Eur J Cancer, Vol. 26, No. 3, pp. 253–260, 1990.
Printed in Great Britain

0277–5379/90$3.00 + 0.00

Reduced Bone Marrow Toxicity of Neocarzinostatin by Conjugation with Divinyl Ether–Maleic Acid Copolymer

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Abstract—*Neocarzinostatin (NCS) was conjugated with divinyl ether–maleic acid/anhydride copolymer (pyran copolymer), and its therapeutic effect was compared with that of NCS. The conjugated NCS (pyran–NCS) with a molecular weight of about* 23,000, *exhibited* in vitro *cytotoxic activity against eight cell lines and bone marrow cells that was similar to the cytotoxic activity of NCS on a molar basis. Furthermore, both drugs had similar effects against a multidrug-resistant Chinese hamster ovary cell line (CH^RC5) and its parent cell line (AUXB1)* in vitro. *However, pharmacological analysis showed that pyran–NCS had reduced accumulation in the spleen, and most important was three times less hematotoxic* in vivo *compared with NCS. Also, pyran–NCS had a* 1.7*-fold higher* 50% *lethal dose* (LD_{50}). *Antitumor activity of pyran–NCS and NCS was tested against two different forms of Meth A tumor. In a solid tumor model, pyran–NCS and NCS suppressed tumor growth at three-fourths of the* LD_{50} *to* 12.8 *and* 19.0% *of the control tumor as evaluated on day* 28, *respectively* ($P < 0.025$). *In an ascitic tumor model, the percentage increase in the median life span caused by pyran–NCS and NCS was more than* 400 *and* 150% *on day* 60, *respectively. Pyran–NCS is more effective than NCS because the reduced acute toxicity permits an increased drug dosage.*

INTRODUCTION

THERE IS IN GENERAL a positive relationship between the dose of an anticancer agent and the degree of tumor cell killing. An increase in the dose of anticancer agents consequently improves the cure rate for cancer [1]. However, high-dose chemotherapy is limited by several toxic side-effects against normal organs, especially bone marrow.

The possibility of reducing the toxic side-effects of anticancer agents by coupling the agents with several carrier systems has been widely explored [2–10]. A critical aspect of reducing dose-limiting toxicity of anticancer agents is thought to be tumor-selective drug delivery. We have reported that a conjugate of styrene–maleic acid copolymer and the antitumor protein neocarzinostatin (NCS), smancs [2], had a much longer half-life than NCS in plasma. The increased tumoritropic character of smancs was attributed to the unique vascular make up of tumor tissue, which shows an enhanced vascular permeability and retention (*EPR*) effect of macromolecules, as demonstrated previously [11]; in addition, the vascular architecture in solid tumors is defective [12].

The biocompatibility of divinylether–maleic acid anhydride (pyran) copolymer had been established previously within some limitations [8, 13, 14]. Therefore, we prepared another macromolecular anticancer agent by conjugating pyran copolymer with NCS to examine its therapeutic effect and to reduce its bone marrow toxicity.

With regard to molecular size, a linear hydrophilic polymer in aqueous solution is known to occupy a larger Stokes's radius than folded globular molecules (most of the plasma proteins). Thus, we chose a molecular mass range of about 23,000 for the conjugate, which would correspond to globular proteins of 30,000 daltons.

MATERIALS AND METHODS

Cell lines

Eight established cell lines were cultured in the growth media containing 10% heat-inactivated fetal

Accepted 24 October 1989.
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bovine serum under 5% CO_2 in humidified air at 37°C. HeLa cells (human cervical cancer) and KB cells (human epidermoid cancer) were cultured in Eagle's minimal essential medium (MEM) (Gibco, Grand Island, NY, U.S.A.). AUXB1 cells (Chinese hamster ovary cells) and CH^RC5 cells (a multi-drug-resistant subline of AUXB1) were generous gifts from Dr Victor Ling, the Ontario Cancer Institute, Toronto, Canada. These cell lines were cultured in MEM (Flow Laboratories, Stanmore, N.S.W., Australia). P388 cells (mouse lymphocytic leukemia), Meth A cells (mouse fibrosarcoma), RLσ1 cells (mouse lymphoma), and K562 cells (human myelogenous leukemia) were maintained in RPMI 1640 medium (Gibco).

