Anti-Cancer Drugs 1995, **6**, pp. 438–442

Therapeutic effects of the angiogenesis inhibitor TNP-470 against carcinomatous peritonitis in mice

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The therapeutic effects of the new anti-angiogenesis factor TNP-470 were examined against carcinomatous peritonitis in mice. In the first experiment using carcinomatous peritonitis caused by i.p. inoculation of 10^6 M5076 tumor cells, TNP-470 solution was injected i.p. in a bolus of 50 mg/kg body weight into two groups of 10 mice either 1 or 8 days after the i.p. inoculation. The administration of TNP-470 on day 1 extended the survival time of the mice compared with 10 control mice receiving no treatment, whereas TNP-470 given on day 8 did not affect the survival time. In the next experiment on the M5076 tumor, TNP-470 solutions at 100 or 300 mg/kg were injected i.p. in a bolus into two groups of 20 mice 1 day after the inoculation 10^6 tumor cells, respectively. The administration of TNP-470 at 100 mg/kg also had an inhibitory effect. However, TNP-470 at 300 mg/kg caused toxic death in half of the mice. Next, we examined the effects of TNP-470 on another type of carcinomatous peritonitis model, which was caused by i.p. inoculation of 10^6 B16 melanoma cells. In this experiment, TNP-470 solutions in a bolus of 150 mg/kg were injected i.p. into six groups of 10 mice each on day 1 only (group 1), on days 1 and 4 (group 2), on days 1, 4 and 7 (group 3), on day 8 only (group 4), on days 8 and 11 (group 5), or on days 8, 11 and 14 (group 6), respectively. The survival time was prolonged only in those groups receiving TNP-470 on days 1 and 4 (group 2), and days 1, 4 and 7 (group 3) as compared with a control group of untreated mice. In both M5076 tumor and B16 melanoma experiments, the early administration of TNP-470 was more effective at extending the survival times of mice receiving these drugs. However, there were significant differences in the relative sensitivities to TNP-470 between these two models.

Key words: Angiogenesis inhibitor, carcinomatous peritonitis, TNP-470.

Introduction

Angiogenesis, which is the formation of new blood vessels, is physiologically limited to certain organs in normal adults.[1] It has been demonstrated that the growth of solid tumors is generally angiogenesis-dependent, and it has been suggested that the growth of micrometastases in target organs and tissues also depends on angiogenesis.[2,3] Therefore, it is feasible that specific angiogenesis inhibitors would have an effective and selective therapeutic activity against the growth and metastasis of a wide range of tumors.[4] TNP-470 (*O*-chloroacetyl-carbamoyl fumagillol), an analog of fumagillin derived from *Aspergillus fumigatus*, is a newly developed potent anti-angiogenetic factor.[5] It has been reported that TNP-470 inhibited the growth and metastasis of several types of cancer in animal tumor models.[6–8] However, there have been no reports that examined the therapeutic effect of TNP-470 against carcinomatous peritonitis. In this study, we investigated the effects of TNP-470 against carcinomatous peritonitis using mice tumor models and characterized its therapeutic actions.

Materials and methods

Drugs and animals

TNP-470 was synthesized at Takeda Chemical Industries Ltd and was kindly donated for these *in vivo* experiments. TNP-470 was dissolved in a 5% glucose solution in all of our experiments.

Seven week old specific-pathogen-free male C57BL/6 mice were purchased from Shimizu Laboratory Animals. The animals were maintained under standard conditions (room temperature 22°C, relative humidity 60%, day–night cycle 12 h), and given standard mouse chow and tap water freely.

Tumor cell lines

The M5076 tumor was maintained i.p. in C57BL/6 mice. The tumor cells were isolated from the ascites

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

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fluid of the mice and was prepared in a suspension of 10^6 cells/ml of Hanks' solution (Nakarai Tesque, Kyoto, Japan). The cell viability was greater than 90%, as determined by the Trypan blue exclusion test. B16 melanoma cells were cultured in RPMI 1670^R medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco, Grand Island NY). The cultured B16 melanoma cells were harvested with phosphate-buffered saline containing 0.01% EDTA and 0.125% trypsin (Nakarai Tesque) and a single cell suspension was then prepared at a concentration of 10^6 cells/ml of Hanks' solution. The Trypan blue exclusion test showed the cell viability to be greater than 90%.

