

# Prevention of Hepatic Metastasis of Human Colon Cancer by Angiogenesis Inhibitor TNP-470<sup>1</sup>

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## ABSTRACT

The antimetastatic effect of a potent angiogenesis inhibitor, *O*-(chloroacetyl-carbamoyl)fumagillol (TNP-470), was investigated in nude mice implanted with human colon cancer. Small pieces of tumors from three established human colon cancer cell lines (TK-3, TK-4, and TK-9), which were maintained in nude mice, were implanted into the cecal wall of nude mice via a small incision in the serosa. TNP-470 (20 or 30 mg/kg) was given s.c. every other day from day 10 after implantation, and the mice were sacrificed after 6 weeks.

There was no difference in the weight of the implanted tumors (control group:  $0.45 \pm 0.29$  g versus treated group:  $0.49 \pm 0.27$  g). An antimetastatic effect of TNP-470 was clearly demonstrated in a dose-dependent manner. In the mice given 20 mg/kg TNP-470, liver metastasis developed in 3 of 10 cases. In the 30-mg/kg group, metastasis developed in only 1 of 17 mice, while it developed in 22 of 32 mice of the control group. The number of metastatic foci was significantly less in the treated groups. TNP-470 effectively prevented liver metastasis, however, but had no effect on the growth of the primary tumor. These results indicate that the angiogenesis inhibitor TNP-470 has a strong inhibitory activity against *in vivo* hepatic metastasis of human colon cancer.

## INTRODUCTION

Although most colon cancer is surgically resectable, hepatic metastasis develops in these patients. Therefore, the control of hepatic metastasis has been attempted in various studies. Tumor neovascularization is part of the complicated process of tumor metastasis (1), and is crucial to the occurrence of hepatic metastasis. Since a small focus of tumor cells cannot grow indefinitely at a secondary site without the induction of angiogenesis, it is to be expected that inhibition of angiogenesis should provide a potent form of therapy for hepatic metastasis of colonic cancer (2).

Protamine has been reported to suppress tumor growth (3), but no clinical trials have yet been performed because of its severe side effects. Recently, we investigated the antitumor effects of TNP-470<sup>3</sup>, a synthetic analogue of fumagillin, which is a naturally secreted antibiotic produced by *Aspergillus fumigatus* fresenius (4, 5). TNP-470 inhibited angiogenesis more potently than its parent compound fumagillin, both *in vivo* and *in vitro* regardless of the presence of angiogenesis factors and the mode of administration, and was also less toxic than fumagillin (6). In addition, it has been reported that TNP-470 has an inhibitory effect on the growth and metastasis of human cell lines (7) and rodent tumors (8).

Implanting human tumor cells orthotopically in the corresponding organ of nude mice results in a much higher rate of metastasis. Human colon cancer cells injected into the cecum of nude mice produce tumors which eventually metastasize to the liver, demonstrating that orthotopic implantation can enhance the metastatic potential of these tumor cells (9-13). Recently, a new model of human colon cancer was developed that avoids the disruption of tumor integrity and

involves the orthotopically implantation of intact tumor tissue (14). Such a model should better reflect the original properties of human cancer and could be of great value in the development of new drugs and treatment strategies. In the present study, the inhibitory effect of TNP-470 on the local growth and hepatic metastasis of human tumors was examined in a nude mouse model of metastatic human colon cancer using the orthotopic implantation of histologically intact tissue.

## MATERIALS AND METHODS

**Materials.** TNP-470 was the kind gift of Takeda Chemical Industries, Ltd., (Osaka, Japan). Its structure has already been reported (4). TNP-470 was suspended a vehicle composed of 1% ethanol plus 5% gum arabic in saline.

**Human Colon Cancer Xenografts.** Three human colon cancer xenografts were used in the study. These were TK-3 (a moderately differentiated adenocarcinoma line), TK-4 (a well-differentiated adenocarcinoma line), and TK-9 (a well-differentiated adenocarcinoma line), which were established in our department. These xenografts were maintained by serial transplantation in nude mice.

