Enhanced Suppression of Tumor Growth by Combination of Angiogenesis Inhibitor
O-(Chloroacetyl-carbamoyl)fumagillol (TNP-470) and Cytotoxic Agents in Mice1

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ABSTRACT

The antitumor effect of the novel angiogenesis inhibitor O-(Chloroacetyl-carbamoyl)fumagillol, TNP-470 (TNP, s.c.), a synthetic analogue of fumagillin, was studied in combination with cytotoxic agents—mitomycin C (MMC, Lp.), Adriamycin (Lp.), cisplatin (Lp.), and 5-fluorouracil (Lp.), using B16BL6 melanoma (B16 m) and Lewis lung carcinoma in C57BL/6 mice. When the mice were treated on days 3 and 5, addition of MMC (total dose, 5 mg/kg) or 5-fluorouracil (140 mg/kg) to TNP (150 mg/kg) maximally reduced s.c. B16 m volume from 60 to 15% or from 68 to 40% of control, respectively, and addition of MMC (5 mg/kg) to TNP (150 mg/kg) reduced s.c. Lewis lung carcinoma volume from 75 to 62% of control (P < 0.02, compared to the corresponding single drug treatments). During treatment on days 3, 5, 7, 9, and 11, addition of MMC (5 mg/kg) to TNP (150 mg/kg) reduced s.c. B16 m volume from 60 to 15% or from 68 to 40% of control and reduced the number of pulmonary metastases of i.v. B16 m from 26 to 5% of control (P < 0.001). For established tumors (>5 mm in maximal diameter), addition of MMC (12-14 mg/kg), Adriamycin (15-17.5 mg/kg), or cisplatin (4 mg/kg, by one shot) to TNP (120-140 mg/kg) with a 6-7 fractionated dosing schedule reduced s.c. B16 m volume from 50 to 20, 24, or 31% of control and reduced s.c. Lewis lung carcinoma volume from 52 to 34, 27, or 34% of control, respectively (P < 0.02). The effect of combination therapy was additive and dose-dependent, and the earlier fractionated dosing schedule exerted more enhanced antitumor effects. TNP reduced the body weight by approximately 10% of control at maximum, but this toxicity was reversible and was not affected by addition of the cytotoxic agents. The results suggest that the combination of angiogenesis inhibitor TNP and standard cytotoxic agents can be a beneficial addition to the treatment of solid tumors.

INTRODUCTION

There is considerable experimental evidence that angiogenesis (induction of new capillary blood vessels) plays a crucial role in the growth of solid tumors over 1-2 mm in diameter (about 10⁶ cells) and in gaining access to the existing vasculature (1-5). Thus, for over 20 years investigations have been focused on angiogenesis inhibitors which could be used as a potent therapeutic measure to control the growth and metastasis of solid tumors. A naturally secreted antibiotic of Aspergillus fumigatus fumigatus (fumagillin) (6, 7), extracts from cartilage (8), vitreous (9) and bovine aorta (10), protamine sulfate (11), medroxyprogesterone (12), cortisone acetate plus heparin (13), a polysulfonated trypsin derived peptide (14), a sulfated polysaccharide-peptidoglycan complex extracted from an Arthrobacter species (15), and others (16-18) have been reported to inhibit angiogenesis and/or tumor growth.

The development of solid tumors, however, is demarcated to 2 stages: the (a) prevascular and (b) vascular phases (1-5, 19). The prevascular phase is usually associated with limited tumor growth and few or no metastases, and may persist for years before the switch to the vascular phase occurs (20-22). Additionally, recent studies have revealed that many angiogenic factors are produced or mediated by tumor cells themselves or tumor-associated cells such as macrophages. Included are basic fibroblast growth factor (23), tumor necrosis factor-α (24), vascular endothelial growth factor (25), a platelet-derived endothelial cell growth factor (26), a heparin binding growth factor (27), and transforming growth factor-α (28). These findings suggest that antiangiogenic modulation should be associated with cytotoxic chemotherapy to attain a sufficient antitumor effect.

Recently it was found that TNP, a synthetic analogue of fumagillin, shows a higher antiangiogenic activity and less toxicity than the parent antibiotic (29, 30). This novel angiogenesis inhibitor has proved to inhibit tumor growth in several in vivo assay systems (29, 31-34). In this study, we treated tumor-bearing mice with TNP in combination with standard cytotoxic agents.