Carcinomatous peritonitis models

The carcinomatous peritonitis models were prepared by i.p. inoculations into mice with 10^6 M5076 tumor or B16 melanoma cells per 1 ml of tumor solution, respectively. In our preliminary study, we found that angiogenesis occurred in the peritoneal metastatic foci of these carcinomatous peritonitis models. When single cell suspensions of M5076 tumor or B16 melanoma were inoculated i.p. at 10^6 cells per mouse, angiogenesis had already occurred by 1 week after the inoculations. Figure 1(a) shows a nest of M5076 tumor in the greater omentum taken from a mouse 4 days after the inoculation of 10^6 tumor cells and Figure 1(b) shows a nest of B16 melanoma in the mesenterium of a mice 7 days after the inoculation of 10^6 cells. Newly-formed blood vessels are clearly visible inside these tumor foci. The M5076 tumors were stained with methyl green and the blood vessels were visualized by an intra-arterial injection of red India ink conjugated to gelatin. The B16 melanoma has a black color due to its intrinsic melanin and the tumor could be easily differentiated from the surrounding tissues.

Therapeutic experiments on carcinomatous peritonitis caused by M5076 tumor

We performed two experiments using the M5076 tumor model. In the first experiment, 10^6 M5076 tumor cells suspended in 1 ml of Hanks' solution were inoculated i.p. into three groups of 10 mice each on day 0. Two of the three groups were given 1 ml mouse of TNP-470 solution, containing 1 mg ml of TNP-470 (equal to 50 mg TNP-470 kg of body weight), on days 1 and 8 after the tumor inoculation,

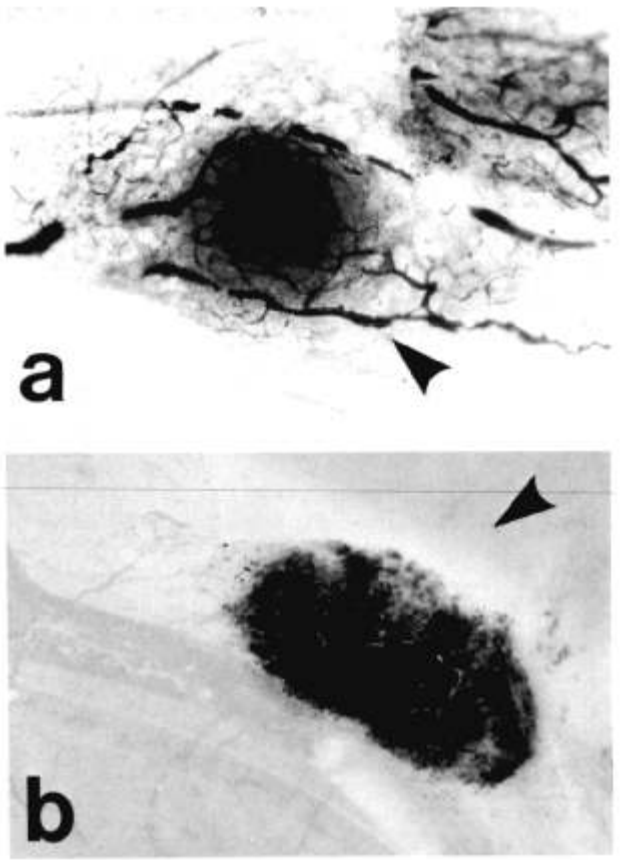

Figure 1. Histology of the peritoneal metastatic foci of the M5076 tumor and the B16 melanoma. (a) The greater omentum of a mouse sacrificed 4 days after an i.p. inoculation of 10^6 M5076 tumor cells (× 20). The main M5076 tumor was stained by methyl green. A lot of black tumor vessels, which were stained by an intra-arterial injection of a red India ink and gelatin mixture, can be seen inside the tumor (large arrows). (b) The mesenterium taken from a mouse sacrificed 7 days after the inoculation of 10^6 B16 melanoma cells (× 10). A large nest of black melanoma cells can be seen with a profusion of new-generated white vessels stained by an injection of red India ink and gelatin (large arrows).

respectively. The third group was untreated as control. The number of surviving mice was checked daily for 60 days, and the dead mice were examined by autopsy to determine the exact cause of death, such as tumor death, toxic death and death from injury at the injections. In the second experiments, 10^6 cells were inoculated i.p. into three groups of 20 mice each on day 0. On day 1 after the inoculation, two groups were given 1 ml per mouse of a TNP-470 solution containing 2 and 6 mg ml (equal to 100 and 300 mg TNP-470 kg of body weight), respectively. The third group received no treatment as control. The survivors were checked daily up to day 60 and the causes of death were also examined in the dead animals.