Mice

Male BALB/c mice and A/Jax mice both 8–9 weeks old weighing about 25 g were purchased from the Shizuoka Agricultural Corp., Hamamatsu, Japan.

Drugs

NCS was obtained from Kayaku Co., Ltd., Tokyo, Japan. Divinyl ether–co-maleic acid/anhydride (abbreviated as pyran copolymer; mean molecular weight 5600) used in the synthesis of polymer conjugate of NCS (pyran–NCS), was prepared at the Research Institute for Polymers and Textiles, Tsukuba, Ibaraki, Japan, by one of us (T.H.) as described previously [8]. Because the toxicity of pyran copolymer was attributed mainly to the relatively high molecular weight fraction [14], a low molecular weight (about 5600) copolymer was chosen for this experiment. All maleic acid residues in pyran copolymer were of the anhydride form.

Preparation of pyran–NCS

Reaction of NCS with the pyran copolymer was performed according to the method described by Maeda *et al.* for smancs [2, 15]. About 250 mg of NCS was dissolved in 50 ml of 0.1 M sodium bicarbonate solution at pH 8.5, and about 500 mg of pyran copolymer was added to give approximately a 2.5 molar excess of pyran copolymer per amino group. The mixture was then stirred in ice-cold water for 3 h in the dark. After the reaction, copolymer was removed by extensive dialysis for 3 days against 0.01 M phosphate buffer (pH 7.5) at 4°C, with several changes of dialysis buffer. The reaction product was concentrated to 10 ml by using an Amicon ultrafiltration apparatus with a YM-10 membrane (Amicon, Danvers, MA, U.S.A.), and it was then applied to a column (25 × 940 mm) of Sephadex G-50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and was eluted with distilled water. Approximately 309 mg of pyran–NCS was obtained. A mean molecular weight of 23,000 was determined by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis; this value agrees with the theoretical value for NCS conjugated with two polymer chains. The schematic structure of pyran-copolymer-conjugated NCS is shown in Fig. 1.

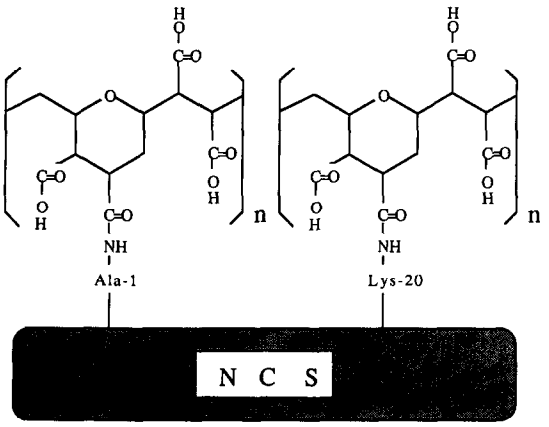

Fig. 1. Structure of pyran–NCS. Pyran copolymer (molecular weight 5600) was conjugated to the free amino groups (Ala-1 and Lys-20) of NCS (molecular weight 11,700). No other amino group exists in NCS.

Cytotoxicity in vitro

Cytotoxicity was determined by measuring colony inhibitory activity for the various cell lines. Adherent cell lines were plated in 96-well tissue culture plates with a flat bottom (No. 3072, Falcon) at a density of 2×10^4 cells/well and were cultured overnight in the growth medium. After removal of the medium, cells were exposed to various concentrations of drugs in the growth medium for 1 h at 37°C under 5% CO_2 in humidified air. After exposure, the medium was removed, the cells were trypsinized, and about 500 cells were seeded onto 35-mm plastic Petri dishes (No. 3001, Falcon) and incubated at 37°C under 5% CO_2 in humidified air. After 7 days, the number of colonies was counted visually after staining with 1% methylene blue solution by using the Digital Colony Counter (Model DC-3, Kayagaki Co. Ltd., Tokyo, Japan). Clusters of 30–50 cells were considered to be a colony of surviving cells.

