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# Differential Neutralizing Effect of Tiopronin on the Toxicity of Neocarzinostatin and SMANCS: A New Rescue Cancer Chemotherapy

Tatsuya Oda,[1] Hirotoshi Yamamoto,[2] Toyohiko Miki[1] and Hiroshi Maeda[1]

*[1]Department of Microbiology and [2]Second Department of Surgery, Kumamoto University Medical School, 2-2-1 Honjo, Kumamoto 860*

**The toxic effect and antitumor activity of neocarzinostatin (NCS) and SMANCS [copoly(styrene-maleic acid)-conjugated NCS] were greatly affected by N-(2-mercaptopropionyl)-glycine [tiopronin] both *in vitro* and *in vivo*, in cultured HeLa cells and RL♀1 tumor-bearing mice. The cytotoxicity of NCS and SMANCS against HeLa cells was remarkably reduced by the addition of tiopronin during drug treatment. Interestingly, the neutralizing effect of tiopronin on the toxicity of SMANCS was greater than that in the case of NCS. In the continuous presence of 10 m*M* tiopronin during a 1 h drug treatment, the 50% cell-killing doses of NCS and SMANCS were increased 72 and 208 times as compared to those without tiopronin, respectively, whereas tiopronin itself has no cytotoxicity to HeLa cells up to 100 m*M*. Furthermore, more effective reduction of the lethal toxicity of SMANCS was observed by the intraperitoneal (ip) administration of tiopronin after ip injection of a lethal dose of SMANCS as compared to the same protocol in the case of NCS in mice. Therapeutic studies on RL♀1 tumor-bearing mice revealed that delayed (time lag) ip administration of tiopronin after high-dose SMANCS administration ip was much superior to the combination of NCS with tiopronin, or SMANCS alone. In this time-lag combination chemotherapy of SMANCS with tiopronin, 60% of treated mice survived more than 60 days after tumor inoculation, while all the untreated control mice died within 20 days.**

Key words: Neocarzinostatin — SMANCS — Tiopronin — High dose chemotherapy

Neocarziostatin (NCS), a proteinaceous antitumor antibiotic, is a unique antineoplastic agent showing a broad therapeutic activity spectrum, against human cancers in the bladder, the liver, the stomach, the brain, and the pancreas, as well as leukemia.[1-4] However, the therapeutic usefulness of NCS is often limited, mainly by its toxic side effects, particularly marrow suppression (low WBC and platelets) and renal toxicity at high dose.[1,5] Most other potent antitumor drugs also have deleterious side effects, such as the cardiotoxicity of adriamycin,[6] and renal toxicity of cisplatin.[7,8] To reduce the toxic side effects of these antitumor drugs, combination chemotherapy of the drugs with appropriate antidotes has been tested. For example, it is well known that leucovorin can effectively prevent methotrexate toxicity to bone marrow and gastrointestinal epithelium if it is administered in sufficient doses following 6- to 36-h infusion of high doses of methotrexate.[9] Furthermore, more recently bismuth subnitrate, which induces a cysteine-rich, low-molecular-weight protein metallothionein, has been proved to suppress significantly the toxicity of cisplatin[10] and adriamycin.[11] Furthermore, Baba *et al.*[12-14] reported that tiopronin is an effective antidote for NCS and that the therapeutic efficacy of NCS was much improved by the combination of high-dose NCS with tiopronin in a rat limb tumor model.

SMANCS is a chemical conjugate of a synthetic styrene maleic acid anhydride copolymer (SMA) and NCS.[15-17] SMANCS shows several improved pharmacologic properties; enhanced *in vivo* stability, decreased antigenicity, tumor- and lympho-tropicity, increased cell-surface affinity, and increased hydrophobicity.[15-19] Its remarkable clinical benefits have been well documented.[20-22] In this study we found that the toxicity of SMANCS was more sensitive to several thiol compounds including tiopronin than that of NCS. Therefore, we carried out experiments to examine the effect of tiopronin on the toxicity and therapeutic effect of SMANCS *in vivo* and *in vitro* in comparison with that in the case of NCS.

In the present studies, we found that treatment of RL♀1 tumor-bearing mice with time-lag chemotherapy involving high-dose SMANCS with tiopronin led to an antitumor effect much superior to that of treatment with NCS and tiopronin according to the same protocol, or SMANCS alone.