Therapeutic experiments on carcinomatous peritonitis caused by B16 melanoma

A suspension containing 10^6 B16 melanoma cells in Hanks' solution was inoculated i.p. into seven groups of 10 mice each on day 0. Three groups received 1 ml/mouse of TNP-470 solutions containing 3 mg/ml of TNP-470 (equal to 150 mg TNP-470/kg of body weight) either once (on day 1), twice (on days 1 and 4) or three times (on days 1, 4 and 7), respectively. Another three groups were given the same volume and concentration of TNP-470 solutions either on days 8 only, on days 8 and 11, or on days 8, 11 and 14, respectively. The final group was an untreated control. The survivors were checked daily for 60 days and the cause of death was examined.

Results

Therapeutic effects against carcinomatous peritonitis caused by the M5076 tumor

In the first experiment on carcinomatous peritonitis caused by the M5076 tumor, TNP-470 at 50 mg/kg given on day 1 significantly extended the survival time of the treated group (mean survival time in the treatment group/mean survival time in the control group (TC%)=113, $p < 0.01$) as compared with the control mice group. However, TNP-470 given on day 8 did not extend the survival time (Table 1). There was neither toxic death nor any severe side effects in both treatment groups. The autopsy findings for the dead mice showed no difference between the two treatment groups and the control group about the volume and appearance of i.p. tumors and ascites fluid. In the next experiment on the M5076 tumor, TNP-470 solution was injected i.p. at 100 mg/kg on day 1. This protocol also prolonged the survival time (TC% = 125, $p < 0.01$). However, TNP-470 at 300 mg/kg on day 1 caused toxic death in about half of the mice (Table 1). Furthermore, the surviving mice of this group had severe body weight loss (about 25% reduction, data not shown). In our preliminary experiment in mice, TNP-470 administered i.p. at 200 mg/kg in a bolus gave no toxic death. However, we limited the doses to 150 mg/kg TNP-470 in a bolus injection in order to prevent toxic death in the next experiment using the B16 melanoma.

Therapeutic effects against the carcinomatous peritonitis model caused by the B16 melanoma

In this experiment, TNP-470 given on days 1 and 4, and on days 1, 4 and 7, extended the survival time of the mice (TC% = 113, $p < 0.05$, and TC% = 124, $p < 0.05$, respectively). However, a single administration of TNP-470 at 150 mg/kg on day 1 did not extend the survival time. Neither single, double nor triple administrations of TNP-470 during the second week (on day 8, on days 8 and 11, and days 8, 11 and 14) significantly extended the survival time of each group compared with control group mice (Table 2). Although no mice died from toxicity of TNP-470, about 10 and 20% weight loss were recognized in the groups of mice receiving TNP-470 twice and three times, respectively (data not shown). There were no significant differences in

Table 1. Therapeutic effects of TNP-470 against carcinomatous peritonitis caused by the M5076 tumor

Treatment	Day of treatment	Dose (mg TNP/kg weight)	No. of mice	Toxic death[a]	Survival time[b] (days)	TC%[c]	p value[d]
Experiment 1							
control			10	0	21.6 ± 2.0	100	
TNP-470	1	50	10	0	24.3 ± 1.3	113	<0.01
	8	50	10	0	22.0 ± 1.5	102	NS
Experiment 2							
control			20	0	22.2 ± 2.1	100	
TNP-470	1	100	20	0	28.8 ± 3.0	130	<0.01
	1	300	20	12	30.5 ± 3.9	137	NS

[a] Number of mice dying from toxicity of TNP-470.
[b] Mean survival time ± SD (excluding animals that died from toxicity).
[c] Mean survival time in the treatment group/mean survival time in the control group.
[d] The p value (each treatment group versus control group) was calculated by the generalized Wilcoxon test.
NS, not significant ($p > 0.05$).

