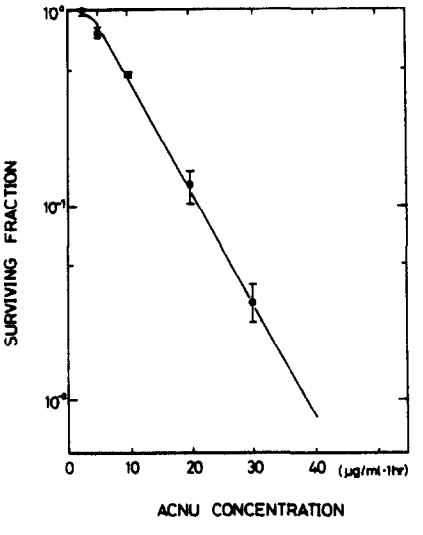

Fig. 2. Dose-survival curve of exponentially growing HeLa S3 cells exposed to increasing concentrations of ACNU for 1 hr. *Bars represent S.D. of three independent experiments.*

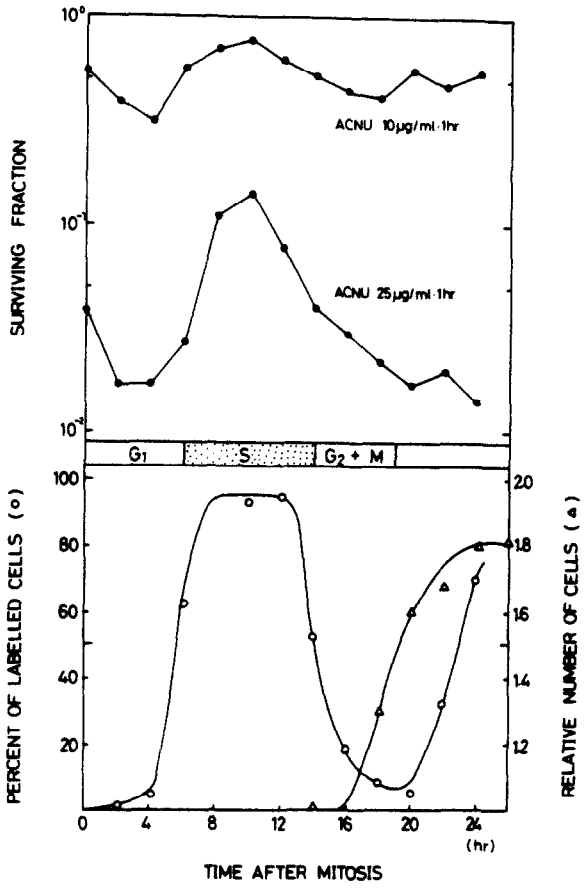

Fig. 3. Age-dependent survival response of HeLa S3 cells for ACNU. Each point is the mean of three replicate dishes. Upper panel, synchronous cells were exposed to either 10 or 25 μg/ml of ACNU for 1 hr at various times after mitotic cell collection; lower panel, the location within the cell cycle at the time of treatment was indicated by using the labeled fraction (○) and relative number of cells (△).

dependent survival curves of synchronous HeLa S3 cells are shown in Fig. 4, indicating that such changes in sensitivity were not due to the width of the shoulder (D_q), but to the exponential slope of each survival curve (D_o).

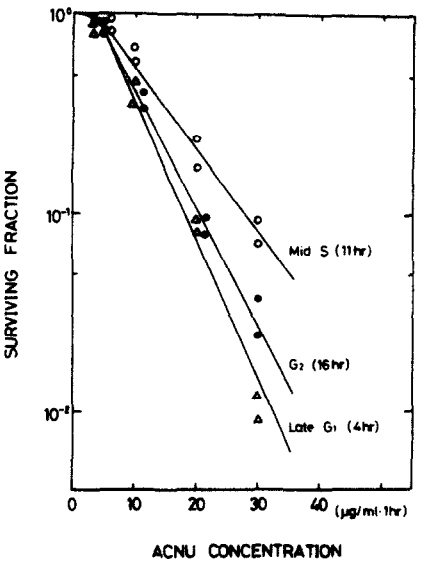

Fig. 4. Dose-survival curves of synchronous HeLa S3 cells for ACNU. Synchronous cells were exposed to increasing concentrations of ACNU at 4 hr (late G_1 phase, △), 11 hr (mid-S phase, ○) and 16 hr (G_2 phase, ●) after mitotic cell collection.