MATERIALS AND METHODS

Animals. Specific pathogen-free female C57BL/6 mice at 6 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used at 8 or 9 weeks of age, weighing 22 to 24 g. The animals were fed with a commercial pellet diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water ad libitum.

Tumors. B16BL6 melanoma and Lewis lung carcinoma were kindly provided by Dr. H. Okada (Takeda Chemical Industries, Ltd., Osaka, Japan). B16BL6 melanoma was maintained by serial i.p. transplantation and Lewis lung carcinoma by serial s.c. transplantation in C57BL/6 mice, respectively.

Tumor nodules of B16BL6 melanoma were collected 10 days after maintenance transplantation to prepare a single cell suspension in 0.9% NaCl solution. A cell suspension of 5 × 10⁶ B16BL6 melanoma cells in 0.1 ml 0.9% NaCl solution was inoculated i.v. into the right flank of mice for the s.c. tumor assay, and a cell suspension of 2 × 10⁵ B16BL6 melanoma cells in 0.1 ml 0.9% NaCl solution was injected i.v. into the lateral tail vein of mice for the pulmonary metastasis assay, on day 0, respectively. Tumor nodules of s.c. Lewis lung carcinoma were resected 14 days after the maintenance transplantation, and a tumor specimen minced in 8 mm³ was transplanted s.c. into the right flank of mice on day 0.

Drugs. The clinical formulation of TNP was the kind gift of Takeda Chemical Industries, Ltd., Osaka, Japan. Its structure and biological characteristics have been reported previously (29, 30). MMC, ADM, and 5-FU were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) and DDP from Bristol-Myers Squibb Co., Ltd. (Tokyo, Japan). All drugs were dissolved in 0.9% NaCl solution just before use, and the concentration was adjusted to make the injection volume 0.1 ml. TNP was administered s.c. in the back of mice and the other drugs were administered i.p. Control animals were given a s.c. injection of 0.1 ml 0.9% NaCl solution. Dosing schedules in each experiment were described in the text, Tables, and/or Figure legends.

Estimation. Tumors and body weight of mice were measured by one person with no knowledge of the treatment. The s.c. tumors were measured 3 times/week using a sliding caliper from the treatment initiation to the time when gross ulceration of the tumors developed in control mice. The tumor size was calculated as:

\[
tumor\ vol.\ (mm^3) = 0.5 \times a \times b^2
\]

where a is the longest diameter and b is the shortest diameter (31). In the metastasis assay, the lungs were excised in all mice after lung tumors were confirmed in control mice. The visible metastatic foci on the bilateral lung

1 The abbreviations used are: TNP, O-(chloroacetyl-carbamoyl)fumagillol (TNP-470); MMC, mitomycin C; ADM, Adriamycin; DDP, cisplatin; 5FU, 5-fluorouracil; CTX, cyclophosphamide; T/C, treated versus control.

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TNP (150 mg/kg) and MMC (5 mg/kg), this fractionated dosing volume). The effects by treatments were represented by: tumor-bearing mice was adjusted by subtracting the tumor weight (tumor surface were counted under a dissecting microscope. The body weight of s.c. tumor-bearing mice was adjusted by subtracting the tumor weight (=tumor volume). The effects by treatments were represented by:

\[
\frac{\text{T/C} \text{ (%)}}{\text{Control}} = \left( \frac{\text{mean value of treated group}}{\text{mean value of control group}} \right) \times 100
\]

**Statistical Analysis.** The significance of differences between the experimental groups was calculated by Student’s t test. \( P < 0.05 \) was considered significant.

**RESULTS**

**Effect of Early Short-Term Treatment on Growth of s.c. Tumors.** TNP (75 mg/kg), MMC (2.5 mg/kg), and 5FU (70 mg/kg), either alone or in combination, were given to the mice on days 3 and 5 of s.c. tumor transplantation. The antitumor effect of the combination treatments was significantly higher than that of the corresponding single drug treatments in all of the experiments (Table 1). The maximal tumor reduction was obtained by the combination of TNP and MMC against B16BL6 melanoma (T/C = 15.4%). Thus, this combination was mainly investigated in the following experiments.