**Animals.** Male BALB/c-*nu/nu* mice were obtained from Clea Japan, Inc., (Tokyo, Japan). Animals were used at 5-6 weeks of age.

**Orthotopic Tumor Tissue Implantation.** Small pieces of tissue were resected aseptically during the exponential growth phase from tumor s.c. implanted in nude mice. Mice were anesthetized, and a small midline incision was made. The colocecum part of the intestine was carefully exteriorized, and the serosa was removed at the site where the tumor pieces were to be implanted. A tumor piece of 5 mm in diameter was then fixed on each injured site of the serosal surface with a 6-0 Dexon (Davis-Geck, Inc., Manati, Puerto Rico) transmuralsuture. The intestine was returned to the abdominal cavity, and the abdominal wall and skin were closed with 6-0 Dexon sutures. The animals were kept in a sterile environment.

**Assay of Tumor Growth and Hepatic Metastasis.** Animals were given TNP-470 s.c. at a dose of 20 or 30 mg/kg every other day from day 10 after implantation. As a control, the same volume of physiological saline was given to other mice.

Mice were sacrificed 6 weeks after implantation. Autopsy was performed immediately, and the tumors growing on the cecal wall were removed and weighed. The liver was processed for routine histological examination to detect metastases after careful macroscopic examination.

**Statistical Analysis.** Student's *t* test and the  $\chi^2$  test were used for statistical analysis.

## RESULTS

**Tumor Growth.** All tumor pieces implanted in the cecum showed local orthotopic growth (Fig. 1). The ability of TNP-470 to inhibit local tumor growth in orthotopic intact tissue implantation was evaluated in nude mice inoculated with the three human colon cancer lines. The effect of TNP-470 at two different doses (20 and 30 mg/kg) was compared with the results in the control group, and no growth inhibitory effect was observed at either dose (Fig. 2).

**Hepatic Metastasis.** To evaluate the inhibitory effect of TNP-470 on hepatic metastasis by TK-3, TK-4, and TK-9, the incidence of hepatic metastasis was examined in nude mice. TNP-470 inhibited hepatic metastasis in a dose-dependent manner (Table 1). The number of metastatic foci in the liver was  $4.00 \pm 3.46$  in the 20-mg/kg group

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<sup>3</sup> The abbreviation used is: TNP-470, *O*-(chloroacetyl-carbamoyl)fumagillol.

