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Efficacy of Two-route Chemotherapy Using Intraperitoneal Neocarzinostatin and Its Antidote, Intravenous Tiopronin, for Peritoneally Disseminated Tumors in Mice

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We assessed the efficacy of "two-route chemotherapy (TRC)" using neocarzinostatin (NCS) given ip and its antidote, N-(2-mercaptopropionyl)-glycine (tiopronin), given iv for peritoneally disseminated tumors in mice. Whether or not the single iv administration of tiopronin (800 mg/kg) at various times after NCS ip would decrease the lethal toxicity induced by NCS ip was given attention. When compared with the LD_{50} (4.4 mg/kg) of NCS ip alone, simultaneous or postadministration of tiopronin together with NCS ip increased the LD_{50} of NCS ip by 2.8 to 7.6 fold in a time-dependent manner. Chemotherapy experiments on ip disseminated tumors in mice were done to compare the antitumor effects of the following treatments, at two dose levels (75 and 100% of LD_{10}) of NCS, with or without tiopronin: treatment with NCS ip alone and combined chemotherapy using NCS ip plus tiopronin iv, simultaneously or postadministered. Based on the survival time of the treated mice, the groups given NCS plus tiopronin (postadministration, 15 or 25 min later) showed a significantly superior survival time to that of the group given NCS ip alone. The side effects, evaluated in terms of the changes in body weight and number of WBC of the mice, were not significantly different among the groups treated with 100% of LD_{10} of NCS.

Key words: Two-route chemotherapy — Intraperitoneal neocarzinostatin — Intravenous tiopronin

Intraperitoneal administration of anticancer drugs has proven to be an effective treatment for peritoneally disseminated cancer,[1-3] presumably because the concentration of the drug in the peritoneal cavity is maintained much higher and for a longer time than it is in the plasma.[2] However, the dose of the drug given ip is limited, as severe side effects occur when an anticancer drug enters the systemic circulation from the peritoneal cavity.[4] To reduce this general toxicity, we developed a combination chemotherapy, termed "two-route chemotherapy (TRC)," in which a high dose of anticancer drug is given locally at the tumor site and its antidote is given systemically to diminish the related side effects.[5,6] TRC, using a combination of *cis*-diamminedichloroplatinum (II) (CDDP) plus sodium thiosulfate (STS) or mechlorethamine N-oxide plus cysteine has been used in cases of liver tumor, peritoneally disseminated tumor, bladder tumor, metastatic lung tumor, and limb tumor in laboratory animals. The therapeutic effects of TRC were remarkable, compared with cases of a single treatment with an anticancer drug alone.[4-14] TRC has been prescribed clinically.[15,16] Other investigators developed combination chemotherapies, using activated cyclophosphamide plus thiol,[17] methotrexate plus leucovorin,[18] and CDDP plus systemic STS[19] to treat peritoneally disseminated tumors.

We used a new combination of neocarzinostatin (NCS) with its antidote, tiopronin,[20-22] and noted effectiveness of TRC to treat a rat limb tumor.[23] NCS is an antitumor antibiotic of high molecular weight, prescribed to treat acute leukemia, gastric cancer, pancreas cancer, and other cancers.[24] Tiopronin, a sulfhydryl compound, has been used clinically to treat mercury poisoning and hepatic disorders.[25,26] We report here that TRC using ip NCS plus iv tiopronin showed greater therapeutic effects than did the ip NCS single treatment for treating peritoneally disseminated tumors in mice.

MATERIALS AND METHODS

Animals Male DDD mice weighing 20–26 g at 6 weeks of age were obtained from the Animal Center of Kyushu University and were maintained on a conventional diet and tap water *ad libitum*.

Chemicals and administrations The original NCS solution (liquid form, Lot. 15404) was kindly provided by Kayaku Co. Ltd., Tokyo. Tiopronin (liquid form) was donated by Santen Pharmaceutical Co. Ltd., Osaka. The original NCS solution (1 mg/ml) was diluted to the desired concentration with cold 0.9% NaCl solution and shielded from light until use. NCS was administered ip in a volume of 2 ml per mouse weighing 25 g. The original

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tiopronin solution (50 mg/ml) was administered iv in a volume of 0.4 ml per mouse weighing 25 g, via the tail vein.

