# UREA TREATMENT AND PRONASE DIGESTION OF ANTITUMOR PROTEIN ANTIBIOTICS, AUROMOMYCIN AND NEOCARZINOSTATIN

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Low molecular weight substances were separated from antitumor protein antibiotics, auromomycin and neocarzinostatin, by Sephadex G50 column chromatography, after denaturation with 8 M urea. The low molecular weight fraction of auromomycin, but not the protein fraction, showed antimicrobial and DNA-cleaving activities.

More than 90% of the auromomycin and neocarzinostatin proteins were digested with a high concentration of pronase E. The digested samples of both antibiotics exhibited the same degree of activities as the original drugs in the inhibition of growth and DNA synthesis of mouse lymphoblastoma L5178Y cells and in causing strand scission of isolated PM2 phage DNA. The low molecular weight chromophores were recovered on Sephadex G50 column from the pronase-digested antibiotics. The results suggest that the *in vitro* biological activity of auromomycin and neocarzinostatin are principally attributed to the non-protein compounds of low molecular weight.

Auromomycin (AUR) is an antitumor protein antibiotic, possessing a chromophore, which shows an absorption maximum at 350~360 nm[1]. We have found that AUR causes DNA strand scission in cultured tumor cells[2], bacterial cells[3] and isolated DNA[2,4], and the DNA breakage is the most important mechanism of action of AUR. Furthermore, we have isolated a non-protein chromophore from AUR by methanol extraction and shown that the chromophore blocks the growth of cultured cells and induces DNA strand cleavage *in vitro*, whereas the chromophore-free protein is biologically inactive[5].

Neocarzinostatin (NCS), another antitumor protein antibiotic[6], also contains a chromophore[7]. It has been demonstrated that the chromophore, extracted from NCS by methanol, exhibits similar biological activities as native NCS[5,8~10].

We have further found that non-protein chromophores of AUR and NCS are separated from their protein moieties on a Sephadex G50 column after the treatment of the antibiotics with 8 M urea, and pronase-treated drugs show the same biological activities as the native ones. The results are presented in this publication.

## Materials and Methods

AUR was generously provided by Dr. K. WATANABE, Kanegafuchi Chemical Industry Co., Takasago, Hyogo-ken, Japan. NCS solution (4,070 units/ml) was a product of Kayaku Antibiotics Research Co., Tokyo, and was used after dialysis to redistilled water. Pronase E (1,000,000 tyrosine units/g) was purchased from Kaken Chemical Co., Tokyo, and [$^3$H]thymidine (20 Ci/mmole) from New England Nuclear, Boston, Mass. All other chemicals were of the highest grade commercially available.

The growth and [$^3$H]thymidine incorporation of mouse lymphoblastoma L5178Y cells were measured by the procedure described previously.[2] Antimicrobial activity was determined by a paper disc method, using *Bacillus subtilis* ATCC 6633 for AUR and *Micrococcus luteus* ATCC 9341 for NCS as test organisms. The assay of strand scission of PM2 phage DNA in agarose gel electrophoresis was

described previously.[4] Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of LAEMMLI and FAVRE[11], using slab gel (16×16×0.2 cm) of 15% polyacrylamide.

Pronase digestion

AUR, NCS or cytochrome C (1 mg/ml) was incubated with pronase E (1 mg or 0.1 mg/ml) in 50 mM Tris-HCl, pH 7.5, at 37°C for 16 hours in the dark.

Sephadex G50 column chromatography

Urea powder was added at 8 M to native or pronase-treated antibiotic solutions, except otherwise noted, and the mixtures were incubated at 37°C for 60 minutes and applied to Sephadex G50 column (1.5×41 cm), which was covered with aluminum foil to avoid light. The elution was performed at room temperature with 8 M urea in 50 mM Tris-HCl, pH 8.0 for AUR and pH 6.0 for NCS, respectively. Fractions of one ml were collected; and the absorbance at 280 and 350 nm, and antibacterial and DNA-cleaving activities of each fraction were assayed immediately after the fractionation.