Effect of ACNU on cell progression

To examine the effects of ACNU on cell progression, a dose of 5.0 μg/ml for 1 hr, a concentration at which 80% of asynchronous cells could survive, was given to synchronous cells at specific points of cell cycle. The fate of cells treated at G_1, G_1/S and mid-S were identified by pulse labeling them with [^{3}H]TdR every 2 hr after treatment, and cell division time was also monitored by microscopic examination. As shown in Fig. 5, synchronous cells treated in the G_1 (LI = 4%) and early S (LI = 45%) phases showed a very similar alteration of cell kinetics; namely, a prolongation of the duration of S phase without any retardation of initiation of DNA synthesis and subsequent division delay. The S and G_2 + M

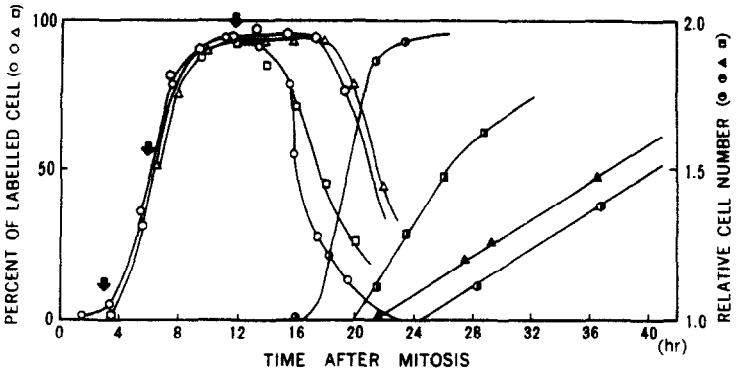

Fig. 5. Effects of ACNU on cell progression of synchronous HeLa S3 cells from the G_1, early S and mid-S phases to mitosis. Synchronous cells were exposed to 5.0 μg/ml of ACNU for 1 hr at 3 hr (G_1 phase), 6 hr (early S phase) and 12 hr (mid-S phase) after mitotic cell collection, and cell progression effects were monitored by pulse labeling of cells with [^{3}H]TdR (control, ○; G_1,○; early S, △; mid-S, □ and measuring the cell number by the window counting technique (control, ●; G_1,◑; early S, ▲; mid-S, ■).

periods measured at the 50% labeling index were 14.5, 20.0 hr for cells treated in G_1 and 14.5, 18.0 hr for cells treated in early S phase, respectively. However, such delays were obviously reduced when treated at mid-S phase in which 90% of cells were labeled: the S and G_2 + M periods were 11.5 and 9.0 hr, respectively.

To clarify the effect on progression when treated during the late S and G_2 phases, asynchronously growing cells either prelabeled or not were exposed to colcemide (0.05 μg/ml) with or without ACNU (5.0 μg/ml) for 7 hr, and the accumulation of mitotic cells was monitored as a function of time in terms of collection function [6] (Fig. 6). Following a short lag, the number of cells in mitosis after ACNU-treatment increased linearly in the same way as with untreated cells for 5 hr after the addition of colcemide. This indicates that ACNU-treated cells located at 5 hr prior to mitosis can progress through G_2 to enter mitosis. Labeled mitotic figures first appeared in cells treated or untreated with ACNU at 4 hr after adding colcemide and increased linearly at least for the next 4 hr. This implied that the some late S cells can also normally progress into mitosis.

The fate of these treated cells in the next cell cycle was then studied. To investigate this, cells when treated in G_2 were analyzed by using the following method: synchronously growing cells pretreated with ACNU in early G_2 (17 hr after mitosis) were exposed to 0.02 μg/ml of colcemide for 4 hr to arrest them in mitosis. Then the cells were harvested by a mitotic collection and reinoculated into new dishes. Their behavior was also monitored using the method used in the first