Pharmacokinetics of NCS and tiopronin For the assay of plasma NCS concentration, groups of 4 mice were given 10 mg/kg of NCS ip and blood samples were taken from the cervical artery 5 to 90 min later. Approximately 0.5 ml of serum was obtained from each blood sample by centrifugation. NCS concentrations in the serum samples were measured by the reverse phytohemagglutinin (RPHA) method.[27] For the assay of blood tiopronin concentration, groups of 4 mice were given 800 mg/kg of tiopronin iv and blood samples were prepared as described above. Sulfhydryl concentrations in the blood samples were measured with 5,5′-dithiobis(2-nitrobenzoic acid) by the method of Ellman.[28] Prior to tiopronin administration, blood samples were taken as a blank to determine the endogenous SH level. To measure the concentration of tiopronin in the peritoneal cavity, groups of 4 mice were given 800 mg/kg of tiopronin iv and killed 1 to 50 min later. Saline was given ip to the mice in a volume of 6 ml per mouse weighing 25 g and recovered immediately after peritoneal massage. The sulfhydryl concentrations in the regained saline samples were measured, as described above.

Lethal toxicity test The protective effect of a single iv administration of tiopronin (800 mg/kg) against the lethal toxicity of NCS in mice was evaluated in terms of the change of the LD_{50} of NCS ip under conditions of a single administration of tiopronin at 0, 8, 15, 25 or 35 min after the ip injection of NCS. At least 5 doses of NCS were tested for each timing of iv administration with tiopronin and at least 8 mice were used for each point. Survival rate was determined 30 days after the treatment. When tiopronin alone (800 mg/kg: approximately 35% of LD_{50} of the drug) was given iv to non-tumor-bearing DDD mice, neither loss of body weight nor toxicity-related death was observed (data not shown).

Tumors and chemotherapy experiments Two syngeneic transplantable tumors of DDD mice were used for chemotherapy experiments. Fibrosarcoma induced with 3-methylcholanthrene (MCFS) and mammary carcinoma (MMK), developed spontaneously in female DDD mice,[29] have been maintained by serial transplantation into the hind limb of DDD mice. Each tumor was excised, minced, suspended in Hanks' balanced salt solution and passed through a metal sieve to obtain a single cell suspension. After a trypan blue exclusion test, 10^6 viable cells per mouse were inoculated ip.

Chemotherapy experiments were carried out 48 h after inoculation. NCS was administered ip in a volume of 2 ml per mouse weighing 25 g. Tiopronin was given iv to mice in the TRC groups at various times after NCS ip. The % increase in life span (ILS) of the treated mice was calculated by comparing the median survival days of the treated groups with that of the untreated control group. Antitumor effects of treatments were evaluated by determining % ILS at 75 and 100% of LD_{10} dose levels of NCS, in each combination with tiopronin.

Side effects To evaluate the general toxicity induced by NCS, mice in each group were weighed on days 3, 6, and 9 after various treatments at 100% of LD_{10} dose level. To determine possible hematological disorders, changes in the number of WBC of the mice in each group were counted with a Coulter counter on days 3, 6, and 9 after the treatments.

RESULTS

Pharmacokinetics of NCS and tiopronin The concentration of NCS in the plasma increased slowly with time after NCS (10 mg/kg) ip administration and reached the peak level (1.23 μg/ml) 30 min later (Fig. 1). The NCS concentration at 15 min after NCS ip was only one-seventh (0.18 μg/kg) of the peak level. On the other hand, the concentration of tiopronin in the blood decreased rapidly soon after the iv administration of the drug (800 mg/kg), the half-life being approximately 6 min (Fig. 2). The concentration of tiopronin in the peritoneal cavity increased rapidly after the iv injection and reached the peak level at 5 min later (Fig. 3).

Lethal toxicity test Table I shows the protective effects of a single iv administration of tiopronin (800 mg/kg) at various times after NCS ip against the lethal toxicity induced by NCS. The effect in diminishing the lethal