## Results

### Separation of Low Molecular Weight Substances from AUR and NCS by the Treatment with 8 M Urea

AUR or NCS gave a single peak on a column of Sephadex G50 chromatography, as assayed by absorbance at 280 and 350 nm as well as by antimicrobial activity (Figs. 1A and 1C). The low molecular weight substances were separated from their proteins on the column after the treatment of the antibiotics with 8 M urea. These substances appeared near DNP-alanine (molecular weight 255) and showed absorbance maxima at 350 and 280 nm. In the case of AUR, antibacterial and DNA-cutting

Fig. 1. Sephadex G50 column chromatography of native and urea-treated AUR and NCS.

The columns of native AUR (A) and NCS (C) were eluted with water and those of urea-treated AUR (B) and NCS (D) were eluted with 8 M urea in 50 mM Tris-HCl buffer. Two mg of each antibiotic was applied to the column.

Arrows indicate the elution positions of 1. blue dextran 2,000 (M.W. $2\times10^6$), 2. cytochrome C (M.W. 12,400) and 3. DNP-alanine (M. W. 255), respectively. —— absorbance at 280 nm, — — absorbance at 350 nm, ------ antimicrobial activities measured by a paper disc method (mm).

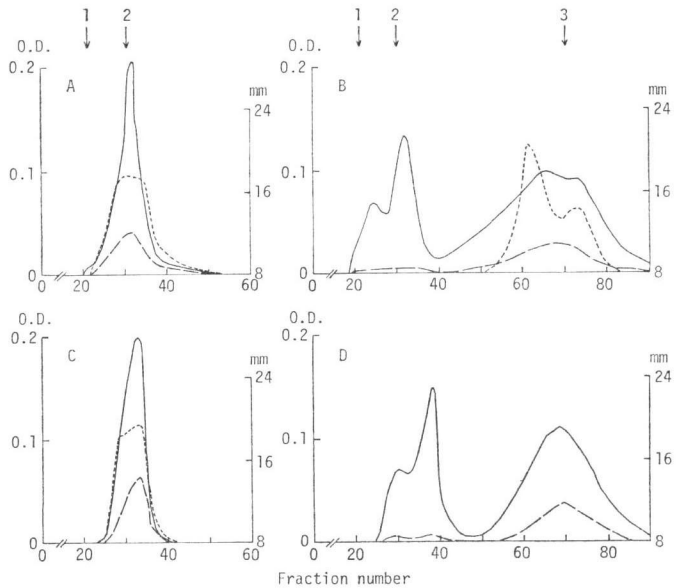
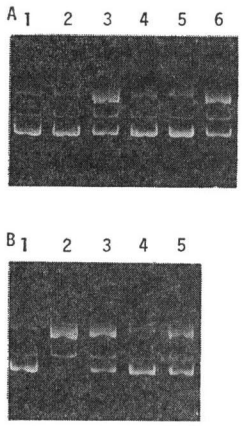


Fig. 2. DNA-cleaving activity of the fractions of AUR obtained from Sephadex G50 column eluted with 8 M urea.

Five $\mu$l of the fractions from the column was added to the incubation mixture (final 25 $\mu$l) and incubated at 37°C for 30 minutes. Urea concentration was adjusted at 1.6 M in all incubation mixture except lane 1.

A. Before pronase treatment (Fig. 1B): 1. no addition, 2. plus urea, 3. AUR 10 $\mu$g/ml, 4. AUR 1 $\mu$g/ml, 5. fraction 32 of Fig. 1B, 6. fraction 62 of Fig. 1B.

B. After pronase treatment (Fig. 4A): 1. no addition, 2. AUR 50 $\mu$g/ml, 3. AUR 10 $\mu$g/ml, 4. fraction 32 of Fig. 4A, 5. fraction 71 of Fig. 4A.




activities were found in these fractions (Figs. 1B and 2A). The protein fractions, appearing near cytochrome C (molecular weight 12,400), did not exhibit an absorbance peak at 350 nm and biological activities. Two peaks of protein fractions appeared on the columns (Figs. 1B and 1D). When each peak was concentrated by dialysis against 15% polyethylene glycol 6,000 and subjected to SDS-PAGE, the materials of both peaks showed the same mobility as that of the original antibiotics (Data are not shown). The results suggested that the peak eluted faster on the column may be an aggregated form of the protein. The elution pattern of NCS was similar to that of AUR. However, by the method employed, antimicrobial and DNA-cleaving activities failed to be detected in all the fractions of NCS (Fig. 1D).