In suspension-type cell lines, cells were placed in 96-well V-bottom plates (No. 3896, Costar) at a density of 2×10^4 cells/well and were exposed to various concentrations of drugs at 37°C. After 1 h, the microplates were centrifuged at 400 ***g*** for 10 min. The medium was then removed and the cells were resuspended in 750 μl of RPMI 1640 medium containing 0.33% agarose (FMC BioProducts, Rockland, ME, U.S.A.) and then were placed onto a base layer of 0.5% agarose (1 ml) in the same medium in 35-mm plastic Petri dishes. The final concentration of cells in each culture was 500 cells/dish. The numbers of colonies of more than 40 cells were counted 1 week after plating by using an

inverted phase contrast microscope (Olympus, Tokyo, Japan).

Determination of 50% *lethal dose* (LD_{50}) *and* 10% *lethal dose* (LD_{10})

For determination of LD_{50} and LD_{10}, groups of 10 male BALB/c mice received intravenous (i.v.) injections of different doses of pyran–NCS or NCS in 0.5 ml of 0.9% NaCl. After 3 weeks of observation, the LD_{50} and LD_{10} values were calculated.

Tissue distribution

The biological activity of drugs in the organs or tissues of treated mice was assayed by using the method of growth inhibition of *Micrococcus luteus* ATCC 9341 on Mueller-Hinton agar as described previously [16]. Both drugs were injected i.v. as a bolus via the tail vein of BALB/c mice that had Meth A tumor (about 8 mm in diameter), at a dose of 4.3 μmol/kg. The mice were killed at 2, 5, 10, 30 and 60 min after injection. A cocktail of protease inhibitors consisting of 0.01% *N*-ethylmaleimide (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 0.1% antipain, 0.1% pepstatin and 0.1% leupeptin (which were generous gifts from Dr T. Aoyagi, Institute of Microbial Chemistry, Tokyo, Japan) was added immediately at 10% in weight to each sample. The drug was allowed to diffuse for 12 h at 4°C, and then the assay plates were incubated for 24 h at 37°C. The minimum detectable concentration of the drugs was about 8.0 nM.

Cytotoxicity against hematopoietic progenitor cells in vitro

Normal A/Jax mice were killed by cervical dislocation, and femurs were obtained. The bone marrow cells were flushed out from the femurs with a 23-gauge needle into RPMI 1640 medium containing 10% fetal bovine serum. After centrifugation, the bone marrow mononuclear cells were placed in 96-well V-bottom plates at a density of 5×10^5 cells/well as described above and exposed to various concentrations of drugs at 37°C under 5% CO_2 in humidified air. After 1 h, the microplates were centrifuged at 400 ***g*** for 10 min, the medium was removed, and the number of colony-forming units in culture (CFU-C) was determined. For the CFU-C assay, 1×10^5 cells were incubated in 1.0 ml of 0.36% semisolid agarose in McCoy's 5 A medium (Gibco) containing 20% fetal bovine serum and 10% (vol/vol) of conditioned medium [pokeweed mitogen (Gibco)-stimulated spleen cells served as a source of colony-stimulating factor]. Marrow cells in culture were maintained for 7 days at 37°C under 5% CO_2 in humidified air, and colonies containing more than 50 cells were counted by using an inverted phase contrast microscope. The 50% inhibitory concentration (IC_{50}) was then determined.

Cytotoxicity to hematopoietic progenitor cells in vivo

Normal A/Jax mice received i.v. bolus injection of increasing doses of pyran–NCS and NCS. Twenty-four hours after the injection, the mice were killed by cervical dislocation, and femurs and spleens were removed. Spleens were diced in the medium and a single-cell suspension was obtained by passage through a stainless steel mesh (300 μm). Spleen cells (2×10^6) and bone marrow cells (1×10^5) were incubated for the CFU-C assay. CFU-C/spleen and CFU-C/femur were determined as described above.