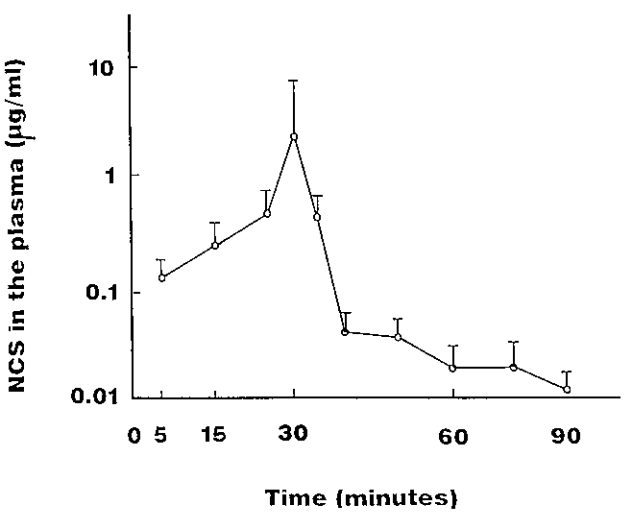

Fig. 1. Plasma NCS concentration following NCS (10 mg/kg) ip administration in DDD mice. Each point indicates the mean of 4 mice. Bars, SE.

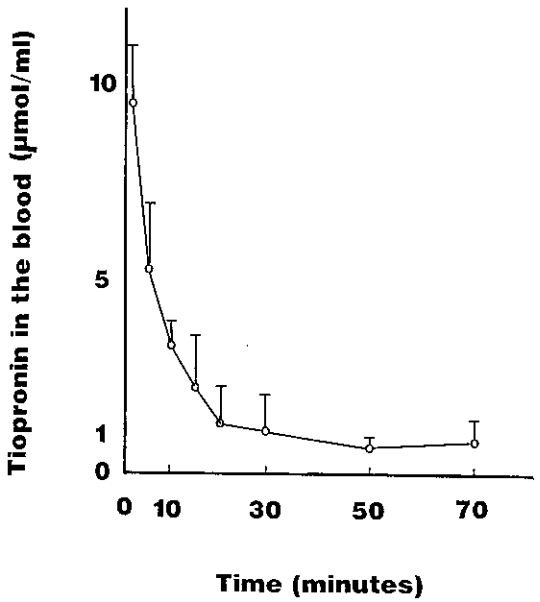

Fig. 2. Blood tiopronin concentration following tiopronin (800 mg/kg) iv administration in DDD mice. Each point indicates the mean of 4 mice. Bars, SE.

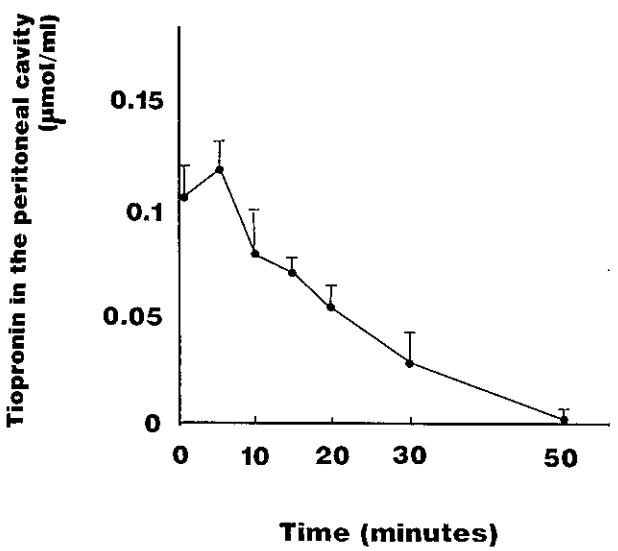

Fig. 3. Peritoneal tiopronin concentration following tiopronin (800 mg/kg) iv administration in DDD mice. Each point indicates the mean of 4 mice. Bars, SE.

Table I. Protective Effects of a Single iv Administration of Tiopronin at Various Times after NCS ip against the Lethal Toxicity of NCS in DDD Mice

Treatment	LD_{50} (mg/kg)[a]	LD_{10} (mg/kg)
NCS ip alone	4.4 (3.2–6.1)[b]	1.4
NCS ip + tiopronin iv[c] simultaneously with NCS ip	33.5 (23.9–46.9)	7.4
NCS ip + tiopronin iv 8 min after NCS ip	30.1 (20.6–43.9)	6.5
NCS ip + tiopronin iv 15 min after NCS ip	26.0 (18.0–37.4)	5.8
NCS ip + tiopronin iv 25 min after NCS ip	22.8 (15.8–32.8)	5.3
NCS ip + tiopronin iv 35 min after NCS ip	12.5 (8.2–19.1)	2.4

a) The LD_{50} values were calculated by the Litchfield and Wilcoxon method.[36]
b) Numbers in parentheses indicate 95% confidence limits of the LD_{50}.
c) Tiopronin (800 mg/kg) was given.