## Pronase Digestion of AUR and NCS

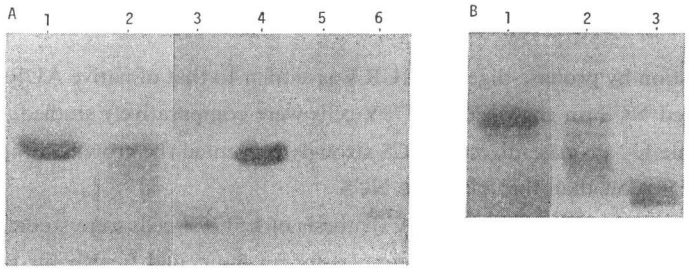
AUR and NCS were reported not to lose their biological activities by the treatment of various proteinases[1,6]. When the antibiotics were incubated at 37°C overnight with pronase E at a substrate-enzyme ratio of 10: 1 (w/w), the digestion seemed to be incomplete and two or more bands with molecular weights more than 6,000 were observed on SDS-PAGE, whereas cytochrome C was completely digested under these conditions (Fig. 3). When a substrate-enzyme ratio of 1: 1 (w/w) was used, the stained bands on SDS-PAGE were found exclusively near bromphenol blue, the front of electrophoresis, indicating that the proteins were digested almost completely (Fig. 3). The same samples were applied

Fig. 3. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of native and pronase-treated AUR and NCS.

A: 1. AUR, 2. pronase-treated AUR (10: 1), 3. pronase-treated AUR (1: 1), 4. cytochrome C, 5. pronase-treated cytochrome C (10: 1), 6. pronase

B: 1. NCS, 2. pronase-treated NCS (10: 1), 3. pronase-treated NCS (1: 1).

The amount of the sample on SDS-PAGE was equivalent to 40 $\mu$g of the original protein. The numbers in parentheses show a substrate-enzyme ratio.



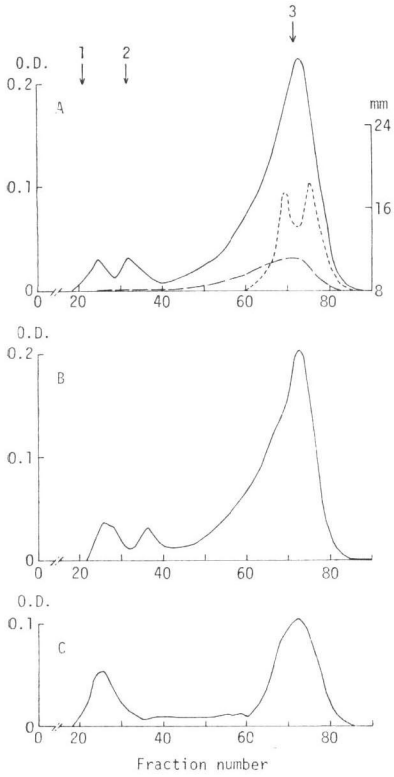
to the column of Sephadex G50 and eluted with 8 M urea solution. As presented in Fig. 4, the low molecular weight substances, appearing near DNP-alanine, were markedly increased and small amounts of the proteins were detected at the position of original proteins. Since pronase E itself gave a peak near the antibiotic proteins (Fig. 4C), more than 90% of the original proteins of AUR and NCS seemed to be digested to low molecular weight peptides or amino acids.

Fig. 4. Sephadex G50 column chromatography of pronase-digested AUR and NCS at a substrate-enzyme ratio of 1 : 1 (w/w).

Two mg of AUR (A) and NCS (B) were applied to the column after digestion with pronase E.

For the control experiment, 2 mg of pronase E (C) was incubated without antibiotic under the same conditions as (A) and (B). The column was eluted with 8 M urea in 50 mM Tris-HCl buffer.