## MATERIALS AND METHODS

**Animals and chemicals** Male ddy mice (Kyudo Co., Ltd., Fukuoka) weighing 25–30 g at 7 weeks of age and male BALB/c mice weighing 20–25 g at 6 to 7 weeks of

Abbreviations used: NCS, neocarzinostatin; SMA, a synthetic copolymer of styrene-maleic acid anhydride or its hydrolyzed form; MEM, Eagle's minimum essential medium; PBS, 0.01 *M* phosphate-buffered 0.15 *M* NaCl, pH 7.4; DTT, dithiothreitol.

age (Shizuoka Laboratory Animal Center, Hamamatsu) were used for toxicity assay and for chemotherapy experiments, respectively. All mice were maintained on a standard diet and water *ad libitum*. NCS was obtained from Kayaku Co., Ltd., Tokyo. SMA with a mean molecular weight of about 1,600 in which 50–70% (mol/mol) of the carboxyl groups of maleic acid were half-esterified with butyl alcohol (Kuraray Co., Ltd., Osaka) was used for conjugation with NCS. SMANCS was prepared as described by Maeda *et al.*[17] Tiopronin (liquid form), N-(2-mercaptopropionyl) glycine was kindly provided by Santen Pharmaceutical Co., Ltd, Osaka and its crystal form was purchased from Sigma Chemical Co., St. Louis, MO. The original concentration of tiopronin was 50 mg/ml in saline. The pH of tiopronin solution was adjusted to 7.2 with 0.1 *M* NaOH before adjustment to the final concentration. Newborn calf serum and Eagle's MEM were obtained from Gibco, Grand Island, NY. All other chemicals were from commercial sources.

**Cells** HeLa cells were maintained in monolayer cultures in Eagle's MEM with 10% heat-inactivated newborn calf serum in a humidified atmosphere containing 5% $CO_2$/95% air at 37°C. The cells were removed from culture flasks 1 day before use by treatment with 0.1% trypsin-0.05% EDTA in PBS and seeded onto plastic wells at a suitable concentration.

***In vitro* cytotoxicity studies** Neutralizing activity of tiopronin against the toxic effect of NCS and SMANCS was measured by colony formation assay using HeLa cells in plastic wells (Falcon 96-well plate, No. 3072) at a density of $2\times10^4$ cells/well. Cells were cultured in 0.2 ml of MEM containing 10% newborn calf serum (growth medium) for 18 h, then the cells were washed twice with PBS, and exposed to various concentrations of drugs in the presence or absence of tiopronin for 1 hr at 37°C in 0.2 ml of serum-free MEM. During the drug treatment, the cells did not become detached. After treatment, cells were washed twice with PBS and trypsinized. An appropriate number of detached cells (usually 250–300 cells) was placed in each 35-mm-diameter plastic Petri dish (Falcon No. 3001) and cultured in 2 ml of fresh growth medium at 37°C in a humidified atmosphere of 5% $CO_2$. After 7–8 days, the numbers of colonies on the plates were counted visually by using a digital colony counter (Model DC-3, Kayagaki Co., Ltd., Tokyo) after staining with 1% methylene blue solution.

**Lethal dose of NCS and SMANCS and rescue of mice with tiopronin** Various doses of tiopronin were given ip in a volume of 10 ml/kg 30 min after ip administration of a lethal dose of NCS (10 mg/kg) or SMANCS (10 mg/kg) ($LD_{50}$ values are about 1.0 and 1.3 mg/kg, respectively) and survival of mice was observed for 30 days. Ten mice were used for each experimental group.

**Tumor cells and chemotherapy experiments** RL♀1 radiation-induced leukemia cells were maintained in ascitic form by serial passage by injecting $3\times10^6$ cells/mouse ip into BALB/c mice. After a trypan blue dye exclusion test, $2\times10^6$ viable cells per mouse were inoculated ip. The high-dose chemotherapy with a lethal dose of NCS or SMANCS with tiopronin was performed once on day two after tumor inoculation. To evaluate the most suitable injection schedule of tiopronin, the doses of NCS or SMANCS and tiopronin were fixed in the present study whereas the time intervals were varied. After ip injection of NCS or SMANCS (both at 5 mg/kg), 1000 mg/kg of tiopronin was given once ip at various intervals. This dose of tiopronin is less than 1/2 $LD_{50}$ and is about 4 times the dose needed for full protection from the lethal toxicity of the drug. The curative efficacy of the treatment was evaluated in terms of the survival rate 60 days after tumor inoculation and the increase in life span.