Table II. Effects of Various NCS Treatments (75% of LD_{10} Dose Level) on Survival Time of DDD Mice Bearing ip Disseminated MCFS Tumor

	Treatment[a]		Survival (days)		% ILS[c]
	NCS dose (mg/kg)	Timing of iv tiopronin[b]	Range	Median	
A	Untreated control		9–11	10.5	—
B	1.1	Not given	12–15	12.0	14
C	5.6	Simultaneously with NCS ip	12–17	14.0	33
D	4.9	8 min after	11–16	13.0	24
E	4.4	15 min after	15–25	16.5	57
F	4.0	25 min after	14–23	16.5	57
G	1.8	35 min after	12–15	12.0	14

a) Treatments were given 48 h after ip inoculation of 10^6 viable MCFS tumor cells. Each group included 10 mice.
b) Tiopronin (800 mg/kg) was given in Groups C, D, E, F and G.
c) % ILS = 100 ($t/c-1$), where t is the median survival time of the treatment group, and c is the median survival time of the control group.
Probability was calculated by means of the generalized Wilcoxon test. P value for Groups, C, D, and G versus B, not significant; P value for Groups E and F versus B, $P<0.05$.

toxicity was time-dependent. The simultaneous administration of tiopronin was the most effective to increase the LD_{50} of NCS and postadministration of tiopronin, even at 15 and 25 min after NCS ip, also led to a marked increase in the LD_{50}.

Chemotherapy experiments Tables II, III and IV show the antitumor effects of various combination treatments at 75 and 100% of LD_{10} of NCS on the survival time of DDD mice bearing peritoneally disseminated MCFS and MMK tumors. As can be seen in Tables II and III, while

the group given NCS ip plus simultaneous iv administration of tiopronin (Group C) showed a moderate increase of % ILS compared with that of the group given NCS ip alone (Group B), the groups given NCS ip plus iv postadministration of tiopronin (15 and 25 min later, Groups E and F) showed significantly greater % ILS than did the NCS single treatment. As shown in Table IV, appreciable increases of the % ILS of the mice bearing MMK tumor were seen in the groups given NCS ip plus iv postadministration of tiopronin.

Side effects Table V shows the changes in number of WBC and body weight after various NCS treatments at 100% of LD_{10} dose level. Neither severe leucopenia nor weight loss was observed in the treated groups. The

Table III. Effects of Various NCS Treatments (100% of LD_{10} Dose Level) on Survival Time of DDD Mice Bearing ip Disseminated MCFS Tumor

Treatment[a]			Survival (days)		% ILS[c]
	NCS dose (mg/kg)	Timing of iv tiopronin[b]	Range	Median	
A	Untreated control		10–12	11.0	—
B	1.4	Not given	13–18	15.0	36
C	7.4	Simultaneously with NCS ip	13–20	15.5	41
D	6.5	8 min after	14–25	15.0	36
E	5.8	15 min after	17–>60	20.0	82
F	5.3	25 min after	17–>60	19.5	77
G	2.4	35 min after	15–25	17.0	55

a) Treatments were given 48 h after ip inoculation of 10^6 viable MCFS tumor cells. Each group included 10 mice.
b) Tiopronin (800 mg/kg) was given in Groups C, D, E, F and G.
c) % ILS=100 $(t/c-1)$, where t is the median survival time of the treatment group, and c is the median survival time of the control group.
Probability was calculated by means of the generalized Wilcoxon test. P value for Groups C, D, and G versus B, not significant; P value for Groups E and F versus B, $P<0.01$.

Table IV. Effects of Various NCS Treatments (100% of LD_{10}) on Survival Time of DDD Mice Bearing ip Disseminated MMK Tumor

Treatment[a]			Survival (days)		% ILS[c]
	NCS dose (mg/kg)	Timing of iv tiopronin[b]	Range	Median	
A	Untreated control		13–15	14.5	—
B	1.4	Not given	16–30	17.5	21
C	5.8	15 min after	18–>60	22.0	51
D	5.3	25 min after	20–>60	21.5	48

a) Treatments were given 48 h after ip inoculation of 10^6 viable MMK tumor cells. Each group included 10 mice.
b) Tiopronin (800 mg/kg) was given in Groups C and D.
c) % ILS=100 $(t/c-1)$, where t is the median survival time of the treatment group, and c is the median survival time of the control group.
Probability was calculated by means of the generalized Wilcoxon test. P value for Groups C and D versus B, $P<0.05$.