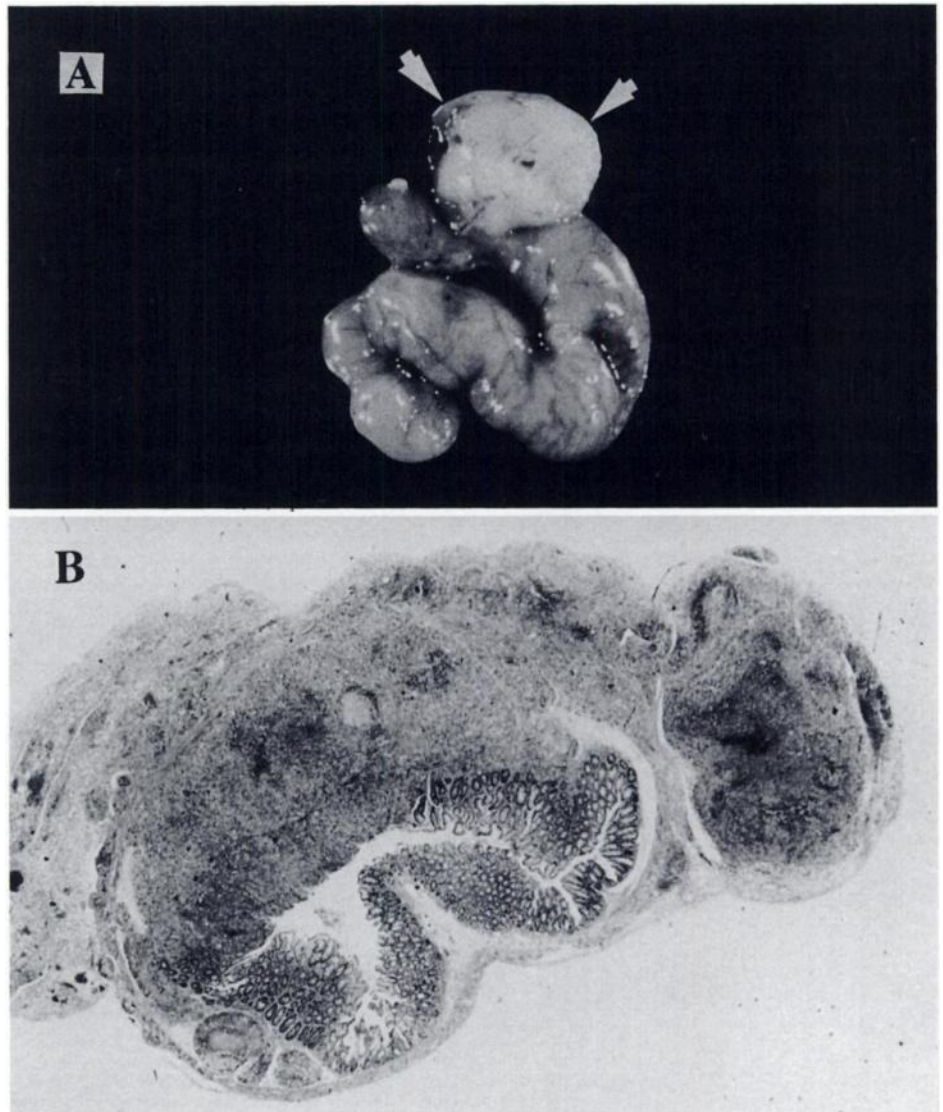


Fig. 1. A, cecum of a nude mouse. Arrows, a locally growing TK-3 tumor following the orthotopic implantation of intact tissue. B, photomicrograph of the TK-3 tumor growing in the cecum shown in A. The tissue was fixed, embedded, sectioned, and stained with H & E using standard procedures.

and 2 in the 30-mg/kg group. In contrast,  $5.61 \pm 3.73$  metastatic foci were found in the control group (Fig. 3).

**Body Weight.** In the mice treated with 30 mg/kg TNP-470, a decrease of weight gain was observed (Fig. 4), whereas the weight gain of the 20-mg/kg group was not decreased compared with the control group.

## DISCUSSION

Since solid tumor growth is reported generally to depend on angiogenesis (2, 15), angiogenesis inhibitors should have an inhibitory effect on *in vivo* tumor growth. The angiogenesis inhibitor TNP-470 has been reported to have an inhibitory activity against both tumor growth and metastasis (4, 6–8, 16). However, its inhibitory effect has not previously been examined in a nude mouse model of human colon cancer constructed using the orthotopic implantation of histologically intact tissue. In this study, we investigated the inhibitory effect of TNP-470 on the local growth and hepatic metastasis of human colon cancer using such a model. We found that TNP-470 had no effect on the local growth of the primary lesion. In previous studies (4, 7, 8, 16), it was reported that TNP-470 had an inhibitory effect of tumor growth. However, because the tumors were inoculated s.c., the model may not have reflected the natural environment of tumor growth. The host

organ microenvironment can profoundly influence the growth of tumor cells (17–19). The orthotopic implantation model used in the present study reflects the actual environment that tumor cells are surrounded by, and thus is more appropriate than heterotopic implantation models. In our previous experiment, the TK-3, TK-4, and TK-9 tumors reached a weight of 1.5–2.0 g in 6 weeks after s.c. inoculation into nude mice (20). On the other hand, when the tumors were implanted into the cecal wall of nude mice, the actual tumor weight was approximately 0.45 g. This difference in tumor growth may have led to the lack of an inhibitory effect of TNP-470 on the local growth of the primary lesion. This is consistent with the hypothesis that the rapidly proliferating tumor is more angiogenesis dependent (21). In addition, it is possible that the timing of TNP-470 administration may influence its antitumor effect.