## RESULTS

**Effect of tiopronin on the cytotoxicity of NCS and SMANCS to HeLa cells** Data presented in Fig. 1

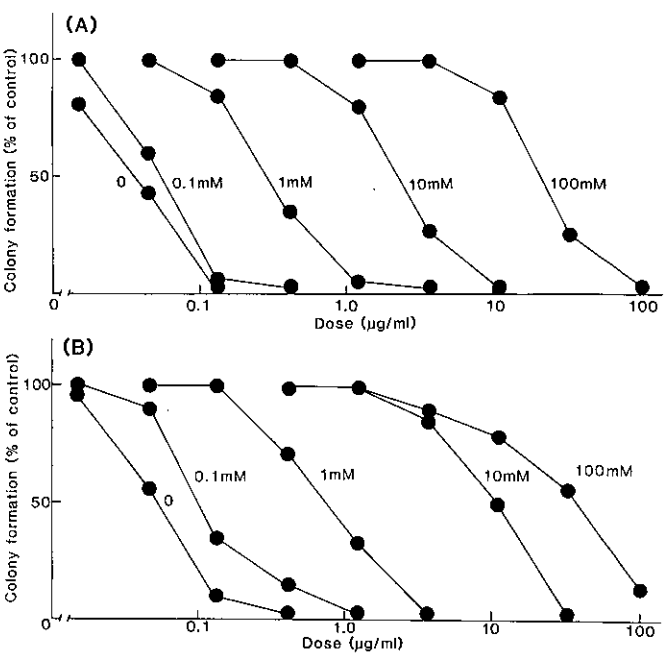


Fig. 1. The effects of tiopronin on NCS- or SMANCS-induced cytotoxicity in HeLa cells. Adherent HeLa cells ($2\times10^4$ cells/well) were treated with various concentrations of NCS (A) or SMANCS (B) for 1 h at 37°C in the presence of the indicated concentrations (0–100 m*M*) of tiopronin, and then assayed for survival by colony formation. Each point represents an average of duplicate determinations.

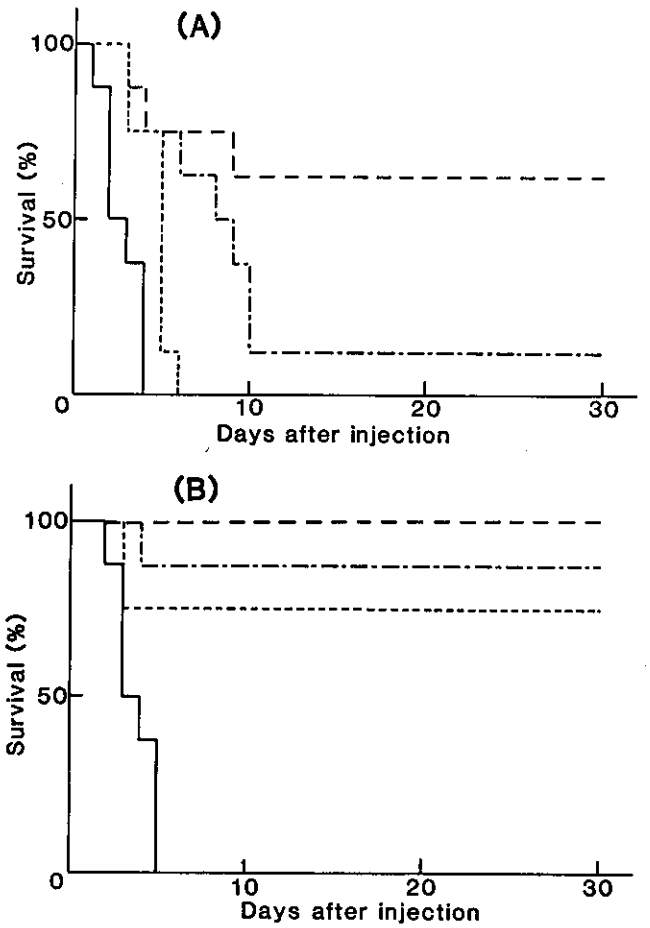


Fig. 2. Protective effect of tiopronin against the lethal toxicity of NCS and SMANCS in mice. A lethal dose of NCS (10 mg/kg) (A) or SMANCS (10 mg/kg) (B) was administered ip. After 30 min, (——), saline alone; (----), 62.5 mg/kg; (— - —), 125 mg/kg; (— — —), or 250 mg/kg of tiopronin was given ip to mice. Ten mice were used for each group. Survival of mice was observed for 30 days.