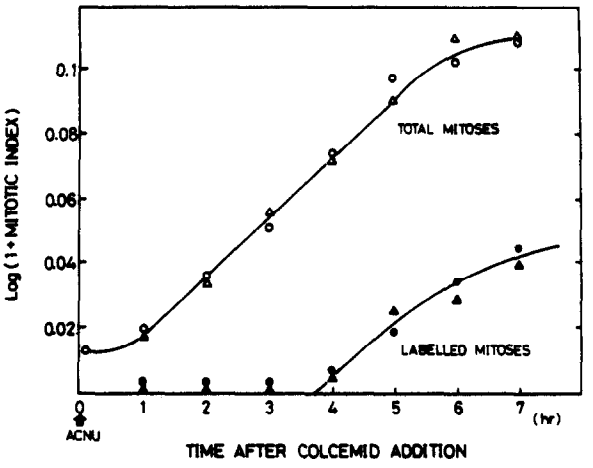

Fig. 6. Effects of ACNU on cell progression of asynchronous HeLa S3 cells from the late S and G_2 phases to mitosis. Asynchronously growing cells prelabeled with [^{3}H]TdR were continuously exposed to ACNU (5.0 μg/ml) and colcemide (0.02 μg/ml). The mitotic frequency in terms of collection function was determined by scoring the accumulated mitotic cells after an addition of colcemide. Total mitotic cells (control, ○; ACNU-treated, △) and labeled mitotic cells (control, ●; ACNU-treated, ▲).

cell cycle. In Fig. 7 the progeny of cells treated in G_2 showed prolongation of the duration of the S phase but without any delay in the start of DNA synthesis, and blockade in G_2 in the next cell cycle; times of the S and G_2 + M periods were 12.0 and 13.0 hr, respectively.

The prolongation of the S and G_2 + M phases of cells treated with ACNU when located at the G_1, early S, mid-S and G_2 phases were divided by the S and G_2 + M phase times of untreated cells to

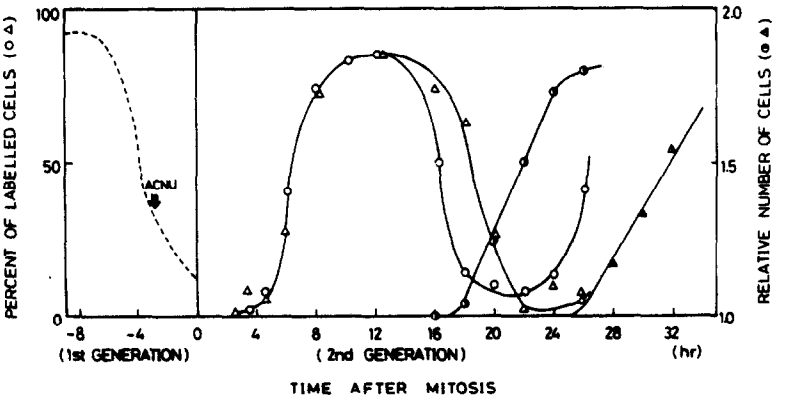

Fig. 7. Effect of ACNU on cell progression of synchronous HeLa S3 cells from G_2 to the next generation. Synchronous G_2 cells were exposed to ACNU (5.0 μg/ml) for 1 hr. After this, cells were incubated in the medium containing 0.02 μg/ml of colcemide for 4 hr. The accumulated mitotic cells were recollected by shaking off, and resynchronized cells were monitored the cell progression in the next generation, as shown in Fig. 5. The left panel shows the time when the cells were treated with ACNU; the right panel shows the effect of the drug on cell progression. Percent of labeled cells (control, ○; ACNU-treated, △ and relative number of cells (control, ●; ACNU-treated, ▲).

Table 1. Effects of ACNU (5.0 μg/ml) for 1 hr on cell progression as a function of the cell cycle phase in which treatment was given

	Cell cycle	phase of drug administration			
		G_1	Early S	Mid-S	G_2
Relative delay time	S	1.5	1.5	1.2	1.2*
(ACNU treated/control)	G_2 + M	6.7	4.5	2.2	2.3*

*Second generation.

obtain the relative delay time. The results are given in Table 1.

DISCUSSION

The dose-response curve for ACNU was characterized by a threshold-exponential curve similar to that of other nitrosourea derivatives [7-10]. It has been assumed that the presence of a shoulder on the survival curve does not have same meaning for chemical-induced damage as it does for irradiation damage [11]. However, we have previously demonstrated using a split dose [12] that the shoulder portion of the survival curve for ACNU was caused by repair from sublethal damage induced by the drug (data not shown). This may be related to the yield of 2-chloroethyl isocyanate [13] during ACNU degradation, which was said to inhibit repair of drug-induced DNA damage [14].