We also evaluated the inhibitory effect of TNP-470 on hepatic metastasis and found that it inhibited hepatic metastasis in a dose-dependent manner (Table 1). Hepatic metastasis of TK-3 and TK-9 tumors was inhibited completely by 30 mg/kg TNP-470. It was recently shown that lung metastasis of human choriocarcinoma could be inhibited in nude mice treated with 30 mg/kg TNP-470 (7). In that study, human choriocarcinoma cells were inoculated s.c. into the axilla, and hepatic metastasis was observed in 4 of 4 (100%) of the

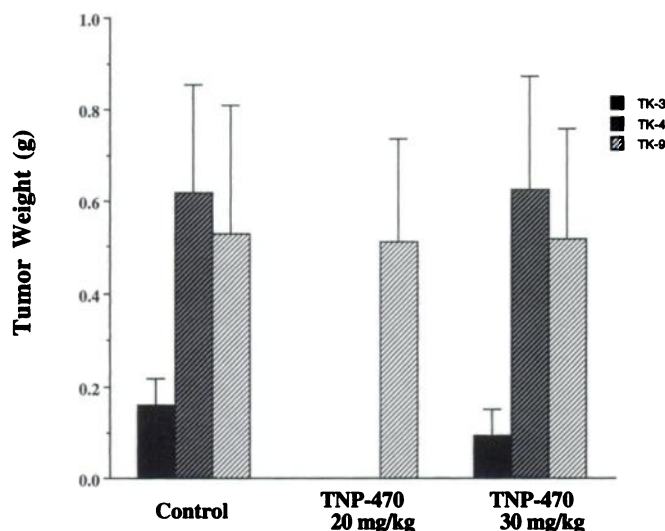


Fig. 2. The actual weight of human colon cancers growing in the nude mouse cecum at 6 weeks after implantation. No inhibitory effect of TNP-470 was observed on local tumor growth. The tumor weight in the 20-mg/kg control and 30-mg/kg groups was  $0.45 \pm 0.29$  g,  $0.51 \pm 0.23$  g, and  $0.49 \pm 0.28$  g, respectively. Error bars, SD.

Table 1 Inhibitory effect of TNP-470 on hepatic metastasis

Drug	Dose (mg/kg)	Tumor	Hepatic metastasis (no. of mice)
Control		TK-3	5/8 (62.5%)
		TK-4	7/9 (77.8%)
		TK-9	10/15 (66.7%)
			22/32 (68.2%)
TNP-470	20	TK-9	3/10 (30%) <sup>a</sup>
TNP-470	30	TK-3	0/4 (0%)
		TK-4	1/10 (10%)
		TK-9	0/3 (0%)
			1/17 (5.9%) <sup>b</sup>

<sup>a</sup>  $P < 0.05$  versus control group.

<sup>b</sup>  $P < 0.01$  versus control group.

30-mg/kg group. TK-4 is the most highly metastatic of the three tumors, and metastasized to the liver when it was inoculated s.c. TNP-470 clearly inhibited the hepatic metastasis of TK-4. Hepatic metastasis was observed in only 1 of 10 (10%) of the 30-mg/kg group<sup>4</sup> and was decreased significantly as compared with the control group.