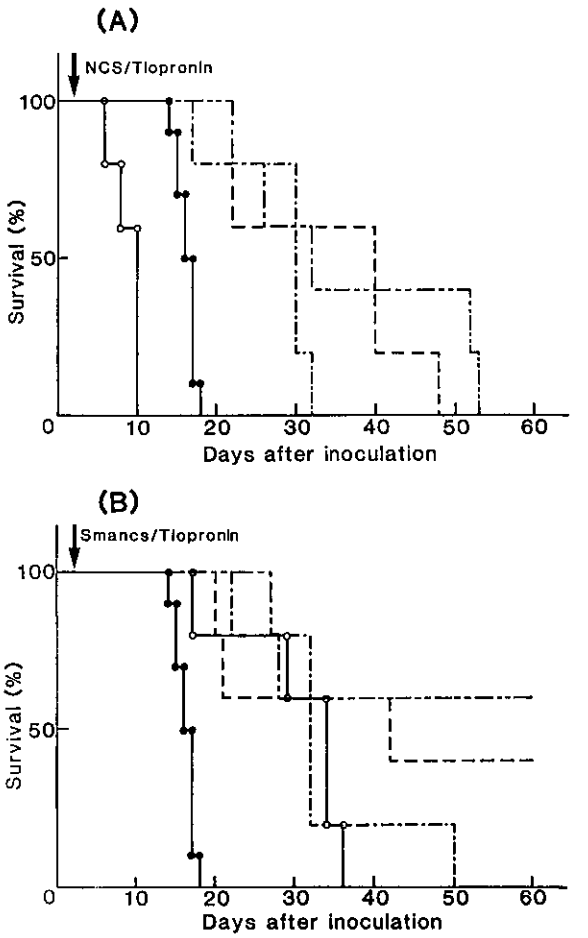


Fig. 3. Antitumor effect against RL♀1 ascites tumor of various time-lagged administrations of tiopronin in combination with NCS (5 mg/kg) (A) or SMANCS (5 mg/kg) (B). No drug (NCS or SMANCS) control, (●——●); anticancer agent alone, (○——○); anticancer agent given ip, subsequently tiopronin (1000 mg/kg) given ip after 10 min (— - —), after 20 min (— - - —) or after 40 min (— — —). Ten mice were used for each group.

demonstrate the protective effects of tiopronin on NCS- and SMANCS-induced cytotoxicity to HeLa cells. This protection was evidenced by a shift of the survival dose response curves to higher concentration of tiopronin. In the presence of 10 m*M* tiopronin, about 72 and 208 times greater amounts of NCS and SMANCS were required to accomplish 50% killing as compared with those of both antitumor agents alone, respectively. These results indicate that the toxicity of SMANCS is more effectively neutralized by tiopronin than that of NCS. Treatment with tiopronin alone had no effect on the survival of HeLa cells up to 100 m*M*. The protective effect of tiopronin was not observed when tiopronin was added to cells after anticancer drug treatment.

**Effect of tiopronin on the lethal toxicity of NCS and SMANCS in mice** A significant protective effect of tiopronin against the lethal toxicity of NCS and SMANCS was observed when tiopronin was administered at 30 min after drug injection, as shown in Fig. 2. Although this protective effect of tiopronin was dose-dependent and a higher dose of tiopronin provided greater protection against both NCS and SMANCS toxicity, the toxicity of SMANCS was more markedly reduced.

**Evaluation of antitumor effect** To find the most effective timing of the administration of tiopronin for enhanced therapeutic effect, chemotherapy experiments with RL♀1 tumor-bearing mice were carried out at a fixed dose of

each drug and tiopronin. After ip administration of NCS or SMANCS at a dose of 5 mg/kg, mice were given ip 1000 mg/kg of tiopronin 10, 20 or 40 min later. The survival curves of all the treated mice are shown in Fig. 3. The untreated control mice died within 18 days after the tumor cell inoculation. In the case of NCS, ip administration of 5 mg/kg without tiopronin produced severe lethal toxicity and all mice died within 8 days after injection. Although all mice treated with a combination of NCS with tiopronin survived longer than untreated control mice after tumor inoculation, there were no 60-day survivors (Fig. 3, A). In contrast, mice treated with SMANCS alone showed an increase of life span to about 206% (Fig. 3, B). The most significant increase in life span was observed in the combination group of SMANCS with administration of tiopronin 20 min later. The survival rate of this group on the 60th day was 60%. These results clearly indicate that a 20 min time lag is the most effective for the combination chemotherapy of SMANCS with tiopronin under the conditions used.

## DISCUSSION

A protective effect of thiol compounds such as DTT and tiopronin on the toxicity of NCS has been described previously,[12-14] and tiopronin itself has been used clinically to treat hepatic disorders.[23] In the present study we have not only confirmed the protecting effect of tiopronin against the toxicity of NCS, but also found that the effect is more pronounced with SMANCS, though the nature of the protecting effect appeared to be different. In our preliminary experiments, we found that among various thiol compounds examined, such as cysteine, DTT, glutathione, cysteamine, penicillamine and tiopronin, tiopronin was the most effective antidote for SMANCS and NCS, exhibiting no toxicity by itself (data not shown).