Antitumor activity against solid tumor

Meth A (2×10^6) cells were injected subcutaneously (s.c.) into the left flank of BALB/c mice. The drugs were injected i.v. once on day 4. Tumor volumes were calculated as $(a \times b^2)/2$, where a and b are the length of the long and short axes of the tumor mass, respectively, measured by a caliper. Antitumor activity was evaluated in terms of T_{rv}/C_{rv}, where T_{rv} is the relative mean tumor volume of tested groups and C_{rv} is that of the control group. Antitumor activity was also evaluated in terms of growth delay (days), that is the time required for the tumors of the treated group to attain four times their pretreatment volume.

Antitumor activity against ascitic Meth A tumor

Meth A cells (2×10^6) were injected intraperitoneally (i.p.) into BALB/c mice on day 0. The drugs were injected i.p. once at 24 h after tumor inoculation. Pyran copolymer (not NCS conjugate) was also injected i.p. to determine any effect of the carrier polymer alone. The doses of pyran copolymer used were equivalent to one-half and one-quarter the LD_{50} of pyran–NCS. The evaluation of anti-tumor activity was made by measuring the percentage increase in median life span (% ILS), $(T/C-1) \times 100$ (%), where T is the median survival time of the drug-treated groups and C is that of the control group.

RESULTS

Cytotoxicity in vitro

Both pyran–NCS and NCS showed similar dose dependent cell killing of various tumor cells, on the basis of a decrease of colony formation *in vitro* and of bone marrow cells as determined by CFU-C. IC_{50} values for these drugs and their relative activity (IC_{50} ratio: pyran–NCS IC_{50}/NSC IC_{50}) are presented in Table 1. A ratio of less than 1.0 indicates that pyran–NCS is more potent than NCS. The relative activity ratios in various cell lines and CFU-C ranged from 0.76 to 1.25. Results indicate that conjugation of NCS with pyran copolymer did not lead to loss of potency of NCS *in vitro*.

Table 1. *Cytotoxicity of pyran-NCS and NCS against cultured cells and hematopoietic progenitor cells* in vitro

	IC_{50} (nM)		
Cell*	Pyran–NCS	NCS	IC_{50} ratio†
CFU-C	19.1	20.0	1.00
HeLa	11.9	9.5	1.25
KB	6.3	5.3	1.19
AUXB1	8.6	7.0	1.23
CH^RC5	47.5	40.0	1.23
Meth A	6.0	5.3	1.14
RL♂ 1	11.0	10.6	1.04
P388	9.4	12.3	0.76
K562	4.3	3.4	1.19

*See text for origin of cells.
†Ratio of pyran–NCS IC_{50} value to NCS IC_{50} value.

We also tested these drugs against the Chinese hamster ovary cell line AUXB1 and its multidrug-resistant cell line CH^RC5 *in vitro*. IC_{50} values of both pyran–NCS and NCS for CH^RC5 cells were only about eight times higher than those for parental AUXB1 cells.

Acute toxicity

LD_{50} values of pyran–NCS and NCS were 0.27 and 0.16 μmol/kg, respectively. Although *in vitro* pyran–NCS was as cytotoxic as NCS on a molar basis, the acute toxicity of pyran–NCS was about 70% of that of NCS. A similar ratio was observed for LD_{10} values for these drugs, namely, 0.17 and 0.11 μmol/kg for pyran–NCS and NCS, respectively.

Tissue distribution

Table 2 shows the tissue distribution of pyran–NCS and NCS in tumor-bearing mice after i.v. injection. When 4.3 μmol/kg of pyran–NCS or NCS was injected i.v., drugs were cleared rapidly, within 20 min. The $t_{1/2}$ values of pyran–NCS and NCS were 1.5 and 1.0 min, respectively. The $t_{1/10}$ of both drugs was about 9 min. The biological activity of pyran–NCS in the tumor was 10 times higher than that of NCS at a peak period (5 min), but it decreased to an undetectable level within 30 min. Pyran–NCS had a much smaller distribution to the spleen compared with NCS. The biological activity of NSC in the spleen was 0.69 nmol/g at 5 min and 0.01 nmol/g at 10 min, whereas that of pyran–NCS was 0.04 nmol/g at 5 min and undetectable after 10 min. Both drugs had low accumulation in the liver. Urinary excretion of both pyran–NCS and NCS was equally fast.