**Effect of Early Fractionated Treatments on Growth of s.c. Tumors.** TNP (30 mg/kg, s.c.) and MMC (1 mg/kg), either alone or in combination at the full or half dose of each drug, were given to i.v. inoculated B16BL6 melanoma-bearing mice on days 3, 5, 7, 9, and 11. An enhancement of antitumor effect by the combination treatments was evident in a dose-dependent manner (Fig. 1 and Table 2). The most prominent tumor reduction was obtained by the full dose combination treatment (T/C = 6.2%). With a combination of the same total dose of TNP (150 mg/kg) and MMC (5 mg/kg), this fractionated dosing schedule exerted a more enhanced antitumor effect as compared with the above-mentioned short-term dosing schedule (T/C = 6.2% versus 15.4%, \( P < 0.008 \); Tables 1 and 2).

**Effect of Delayed Fractionated Treatments on Growth of Established s.c. Tumors.** Treatments were initiated when the s.c. transplanted tumors reached at least 5 mm in the maximal diameter. TNP (20 mg/kg), MMC (2 mg/kg), and ADM (2.5 mg/kg) were given to B16BL6 melanoma-bearing mice on days 10, 12, 14, 16, 18, and 21, and to Lewis lung carcinoma-bearing mice on days 5, 7, 9, 11, 14, 16, and 18, but DDP (4 mg/kg) was given once on the day of treatment initiation. Both tumors showed a similar degree of sensitivity to TNP. The antitumor effect of TNP was significantly enhanced by combination with MMC, ADM, or DDP, irrespective of the difference in sensitivity of the tumors to these cytotoxic agents. But T/C by this delayed combination treatment protocol remained in a range of approximately 20 to 35% (Table 3; and Figs. 2 and 3).

**Effect on Pulmonary Metastasis.** TNP (30 mg/kg) and MMC (1 mg/kg), either alone or in combination at the full or half dose of each drug, were given to i.v. inoculated B16BL6 melanoma-bearing mice on days 3, 5, 7, 9, and 11. The full dose combination therapy provided a T/C of 5.2% on day 28, which could be significantly contrasted with the T/C of 26.1% by TNP alone, 33.9% by MMC alone, and 13.6% by the half dose combination therapy (Table 4). Decrease in the size of the metastatic foci was found in all of the treated mice, but it was most prominent with the full dose combination therapy (data not shown).

**Effect on Body Weight.** In all experiments, TNP with a total dose of 120–150 mg/kg maximally decreased the body weight by approx-
Table 2 Suppression of s.c. transplanted B16BL6 melanoma by early fractionated treatments

<table>
<thead>
<tr>
<th>Drug (dose, mg/kg)</th>
<th>No. of mice</th>
<th>Tumor size (T/C, %)</th>
<th>P value versus:</th>
<th>Body wt (T/C, %)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>TNP (30)</td>
<td>11</td>
<td>42.9</td>
<td>&lt;0.001</td>
<td>104.6</td>
</tr>
<tr>
<td>MMC (1)</td>
<td>13</td>
<td>17.9</td>
<td>&lt;0.001&lt;0.001</td>
<td>102.7</td>
</tr>
<tr>
<td>TNP (30) + MMC (1)</td>
<td>12</td>
<td>6.2</td>
<td>&lt;0.001&lt;0.001</td>
<td>103.7</td>
</tr>
<tr>
<td>TNP (15) + MMC (0.5)</td>
<td>13</td>
<td>18.1</td>
<td>&lt;0.001&lt;0.001&lt;0.001*</td>
<td>101.6</td>
</tr>
</tbody>
</table>

a Adjusted by tumor volume.
b P < 0.006, compared to TNP (15) + MMC (0.5).

discussed above, but this toxicity was not affected by adding the cytotoxic agents (representative data are shown in Table 3). The body weight loss by TNP was gradually improved to the range of control approximately 2 weeks after the termination of TNP administration (Tables 2 and 4).

DISCUSSION

TNP has been demonstrated to exert inhibitory effects on a considerably wide range of in vivo tumor systems (29, 31—34). In culture systems, the human umbilical vein endothelial cells have been extremely more sensitive to TNP than the nonendothelial cells and human tumor cells (29, 30), and the choriocarcinoma cells have been much more sensitive to TNP than the ovarian epithelial cells and uterine endometrial cancer cells (32). TNP has significantly decreased the number of blood vessels in tumor tissues in mice (32). While PC-3 prostate cancer cells and MDA-MB-231 breast cancer cells have been considerably insensitive to TNP in monolayer culture, the growth of these cells transplanted in mice has been remarkably suppressed by TNP (34). PC-3 cells have proved to produce large quantities of basic fibroblast growth factor (23) and MDA-MB-231 cells to secrete pleiotrophin, a possible angiogenic factor (27). Consequently, the antitumor activities of TNP are most likely to come from interfering with angiogenesis and/or other biological actions of angiogenic factors.