Interestingly, the cytotoxicity of SMANCS was more effectively reduced by tiopronin than that of NCS *in vitro*. In addition, it was confirmed that the lethal toxicity of SMANCS was more effectively reduced by tiopronin than that of NCS *in vivo* as well. The mechanism involved is unclear, but it may be speculated that increased hydrophobicity of SMANCS introduced by the conjugation with SMA polymer may facilitate interaction between SMANCS and tiopronin. Another possible explanation is that SMANCS tends to be retained longer in the blood (and in tumor, ip) compartment. Thus, the longer plasma $t_{1/2}$ due to lower excretion and absorption made it more accessible to tiopronin in the same compartment. Further, SMANCS or another polymer (pyran-copolymer)-conjugated NCS which has a large molecular size seems to leak out from the blood vessels into the bone marrow less effectively than NCS, resulting in lower marrow toxicity of the macromolecular drug (Yamamoto *et al.*, unpublished data). A more plausible detoxifying mechanism may be as follows: tiopronin is known to accumulate in the kidney, the epidermal adipose tissue, the adrenal tissue, the liver, the pancreas, and the diaphragm,[24] while SMANCS accumulates, in the kidney, the liver, the lymph nodes and the bone marrow, etc.[25] Thus, both drugs tend to meet in the kidney, which is a major target organ for the toxicity.

Taniguchi and Baba[26] proposed that the selection of a combination of a potent anticancer drug and an effective antidote was important for promising combination chemotherapy; the combination of an anticancer drug possessing a rapid cytocidal effect with an effective antidote possessing no toxicity was ideal. Recently we found that SMANCS exhibited more rapid toxicity to cultured cells due to increased cell-surface binding affinity compared with that of NCS.[18, 19] Namely, at the same dose of 30 n*M*, SMANCS achieved 50% inhibition of colony formation relative to the control (no drug) within 5 min, whereas NCS required more than 90 min to achieve the same toxic effect.[18] Thus, SMANCS appears to meet the criterion for effective combination chemotherapy with an antidote, tiopronin.

The results of the present study clearly indicated that time-lagged high-dose chemotherapy with a combination of SMANCS, ip and tiopronin, ip produced a remarkable therapeutic effect in RL♀1 tumor-bearing mice. In this time-lag chemotherapy, it was found that administration of tiopronin 20 min after SMANCS, ip injection was the most suitable, and 60% of treated mice survived over 60 days after tumor inoculation, while no 60-day survivors were obtained by the combination therapy of NCS with tiopronin under the same protocol or by SMANCS alone.

In this time-lag high-dose chemotherapy, it is speculated that the intraperitoneally disseminated tumor cells were effectively killed by the high dose of SMANCS administered first, while the effect of the drug on normal tissues was readily inactivated by subsequent administration of tiopronin.

With respect to the protective action of thiol compounds, it is well known that thiols have a protective effect against alkylating agents such as nitrogen mustard.[27, 28] In addition, radioprotector thiols such as 2-[(aminopropyl)amino]ethanethiol can protect against cisplatin-induced cytotoxicity and mutagenicity.[29] In general, the protective effect of thiols has been proposed to be mediated through scavenging of free radicals,[30, 31] by donating hydrogen atoms, etc.[32]

The molecular mechanism of NCS cytotoxicity is complex. NCS requires thiol compounds for cleavage of DNA *in vitro*, but thiols also inactivate it. Sheridan and Gupta reported involvement of free radicals in the action of NCS.[33] This implies that thiol (tiopronin) may nullify

free radicals generated by NCS and SMANCS. The mode of action of tiopronin against NCS remains unclear. Iwamoto *et al.*[12] postulated that the scission of disulfide bonds of the apoprotein of NCS by tiopronin may cause dissociation of the chromophore, a biologically active portion of NCS, from the apoprotein, and the chromophore may by immediately inactivated due to its instability. While further studies on the relationship between SMANCS and tiopronin are needed, the time-lag chemotherapy presented here using the combination of SMANCS with tiopronin may be applicable to the clinical situation. By the judicious use of SMANCS with tiopronin as an adjuvant in well-designed therapeutic protocols involving a time-lag schedule, it may be possible not only to obtain a therapeutic advantage but also to reduce significantly the drug-induced toxic effects.

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