Table 2. Therapeutic effects of TNP-470 against carcinomatous peritonitis caused by the B16 melanoma

Treatment	Day of treatment	Dose (mg TNP/kg weight)	No. of mice	Toxic death[a]	Survival time[b] (days)	TC%[c]	*p* value[d]
Control			10	0	27.5 ± 3.0	100	
TNP-470	1	150	10	0	27.1 ± 2.1	99	NS
	1,4	150 × 2	10	0	31.0 ± 5.9	113	<0.05
	1,4,7	150 × 3	10	0	34.0 ± 8.6	124	<0.05
	8	150	10	0	27.4 ± 2.2	100	NS
	8,11	150 × 2	10	0	28.5 ± 5.1	104	NS
	8,11,14	150 × 3	10	0	29.8 ± 4.3	108	NS

[a] Number of mice dying from toxicity of TNP-470.
[b] Mean survival time ± SD (excluding animals that died from toxicity).
[c] Mean survival time in the treatment group/mean survival time in the control group.
[d] The *p* value (each treatment group versus control group) was calculated by the generalized Wilcoxon test.
NS, not significant ($p > 0.05$).

the autopsy findings of mice dying from tumors between the six treatment groups and control group.

Discussion

In normal adults, the vascular system in most organs is very stable and the turnover rate of endothelial cells is measured in years. Physiologic angiogenesis occurs only in some reproductive organs, such as during ovulation, menstruation and the development of the placenta.[1] A lot of evidence suggests that solid tumors are angiogenesis-dependent. The growth of a tumor beyond a certain size requires new blood vessles in order to supply nutrition and oxygen.[2] It has been also suggested that the growth of micrometastases in target organs and tissues depends on angiogenesis.[3] For these reasons, it is expected that specific angiogenesis inhibitors will provide a more effective and selective therapy against the growth and metastasis of a wide variety of tumors with fewer side effects than conventionally-used chemotherapeutic agents.[4] TNP-470 is a newly-developed potent anti-angiogenetic factor that has a very strong inhibitory action against endothelial cell growth and a weaker anti-tumor action.[5-9] It has been reported that TNP-470 inhibited the growth and metastasis of several animal tumor models by its anti-angiogenesis action with less toxicity.[6-8]

There has been a previous report examining the therapeutic effects of a high dose of protamine as an anti-angiogenesis factor against carcinomatous peritonitis caused by Walker 256 sarcoma in rat.[9] However, there have been no reports examining the inhibitory efficacy of TNP-470 against carcinomatous peritonitis. Therefore, in this study, we examined the effects of TNP-470 by i.p. injections against carcinomatous peritonitis using mice tumor models. We used two models of carcinomatous peritonitis caused by two mice tumors: a model caused by the M5075 tumor and another caused by the B16 melanoma. In our preliminary study, we found that angiogenesis had occurred in the peritoneal foci of these carcinomatous peritonitis models by 1 week after the i.p. inoculation of 10^6 M5076 tumor or B16 melanoma cells. Therefore, in the present study, we gave TNP-470 during this first week or later, and then compared the effects between them.

In the experiments using the M5076 tumor, i.p. administration of TNP-470 on day 1 after inoculation prolonged the survival time of these mice, whereas the mice receiving an administration of TNP-470 on day 8 did not survive longer. This result suggests that the administration of TNP-470 was more effective during the first week after innoculation in the M5076 tumor model. In the experiment with the B16 melanoma, double or triple administrations of TNP-470 in a bolus of 150 mg/kg each during the first week prolonged the survival times of these mice, whereas administration of the same doses in the second week did not extend the survival time for either group. This result also showed that treatment with TNP-470 against carcinomatous peritonitis caused by the B16 melanoma was more effective within 1 week after inoculations. However, a single adiminstration of TNP-470 at 150 mg/kg on day 1 did not prolong the survival time of the mice. This result indicated that carcinomatous peritonitis caused by the B16 melanoma was more resistant to the inhibitory effects of TNP-470 than the M5076 tumor. These differences in the sensitivity to the inhibitory or therapeutic effects of TNP-470 have been recognized among the various tumor models. Such differences in the sensitivity to TNP-

470 *in vivo* did not correlate with differences in sensitivity *in vitro*.

We conclude that TNP-470 has a positive therapeutic effect against carcinomatous peritonitis caused by the mice tumors.

Acknowledgments

We would like to express our gratitude to Dr K Sudo, Dr H Okada and other members of Pharmaceutical Research Laboratories III and DDS Research Laboratories in Takeda Chemical Industries Ltd for providing TNP-470 and valuable advice.

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(Received 24 January 1995; accepted 23 February 1995)