Tumor cell metastasis occurs through a complicated process that involves five main steps: (a) angiogenesis, (b) adhesion to endothelial cell basement membrane components, (c) local proteolytic destruction of the basement membrane, (d) migration into secondary sites, and (e) proliferation at the secondary sites (1). Tumor growth and metastasis require the development of new vessels (22–24). Efficacy for hepatic metastasis by TNP-470 may depend on the inhibition of angiogenesis at the first and the final step of the metastatic process. Mature capillaries have a thickened basement membrane but growing capillaries have fragmented basement membrane (25). Because growing capillaries are leaky, these new vessels also increase the opportunity for tumor cells to enter the circulation (26). Tumor cells rarely enter the circulation because of inhibition of capillaries growth by TNP-470. Tumor cells that arrived at the target organ cannot grow without the induction of angiogenesis. After a new tumor has attained a size of a few millimeters in diameter, further expansion requires the development of new capillaries. Tumor cells that arrived at the liver could not grow to the detectable mass since angiogenesis was inhibited

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by TNP-470. Tumor angiogenesis has been shown to correlate with metastasis in breast carcinoma (27) and the present study indicated that hepatic metastasis of human colon cancer was prevented by inhibiting tumor angiogenesis. Thus, hepatic metastasis may have been prevented by TNP-470 both the opportunity for tumor cells to enter the circulation and by inhibiting the growth of tumor cells arriving in the liver.

A decrease of weight gain was observed in the 30-mg/kg group in this study. Yanase *et al.* (7) did not observe weight loss, and Yamaoka *et al.* (8) said that the effect of TNP-470 on weight gain seemed to depend on the type of tumor. Decreased weight gain was the only side effect of TNP-470 noted in this study, but investigation may be necessary.

The angiogenesis inhibitor TNP-470 seems to be a potent antimetastatic agent for colon cancer, and it may be clinically applicable after further study.

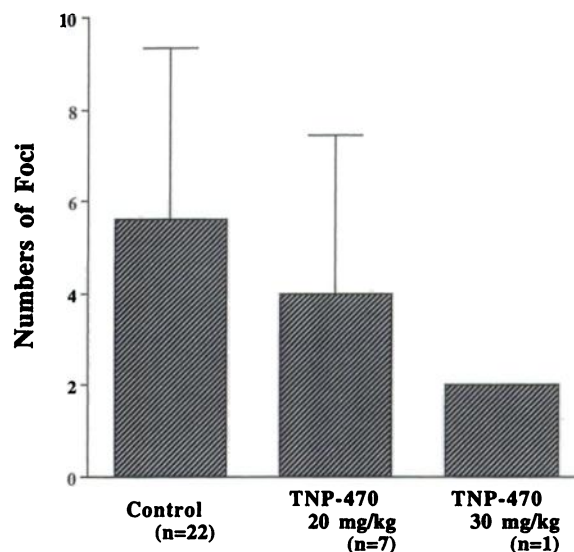


Fig. 3. Number of metastatic foci in the nude mouse liver. The inhibitory effect of TNP-470 on hepatic metastasis was dose dependent. Error bars, SD. Because there was only one mouse in which hepatic metastasis was found in the 30-mg/kg group, statistical analysis is impossible.

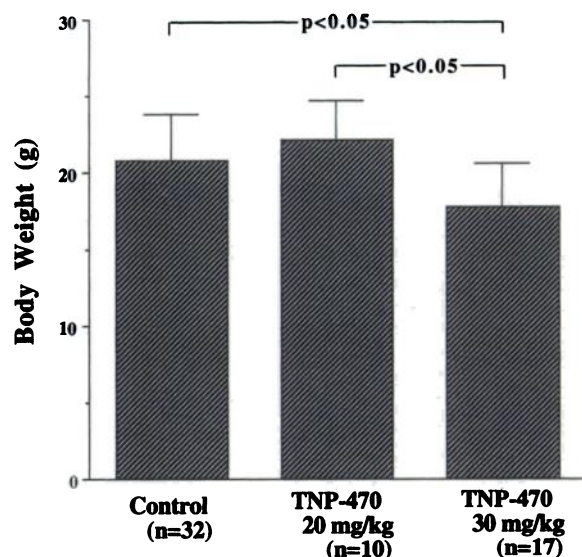


Fig. 4. Body weight of nude mice 6 weeks after tumor implantation. When TNP-470 was administered to tumor-bearing mice at a dose of 30 mg/kg, it affected body weight gain. Error bars, SD.

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