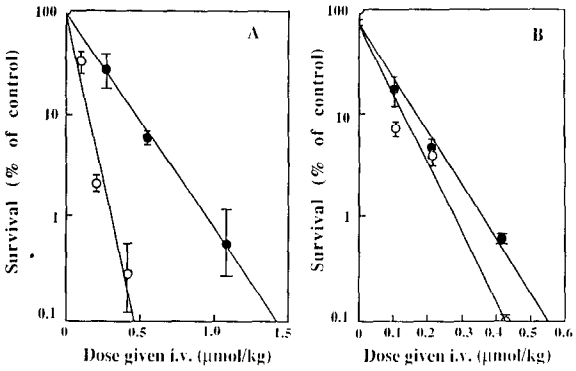

Fig. 2. *Dose–survival curves of CFU-C* in situ. *Pyran–NCS and NCS were injected i.v. into normal A/Jax mice. After* 24 h, *bone marrow cells and spleen cells were separated; CFU-C/femur and CFU-C/spleen were determined on day* 7. *Survival of bone marrow cells (A) and spleen cells (B). Each experimental point is a mean; vertical bars indicate* ± *S.E.M.* (n = 5). (●) *Pyran–NCS*; (○) *NCS. (See text for details.)*

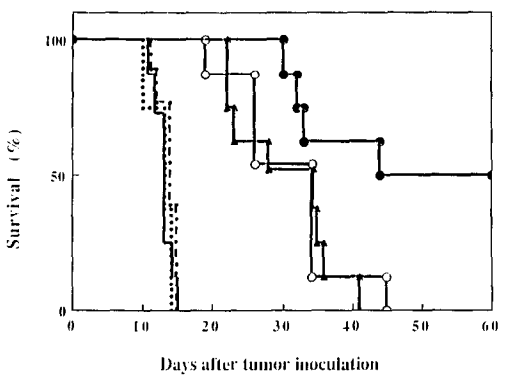

Fig. 3. *Survival profile of Meth A ascitic tumor-bearing mice. Meth A cells* (2×10^6) *were injected into BALB/c mice i.p., and mice were treated with drug by i.p. injection once at* 24 h *after inoculation.* (——), *untreated control*; (·····), *pyran copolymer alone*, 0.14 μmol/kg; (–·–), *pyran copolymer alone*, 0.07 μmol/kg; (-●-), *pyran–NCS*, 0.14 μmol/kg; (-▲-), *pyran–NCS*, 0.07 μmol/kg; (-○-), *NCS*, 0.08 μmol/kg. *Eight mice were used for each group.*

Cytotoxicity against hematopoietic progenitor cells in vivo *as assayed by CFU-C* in vitro

The survival of *in situ* CFU-C/femur and CFU-C/spleen decreased almost exponentially and did not reach saturation values with increasing doses (Fig. 2). Survival curves of NCS in CFU-C/femur bone marrow (Fig. 2A) and CFU-C/spleen (Fig. 2B) were 3 and 1.3 times steeper than those of pyran–NCS, respectively; this result indicates that pyran–NCS was less toxic than NCS to normal hematopoietic progenitor cells *in vivo*.

Antitumor activity

In the solid tumor model, pyran–NCS was more effective than NCS at the same toxic dose level. As shown in Table 3, pyran–NCS and NCS suppressed tumor growth to 12.8 and 19.0% compared with controls at three-fourths of the LD_{50}, as evaluated on day 28, respectively ($P < 0.025$). In the ascitic tumor model, one-half of the LD_{50} of pyran–NCS

*Table 2. Tissue distribution of pyran–NCS and NCS in tumor-bearing mice after i.v. injection**