—— absorbance at 280 nm, — — absorbance at 350 nm, ------ antimicrobial activity measured by a paper disc method (mm).

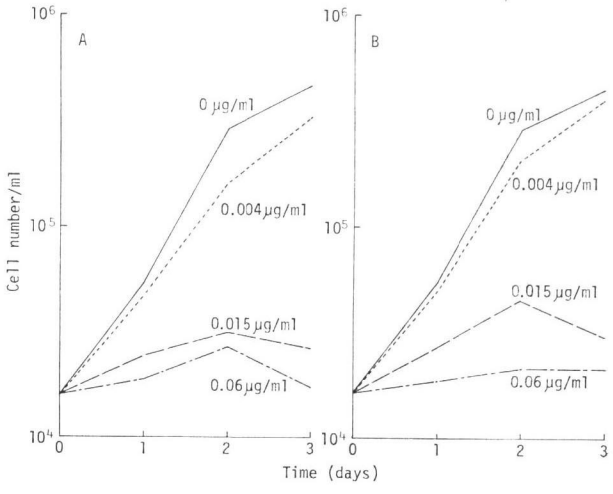



## *In Vitro* Biological Activities of Pronase-digested AUR and NCS

Pronase-digested materials of AUR or NCS (1 : 1 substrate-enzyme ratio) were diluted with sterilized redistilled water, and added to the culture of L5178Y cells. The growth was observed for 3 days. Pronase-digested AUR and NCS were observed to block the cell growth. In a parallel experiment, pronase solution, incubated under the same conditions without antibiotics, showed no significant growth-inhibitory activity even at a concentration of 1 μg/ml (Data are not shown). As illustrated in Fig. 5,

Fig. 5. Effects of native and pronase-digested AUR on the growth of L5178Y cells.

A. AUR, B. pronase-digested AUR (1 : 1)




the degree of inhibition by pronase-digested AUR was similar to that of native AUR. Effects of native and pronase-digested NCS on growth of L5178Y cells were comparatively studied, and the results are summarized in Table 1. Pronase-digested NCS strongly prevented the growth, although the inhibition degree was slightly weaker than that of native NCS.

Effects of pronase-digested NCS on DNA synthesis of L5178Y cells were studied by [$^3$H]thymidine uptake. The cells were incubated with the samples for an hour, and further for another hour in the

Fig. 6. DNA-cleaving activities of pronase-digested AUR and NCS (1: 1). A: AUR, B: NCS.

1. no addition, 2. native antibiotic 100 μg/ml, 3. 20 μg/ml, 4. 4 μg/ml, 5. pronase-digested antibiotic 100 μg/ml, 6. 20 μg/ml, 7. 4 μg/ml, 8. pronase E 100 μg/ml.

In the case of NCS, 10 mM of 2-mercaptoethanol was added to the incubation mixture.

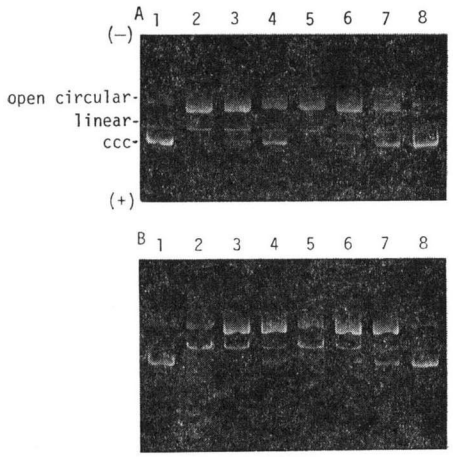



Table 1. Effects of NCS and pronase-digested NCS on growth of L5178Y cells.

| Concentration of materials μg/ml | Native NCS cells/ml (%) | Pronase-digested NCS cells/ml (%) |
|---|---|---|
| 1 | 27,600 ( 7.9) | 25,500 ( 7.3) |
| 0.2 | 31,800 ( 9.1) | 44,100 (12.7) |
| 0.04 | 164,400 ( 47.3) | 267,000 (76.9) |
| 0 | 347,400 (100 ) | |

The cell number on day 0 was 15,200/ml and those on day 3 were presented in the Table.