Table V. Changes in Number of WBC and Body Weight after Various NCS Treatments (100% of LD_{10} Dose Level)

Treatment[a]			Days after treatment	WBC (mean±SE)	% of initial body weight (mean)
	NCS dose (mg/kg)	Timing of iv tiopronin[b]			
A	Untreated control		3	6942±326	3.1
			6	7107±103	6.1
			9	7888±269	7.2
B	1.4	Not given	3	5970±312	−1.2
			6	6444±290	3.8
			9	6901±632	4.0
C	5.8	15 min after	3	6122±188	−2.1
			6	7419±547	3.2
			9	7004±370	4.6
D	5.3	25 min after	3	6495±109	−1.7
			6	6691±505	2.7
			9	7114±495	3.7

a) Treatments were given 48 h after ip inoculation of 10^6 viable MMK tumor cells. Each group included 5 mice.
b) Tiopronin (800 mg/kg) was given in Groups C and D.

changes in number of WBC and body weight were not significantly different among the treated groups.

DISCUSSION

The TRC using NCS ip and its antidote, tiopronin iv, showed greater therapeutic effects against ip disseminated tumors in mice than did the NCS single treatment. A characteristic point in this TRC using NCS plus tiopronin is that tiopronin was postadministered to NCS ip. The iv postadministration of tiopronin with NCS ip was effective in reducing the general toxicity induced by NCS, as well as the simultaneous administration of tiopronin (Table I). However, in the combination chemotherapy using a high dose of CDDP ip plus STS iv STS had to be administered simultaneously with CDDP ip for effective detoxification of CDDP.[4] Postadministration of STS with a high dose of CDDP ip was not effective in reducing the general toxicity induced by CDDP.[4] The difference in the effectiveness of postadministration of these antidotes to detoxify CDDP or NCS is considered to be due to the difference in the rate of absorption of each anticancer drug from the peritoneal cavity into the systemic circulation. Whereas 30 min was required for NCS (10 mg/kg) ip to reach the maximun concentration in the plasma (Fig. 1), only 10 min was required for CDDP (10 mg/kg) ip.[4] This slower absorption of NCS seems to be attributable to the difference in molecular weight: NCS has a much higher molecular weight (Mw 10,700)[24] than CDDP (Mw 300).

In the chemotherapy experiments comparing TRC with NCS ip single treatment, TRC using NCS ip plus simultaneous administration of tiopronin resulted in a moderate increase in % ILS (Tables II and III). TRC using NCS ip plus postadministration (15 or 25 min later) of tiopronin led to significantly greater antitumor effects on MCFS and MMK tumors than did the NCS ip single treatment (Tables II, III and IV). These results show the importance of postadministration of tiopronin in the TRC using NCS and tiopronin for ip disseminated tumors. The difference in antitumor effects between the two combination chemotherapies, NCS plus simultaneous or postadministration of tiopronin, may be explained by the difference in the exposure time of the tumor cells to active NCS. In the former TRC, simultaneously iv administered tiopronin rapidly passed into the peritoneal cavity from the systemic circulation and reduced the cancerotoxic activity of NCS (Fig. 3). In the latter TRC, tumor cells in the peritoneal cavity were exposed to active NCS for 15 or 25 min without tiopronin. It has been reported that, in the case of treatment of mice bearing ip sarcoma 180 ascites tumor, NCS became bound to the tumor cells within 10 min after ip administration of the drug.[30] This finding also provides a rationale for the timing of postadministration (15 or 25 min later) of tiopronin with respect to NCS.

The results of postadministration of an antidote with an anticancer drug obtained in this experimental study suggest that sufficient therapeutic effects may be obtained in the clinical situation by using a potent anticancer drug in combination with postadministration of its antidote. Therefore, it is considered of importance to screen not only for new anticancer drugs but also for appropriate antidotes for the drugs. For example, SMANCS, a conjugate of NCS with a synthetic styrene maleic acid anhydride copolymer (SMA),[31] has superior pharmacological properties in several respects,[32,33] is prescribed clinically,[34] and is detoxified by thiol compounds.[35] Combination chemotherapy using an anticancer drug such as SMANCS plus its antidote may be effective for treating human cancers.

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