TNP has been reported remarkably less toxic than fumagillin, the parent antibiotic of TNP (29, 30), but a considerable body weight loss by TNP has been mentioned in a dose-dependent manner (30) (data not shown). Our study showed that a total s.c. TNP dose of 120—150 mg/kg led to a body weight loss by approximately 10% of control mice, even though this toxicity was reversible after the treatment was terminated. These findings indicate that the major dose limiting factor of TNP still is the considerable weight loss. In addition, the data from previous investigations (29, 31—34) and ours have shown that a complete regression of tumors could be rarely attained by a nontoxic dose of TNP alone, as is the case with other antiangiogenic modulators (8—18) and conventional cytotoxic agents.

The antiangiogenic activity of cortisone acetate has enhanced the antitumor effect of ADM, DDP, MMC, and CTX on MBT-2 transitional cell carcinoma in mice (36), and the antiangiogenic modulation with tetrahydrocortisol, @3-cyclodextrin tetradecasulfate, and tetracycline derivative minocycline has potentiated the antitumor effects of DDP, ADM, CTX, and bleomycin on Lewis lung carcinoma in mice (37). As for TNP, Yamaoka et al. (34) have briefly described how the inhibitory effect of TNP on PC-3 prostate carcinoma has been increased by combination with DDP. More recently, Teicher et al. (38, 39) have reported that TNP has exerted a dose-modifying effect on the toxicity of CTX against FSA11C fibrosarcoma, resulting in an 8-fold increase in tumor cell killing at a CTX dose of 500 mg/kg, and that the
Table 4 Suppression of pulmonary metastasis of i.v. inoculated B16BL6 melanoma

TNP (30 mg/kg, s.c.) and MMC (1 mg/kg, i.p.) were given on days 3, 5, 7, 9, and 11 after tumor transplantation, either alone or in combination at the full or half dose of each drug. The data on day 28 are listed in Table 2.

<table>
<thead>
<tr>
<th>Drug (dose, mg/kg)</th>
<th>No. of mice</th>
<th>Tumor Volume (mm³)</th>
<th>P value versus:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>28.7 ± 3.93</td>
<td>Control</td>
</tr>
<tr>
<td>TNP (30)</td>
<td>12</td>
<td>7.5 ± 1.35</td>
<td>MMC</td>
</tr>
<tr>
<td>MMC (1)</td>
<td>12</td>
<td>9.7 ± 1.92</td>
<td>Control</td>
</tr>
<tr>
<td>TNP (30) + MMC (1)</td>
<td>12</td>
<td>1.5 ± 0.57</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNP (15) + MMC (0.5)</td>
<td>10</td>
<td>3.9 ± 1.53</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Visible tumors on surface of bilateral lungs, mean ± SE.

combination of TNP, minocycline, and CTX has led to 40 to 50% long-term survivors in Lewis lung carcinoma-bearing mice (38, 39). In those situations, inhibition of angiogenesis may have led to death of tumor cells most distal from the established vasculature, thereby decreasing the tumor volume and facilitating the permeation of given chemotherapeutic agents throughout the tumor tissues. The cytotoxic action of chemotherapeutic agents, at the same time, could directly suppress the viability of tumor cells and thus reduce the possible production of angiogenic factors by tumor cells themselves and/or tumor-associated cells (23–28).

Our study showed that the combination of TNP and standard cytotoxic agents including MMC, ADM, DDP, and 5FU exerted a substantially enhanced antitumor effect as compared with the corresponding single drug treatments, that the combination therapy was
effective on both s.c. transplanted tumors and pulmonary metastasis by i.v. inoculation, and that the body weight loss by TNP was hardly affected by adding these cytotoxic agents. The combination effect was additive and dose-dependent. The early initiation of a prolonged-fractionated dosing schedule was more effective than a short-term high dose treatment or a delayed treatment. In conclusion, combination therapy with the novel angiogenesis inhibitor TNP and standard cytotoxic agents can be therapeutic additions to the treatment of solid tumors. Further investigations on optimal dosing schedules and optimal drug combinations will elucidate the clinical availability of such an angiotoxic chemotherapy.

REFERENCES


Enhanced Suppression of Tumor Growth by Combination of Angiogenesis Inhibitor O-(Chloroacetyl-carbamoyl)fumagillol (TNP-470) and Cytotoxic Agents in Mice

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