	Biological activity† (nmol/g of tissue)									
	Pyran–NCS					NCS				
Specimen	2 min	5 min	10 min	30 min	60 min	2 min	5 min	10 min	30 min	60 min
Plasma	11.74 ± 6.96	4.49 ± 0.90	0.58 ± 0.18	UD	UD	3.50 ± 0.09	2.05 ± 0.65	0.69 ± 0.26	UD	UD
Tumor	0.44 ± 0.22	3.14 ± 0.24	0.34 ± 0.15	0.05 ± 0.05	UD	1.62 ± 0.65	0.33 ± 0.07	0.24 ± 0.19	UD	UD
Spleen	0.30 ± 0.19	0.04 ± 0.02	UD‡	UD	UD	0.55 ± 0.22	0.69 ± 0.43	0.01 ± 0.01	UD	UD
Lung	1.33 ± 0.15	0.83 ± 0.15	0.26 ± 0.04	0.12 ± 0.11	UD	1.62 ± 0.65	0.42 ± 0.16	0.21 ± 0.08	0.07 ± 0.07	UD
Liver	0.03 ± 0.03	UD	UD	UD	UD	0.06 ± 0.06	UD	UD	UD	UD
Kidney	13.74 ± 6.88	7.78 ± 2.96	0.16 ± 0.05	0.01 ± 0.01	UD	2.74 ± 0.47	3.68 ± 0.40	0.37 ± 0.19	0.16 ± 0.13	UD
Urine§	11.17 ± 2.04	30.65‖	27.46 ± 0.07	147.33 ± 11.37	110.57‖	2.63 ± 0.53	70.61 ± 1.66	49.51 ± 1.07	140.81 ± 17.74	154.6 ± 13.12

*Drug at a dose of 4.3 μmol/kg was injected i.v. through the tail vein of BALB/c mice with Meth A tumor.
†Activity of drugs was measured by bioassay using growth inhibition of *Micrococcus luteus* ATCC 9341 (see text). Values are means ± S.E.M. (n=5). Minutes are times after injection.
§Values are cumulative mean.
‡Drug concentration was below the limit of detection.
‖Only one specimen available.

Table 3. Antitumor effects of pyran–NCS and NCS on Meth A solid tumor

Drug and dose	RV*	Trv/Crv (%)†	Tumor growth delay (days)‡
Control	85.7 ± 10.3	100	8.5
Pyran			
0.20 μmol/kg	74.9 ± 15.7	87.4	9.0
Pyran–NCS			
0.20 μmol/kg ($3/4LD_{50}$)	11.0 ± 1.9§‖	12.8	20.0
0.12 μmol/kg	17.3 ± 1.5§	20.2	18.5
NCS			
0.12 μmol/kg ($3/4LD_{50}$)	16.3 ± 2.2§	19.0	18.0

*Relative mean tumor volume on day 28. Values are means ± S.E.M. (n=5).
†Comparison of the relative tumor volume of treated group and of control on day 28.
‡Time to reach 4 times the pretreatment tumor volume.
§Significantly different from control, $P < 0.0005$.
‖Significantly different from $3/4LD_{50}$ of NCS, $P < 0.025$.

was more effective than the same dose of NCS; there was a large increase in ILS (more than 400% for pyran–NCS and 150% for NCS, both on day 60; see Fig. 3). Antitumor activity of pyran–NCS was as great as that of NCS on a molar basis, and pyran copolymer alone was found to have no effect on solid and ascitic tumors (Fig. 3, Table 3).

DISCUSSION

We report here preparation of another macromolecular anticancer agent by conjugating pyran copolymer to NCS. The conjugate pyran–NCS had similar cytotoxic activity to NCS *in vitro* on a molar basis (Table 1), as expected because *in vitro* there are no pharmacokinetics or effects of tumor vascular permeability. In addition, the results show that the preparative procedure did not affect the intrinsic activity of NCS.

In general, *in vivo* toxicity of biocompatible conjugated polymer drugs becomes lower than that of parental agents [2–9]. This was also the case with pyran–NCS. Namely, the LD_{50} of this conjugate was 1.7-fold higher than that of NCS. Because the dose-limiting toxicity of NCS is primarily marrow suppression, the hematotoxicity of the two drugs was compared. Although both pyran–NCS and NCS showed the same cytotoxicity by CFU-C assay *in vitro*, the *in situ* toxicity of pyran–NCS against bone marrow cells and spleen cells was much lower than that of NCS (Fig. 2). Furthermore, pyran–NCS had a lower distribution to the spleen compared with that of NCS (Table 2). Thus, pyran–NCS may have a lower distribution to the bone marrow than NCS. In addition, both drugs accumulated very little in the liver (Table 2). The vascular permeability barrier in the brain is well known. A permeability barrier may exist for pyran–NCS in the blood circulation of the bone marrow and spleen and to some extent other organs. The estimated area under the curve of biological activity of pyran–NCS in plasma was about 2.2-fold higher than that of NCS (Table 2). The pyran copolymer seems to have caused less accumulation in bone marrow.