ACNU exerted its main killing effect on HeLa cells in the G_1 and G_2 + M phases, whereas cells in S were resistant to the drug (Fig. 3). The pattern of age-dependent sensitivity of ACNU is not entirely consisted with that of other nitrosoureas, which have been reported to be sensitive to the G_1/S and early S phase of the cell cycle [8, 9, 15, 16]. The specific feature of being deficient of a resistant peak in the G_1 phase is rather similar to that of mitomycin-C [MMC] [17, 18] and cisplatin(II) [19]. In the case of MMC, it has been postulated by Djordjevic and Kim [17] that MMC cross-links DNA to a greater extent earlier in G_1, where the strands of the macromolecule are in a more favorable juxtaposition. It has been proposed by Kohn [20] and Erickson *et al.* [21] that chloroethyl nitrosourea produces DNA interstrand cross-links in a two-step reaction sequence; the first step is a formation of chloroethyl monoadducts, and the second step is the formation of interstrand cross-links during the next few hours. Therefore it may be assumed that ACNU can easily bind DNA strands in G_1 to produce DNA interstrand cross-links without removing monoadducts by some repair mechanism [21].

Nitrosoureas have generally been reported to cause a prolongation of the S phase and a G_2 block [7, 22-25]. Our study also demonstrated that ACNU prolonged the duration of the S and G_2 phases in HeLa S3 cells. However, the magnitude of perturbation in S and G_2 depended on the position in the cell cycle when the drug was given. Cells treated in G_1 and early S were not prevented from entering S but prolonged the duration of the S phase, followed by a marked delay in progression through G_2. Treatment in mid-S caused less delay (Fig. 5). When cells were treated in late S or G_2, the progression into mitosis was not affected by the treatment, but a marked lengthening of the S and G_2 phases occurred in the next generation (Figs 6 and 7).

In the ACNU-induced S prolongation it may be assumed that cells, when treated in G_1 and early S, fail to complete the replication of alkylated-template DNA or retard DNA synthesis, resulting in prolongation of S phase.

Two causes for drug-induced G_2 delay have been postulated: (1) a surveillance mechanism operates throughout G_2 to eliminate cells that have suffered irreparable damage or alteration to their DNA [23]; and (2) loss or modification of a gene or genes necessary for the synthesis of specific proteins required for the initiation of chromosome condensation required to enter mitosis [24].

Ehmann and Wheeler [25] observed that in BCNU-treated 9L cells late S- and G_2-treated cells progress normally into mitosis without any delay. They explained this phenomenon by saying that cells in late S and G_2 did not have enough time to convert BCNU-induced monoadducts of DNA to cross-links during the treatment generation. The cross-linking of DNA by the drug might be primarily responsible for G_2-delay.

Barlogie and Drewinko [26] suggested that the cell progression delay by various antitumor agents might be the result of either DNA damage or inhibition of RNA and/or protein synthesis needed to produce certain postulated division-specific enzymes. It was reported that ACNU treatment of 10 μg/ml for 30 min only slightly affected the synthesis of RNA and protein [4]. Thus the latter case does not seem to be true with ACNU.

Figure 3 shows that ACNU at doses of 10 and 25 μg/ml killed cells effectively during the G_1 and G_2 + M phases. At the lower concentration of 5 μg/ml, the treated G_1 cells scarcely lost their clonogenic integrity but resulted in a remarkable G_2 delay. This discrepancy might be because the G_2 delay is caused by the time to repair the alkylated DNA damage. The greater the damage, the longer the delayed time. In contrast, cells treated in G_2 could divide normally but exhibited during the next generation cycle the same type of effect as that found to be exerted in cells treated in G_1. This implies that the damage on G_2 cells produced by ACNU is not expressed in the treatment cycle but carried over the next generation, in which DNA synthesis is retarded and the subsequent G_2 delay is produced. Being different from the damage caused in the G_1 phase, however, the damage produced in the G_2 phase might be divided equally between the daughter cells, so that the lethal and cell progression effects would be reduced by half in the next cell cycle.

We observed the intimate correlation between the killing and progression effects of the cells and cell age when treated with ACNU, as shown in Fig. 3 and Table 1. As a result, we suggest that these two effects of ACNU may be caused by damage of the same target, namely the formation of cross-linking of parent DNA.

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