ANTIMETASTATIC AND ANTITUMOR ACTIVITY OF A DERIVATIVE OF NEO-
CARZINOSTATIN: AN ORGANIC SOLVENT- AND WATER-SOLUBLE POLY-
MER-CONJUGATED PROTEIN*1

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A highly lymphotropic derivative of a proteinaceous antitumor agent, Neo-
carzinostatin, was prepared by chemical conjugation of a water-soluble syn-
thetic poly(maleic acid)-styrene oligomer. The derivative of 2.5×10^4 dalton
exhibited a strong antitumor activity against AH109A and DBLA-6 as well as
antimetastatic activity against metastatic AH109A with which experimental
lymphatic metastasis was produced in rats. An increased lipophilicity and
molecular weight of the derivative appear to be responsible for its improvement
as lymphotropic antimetastatic agent.

Tumor metastasis is one of the major
problems in cancer therapy and it is indeed
responsible for many therapeutic failures.
Metastasis is known to progress very fre-
quently via the lymphatic system. In lym-
phology, high molecular weight proteins
and lipophilic substances are known to be
recovered via the lymphatic system when
administered in the tissue or when protein
molecules leak out of blood circulation.1)

Based on these facts and principles, we
have synthesized a derivative of the
proteinaceous antitumor agent, Neocar-
zinostatin (NCS), of which the amino
acid sequence is known.6) The method
of chemical synthesis utilizes a conjugation
reaction of a synthetic water-soluble poly-
mer, [(styrene)_{1-3}-(maleic acid)_{4-7} anhy-
dride] abbreviated SMA, with NCS, where
two of the free amino groups in NCS
(alanine 1 and lysine 20) reacted with
the anhydride component in SMA in a
relatively mild condition (pH 8.6, 24°, 4
hr). After the reaction, the mixture was
purified on a column of Sephadex G-100
with 10mM ammonium carbonate. A
major component thus obtained was almost
a single peak which was lypophilized and
designated as SMANCS.8)

MATERIALS AND METHODS

Materials SMANCS was prepared as described
recently8) and it exhibited completely different
chemical and biological properties as shown in
Table I. NCS was obtained from Kayaku Anti-
biotic Research Co., Tokyo. SMA, type 1440H,
was obtained from ARCO Chemical Co., Phil-
adelphia, U.S.A. Other chemicals were from
commercial sources.

Biological Activity in vitro Biological activi-
ties of SMANCS or NCS were usually assayed
using a gram-positive bacterium, Sarcina lutea, by
the cup method with a glass cylinder on sensitivity-
test agar (Eiken Chemical Co., Tokyo) which
was diluted with the culture broth to 1% in
agar.11) Since the diffusion of proteinous anti-
biotics is much slower on an agar plate than low mol.
wt. antibiotics, a long period of diffusion
(4 hr) at 4° and a low concentration of agar (1%)

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were used so as to facilitate effective diffusion and accurate assays. A small inoculum size was employed for the same reason, in which 0.05 ml of a culture of Sarcina lutea in full growth was added to 100 ml of the agar medium. The sensitivity of the assay is as low as 0.01 μg/ml for NCS which was used as a standard. They were also assayed with a lymphoblastoid cell line (XPL-15) in a test tube in RPMI-1640 medium with 10% fetal calf serum, based on the cell killing effect at 37°C. The cell killing rate was measured by the Trypan Blue dye exclusion method 3 days after cell inoculation and drug treatment. In the control (without the drug), the cells reached a maximum density of 2.0 × 10^6/ml.

**Arbitrary Toxicity in vivo** Maximum tolerable dose of SMANCS and NCS was examined with a single intravenous dose of each drug to healthy Donryu rats, with average body weight of about 200 g, and six rats per group were used.

**Antitumor Activity** The antitumor effect of SMANCS was compared with that of parental NCS using rat leukemia DBLA-6 cells based on survival days of Donryu rats. Ten rats were used per group. Each drug was administered five separate times, and one-fifth of the maximum tolerable dose per day was injected intravenously each time between day 3 and 7 after tumor cell inoculation (5 × 10^6, iv).

SMANCS was also administered ip at 5.0 mg/kg to Donryu rats bearing ascitic AH109A tumor, inoculated with 2 × 10^6 tumor cells 96 hr before drug treatment, and histological examination of the smear specimen was made under a microscope 24 hr later.

**Antimetastatic Activity** The antimetastatic effect of SMANCS and other drugs was evaluated and compared in the experimental lymphotropic metastasis with AH109A tumor cells. The tumor cells for inoculation were obtained 5 days after ip inoculation in rats. An inoculum of 10^6 AH109A tumor cells was implanted inside the right thigh subcutaneously in Donryu rats. Seven days after the implantation, the right leg was amputated to remove the primary tumor, and then the rat was treated with the drugs by subcutaneous injection inside the thigh of the left leg. Drugs were administered sc on days 9, 10, and 11 at a dose of 1/5 of the maximal tolerable dose, once daily. The animals were sacrificed on day 12 for evaluation of metastasis in the lymph nodes (inguinal and axillary) based on the weight. In this experiment the primary regional lymph node (popliteal) was removed by amputation and only remote lymph nodes (inguinal and axillary) were evaluated. Drug injection (left thigh) was remote from the tumor inoculation site (right thigh) which was amputated later to ascertain complete removal of the inoculated tumors because our experiment is designed to evaluate primarily antimetastatic activity.