We have reported that the LD_{50} of smancs was about 50% of that of NCS [15], indicating reduced marrow toxicity by increased molecular weight. Since there is no detailed study of smancs on the bone marrow toxicity, it is difficult to compare smancs with pyran–NCS.

The antitumor activity of pyran–NCS was not increased in the i.p. (tumor)/i.p. (drug) and s.c. (tumor)/i.v. (drug) systems at the same dose on a molar basis when compared with NCS. However, at a high dose pyran–NCS was more effective than NCS because the reduced acute toxicity permitted an increase in drug dosage (Fig. 3 and Table 3). We previously reported that biocompatible macromolecules such as albumin, IgG or smancs can accumulate more in tumor tissue than in normal tissue [11]. This phenomenon was based on the EPR effect of macromolecules [11] and lipids [17] in solid tumors. In fact, the biological activity of pyran–NCS in the solid tumor was 10 times higher than that of NCS at 5 min after injection (Table 2). However, pyran–NCS was excreted so rapidly into the urine that there was not enough time to build up a high concentration in the tumor after 30 min or longer (Table 2). Thus, the increase in molecular weight from 11,700 to about 23,000 (pyran–NCS) did not affect the renal clearance rate, and pyran–NCS could not use the EPR effect for greater tumor targeting compared with NCS, although a slight EPR effect was seen (Table 2). It was hypothesized that the good EPR effect of smancs is based on its relatively large apparent molecular size. Smancs, with its physical mass of 15,500 daltons, can bind to serum albumin (68 k), and thus

its apparent size can be 84 kdaltons [18]. These biocompatible macromolecules have a longer plasma half-life, and the EPR effect in solid tumors and tumor targeting became apparent [11].

The present experiment has provided an example of creation of another macromolecular anticancer agent by using pyran copolymer. Again, the EPR effect on tumor tissue was noted with lessened marrow toxicity. As shown in Table 2, pyran–NCS, with its molecular mass of 23,000, could be considered to be as small as NCS in view of its renal clearance or retention period of intratumor concentration. Namely, this molecular size is too small to use the EPR effect. For example, the plasma half-life of native superoxide dismutase (SOD) (molecular weight 32,000) with a $t_{1/2}$ of 5 min, was nearly equal to that of pyran–NCS. However, a conjugate of pyran copolymer with SOD (molecular weight about 40,000–50,000) had a prolonged plasma half-life compared with native SOD ($t_{1/10}$ of native SOD and pyran–SOD was 9 and 55 min, respectively) [13]. It is suggested that macromolecules that are conjugates of larger pyran copolymer, with a molecular weight of more than 50,000, may be more suitable for utilizing the EPR effect.

An interesting observation is that both NCS and pyran–NCS seemed less affected by multidrug-resistant cells (parental AUXB1 vs. resistant CH^RC5 Chinese hamster ovary cells), and the IC_{50} ratio for Adriamycin® was 720 times higher for CH^RC5 (not shown). This result may be explained by the fact that pyran–NCS is internalized via endocytosis, as found for NCS [19, 20] and smancs [21, 22]. Thus these macromolecules are not subject to a *p*-glycoprotein-dependent efflux mechanism, as observed for Adriamycin® [23]. Present results thus provide another advantage of pyran–NCS: less prone to drug resistance.

Furthermore, pyran copolymer itself is known to induce interferon *in vivo* [24, 25]. Whether pyran–NCS can do so should be tested. A similar polyanion conjugate, smancs, was also recently reported to induce interferon, of which about 60% was the γ-type [26], which indicates an adjuvant effect of the polyanion conjugated anticancer agents.

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