Table 2. Effects of NCS and pronase-digested NCS on DNA synthesis of L5178Y cells.

| Concentration of materials μg/ml | Native NCS cpm (%) | Pronase-digested NCS cpm (%) |
|---|---|---|
| 5 | 14,200 ( 35.5) | 23,900 (59.8) |
| 1 | 28,800 ( 72.0) | 28,600 (71.5) |
| 0.2 | 32,100 ( 80.3) | 29,600 (74.0) |
| 0 | 40,000 (100 ) | |

L5178Y cells in 1 ml cell suspension (115,000 cells/ml) were incubated with native or pronase-digested NCS at various concentrations for 60 minutes at 37°C, and then [$^3$H]thymidine (final 1 μCi/ml) was added. After further 60 minutes incubation, trichloroacetic acid-insoluble radioactivity was determined in a liquid scintillation counter. The value at zero time (1,400 cpm) was subtracted from each value.

presence of [$^3$H]thymidine. As shown in Table 2, pronase-digested NCS inhibited DNA synthesis of L5178Y cells at a similar degree to native NCS. Pronase alone, incubated under the same conditions in the absence of the antibiotics, exhibited no significant inhibition at 5 μg/ml (Data are not shown).

*In vitro* DNA-cleaving activities of pronase-digested AUR and NCS were compared with those of their native substances, using PM2 phage DNA. Pronase-digested AUR at concentrations of 100, 20 and 4 μg/ml converted covalently-closed circular DNA (the fastest moving band) to open circular DNA (the most slowly moving band) without any supplement. The results are in accord with the previous reports[2,4]. The degree of DNA strand scission was dependent upon the concentrations, and the activity seemed to be similar to that of native AUR (Fig. 6A). In the presence of 10 mM 2-mercaptoethanol, pronase-digested NCS also exhibited DNA-cutting activity as native NCS (Fig. 6B). Pronase itself did not cause DNA strand breaks at 100 μg/ml (Fig. 6). When pronase-digested AUR was applied to Sephadex G50 column and eluted with 8 M urea, the antimicrobial and DNA-cleaving activities were found in the low molecular weight fractions on the column (Figs. 2B and 4A).

## Discussion

Biologically active non-protein chromophores have been extracted by methanol from AUR[5] and NCS[5,8~10]. In the current experiment, a biologically active substance is separated from AUR protein on Sephadex G50 column by denaturation of the antibiotic with 8 M urea, and shown to possess a low molecular weight. The results are in accord with the previous ones.

AUR and NCS have been reported not to lose their biological activities by the treatment of various proteolytic enzymes[1,6]. However, it has remained to be determined whether the antibiotics are digested with these enzymes. Pronase E is one of the most potent proteolytic enzyme and has a broad substrate specificity[12]. NOMOTO *et al*[13]. have demonstrated that the extent of hydrolysis of casein, ovalbumin and wheat gluten by pronase reaches 75~87% even at a substrate-enzyme ratio of 200 : 1 (w/w). We have found in the present experiment that both proteins of AUR and NCS are resistant to pronase at a substrate-enzyme ratio of 10 : 1, but sensitive to the enzyme at a 1 : 1 ratio. The reason why the proteins of AUR and NCS are more resistant to proteolytic enzymes than ordinary proteins remains open to discussion. A preliminary experiment shows that the AUR protein moiety, after removal of the chromophore by methanol extraction or by fractionation on Sephadex G50 column with 8 M urea, is still resistant to pronase E digestion (Data are not shown).

Pronase-digested NCS, as well as methanol-extracted NCS chromophore[5,8~10], requires 2-mercaptoethanol to cleave DNA strands *in vitro*, whereas those of AUR do not. The results suggest that the chromophores or biologically active components of AUR and NCS are similar to, but different from each other. Recently, naphthalenecarboxylic acid derivatives have been isolated from NCS, but they possess no biological activity[14].

The current experiments suggest that the *in vitro* biological activity of AUR or NCS is attributed to the chromophore component. However, the role of the protein moiety in the antitumor activity remains to be determined.

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