**RESULTS**

**In vitro Activity** SMANCS exhibited a similar degree of antibacterial activity to that of NCS on molar basis; SMANCS is about 3 times less potent than NCS on the basis of weight (Table I). Growth inhibitory activity of SMANCS against Epstein-Barr virus-transformed lymphoblastoid cell line (XPL-15) tested in a test tube showed that the minimum inhibitory concentration was less than 0.025 μg/ml compared to less than 0.01 μg/ml of that of NCS (Fig. 1). Both these results indicate that the molar specific activity of SMANCS and NCS is about the same in vitro.

| Table I. Physical and Biological Properties of SMANCS and Neocarzinostatin |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Elemental analysis (found, %)** | **Mol. wt. (× 10^4)** | **Solubility\(^a\) (2 mg/ml) at 25°C in** | **Minimum inhibitory concentration (μg/ml)** |
| C | H | N | pyridine | acetone | saline (pH 7.2) | 602 Gann |
| SMANCS | 63.49 | 7.23 | 6.55 | 2.5 | + | + | 0.03 |
| NCS | 46.94 | 7.21 | 14.24 | 1.07 | — | — | 0.01 |

\(^a\) Practically insoluble or slightly soluble, <5%; + sparingly soluble, 5~70%; + soluble, >70%.

\(^b\) Agar diffusion method (cup) was used.
A NEW ANTIMETASTATIC AGENT, SMANCS

Fig. 1. Arbitrarry cell killing effect of NCS (○—○) and SMANCS (●—●) at various dose levels on lymphoblastoid cells (XPL-15)

Viable cells (%) = viable cell number with drug / viable cell number without drug × 100

The growth inhibitory effect was similar to this result.

Arbitrary Maximum Tolerable Dose
The preliminary acute toxicity examined in rats revealed that maximum tolerable dose of SMANCS and NCS is 4.4 and 1.1 mg/kg, respectively. All six rats survived with this dose. This result may indicate slightly less toxicity in SMANCS than NCS on molar basis.

Antitumor Activity in vivo The result of antitumor activity against rat leukemia DBLA-6 based on survival days is shown in Fig. 2. SMANCS became more effective than NCS against DBLA-6. The antitumor effect of SMANCS against ascitic AH109A tumor (5 mg/kg ip once) is shown in Photo 1, A and B. The tumor cells not only decreased in number but also exhibited a degenerated, pycnotic, and some swollen morphology.

Antimetastatic Activity Result from SMANCS in this assay system with AH109A revealed a significant effect against metastatic lymph nodes in comparison with tumor weight of control group. Among two other antitumor agents used, NCS showed only a slight effect while mitomycin-C exhibited no suppressive effect against the metastatic tumor growth (Table II). Histological examination of the metastatic lymph nodes, with or without SMANCS treatment, showed that the changes were mainly enlarged cell structure, and vacuolated and degenerative morphology when observed under a microscope.

DISCUSSION
The uniqueness of the distribution of SMANCS in vivo resides in its high accumulation in the lymph nodes; it is much higher than that of the original NCS.5,9,13)
This finding is in concordance with the result obtained in the assay of antimetastatic activity based on the weight of metastatic lymph nodes in experimental rats (Table II), in which the suppression of tumor growth is evident. The inguinal and axillary lymph nodes were not the primary regional lymph nodes, and they were remote from either the site of the drug injection or that of tumor inoculation. Histological observation of the metastatic lymph nodes confirmed that the tumor cells exhibited enlarged and vacuolated cell morphology, a typical cell toxicity caused by NCS or SMANCS (not shown). Other drugs, mitomycin-C and NCS, did not show such a significant suppression. Furthermore, the antitumor activity of SMANCS became effective in AH109A which was not susceptible to original NCS (Photo 1). In addition, SMANCS exhibited an increased chemotherapeutic activity than NCS when tested in DBLA-6 leukemia in rats (Fig. 2). This may indicate that the lipophilic nature of SMANCS may have increased its affinity to tumor cells in addition to the increased stability in vitro comparable to that of succinyl derivative. The lipophilicity of SMANCS can be envisaged from its solubility in some organic solvents such as pyridine and acetone, while NCS is insoluble in these solvents (Table I). These altered characteristics (increased molecular wt. and lipophilicity) indeed verified the lymphological requirements for pronounced lymphotropicity. Although the acute toxicity of SMANCS and NCS is similar on the molar basis, these lymphotropic character of SMANCS will benefit in the control of lymphotropic metastasis. Detailed comparisons will, however, require more elaborate experiments considering route and intervals of drug administration, inoculum size, and types of tumors.

Previously several attempts were made to improve the therapeutic efficacy of anti-cancer agents by means of liposome emulsion. Cytosine arabinoside, 5-fluorouracil, and other drugs have shown considerable success in the control of metastasis. The results presented here indicate an alternative approach for a similar purpose.

The original NCS, for example, is unique in its pharmacokinetics. It is very labile in vivo and is excreted into the urine extremely rapidly (t1/2 = 5 min) and a slower renal clearance rate than NCS (unpublished). Nevertheless, SMANCS appears to have a similar mode of action to NCS because NCS was recovered from the urine of rabbits injected with SMANCS as revealed by Sephadex G-100 (not shown). The poly-maleic residues, therefore, seem to be hydrolyzed fairly rapidly in vivo.

The present results encourage further application of the chemical derivatization of known antitumor agents for the above objective.

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References

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EXPLANATION OF PLATE

Photo 1. Photomicrographs of ascitic AH109A tumor cells in rats. (A) Control cells (no drug), (B) SMANCS-treated cells 24 hr after drug administration (5 mg/kg, ip). Stained with Giemsa. ×400.