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Antitumour effect of a synthetic analogue of fumagillin on murine renal carcinoma

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Objective To evaluate the antitumour effect of an angio-inhibitory drug, a synthetic analogue of fumagillin (TNP-470), on murine renal carcinoma (Renca) *in vivo* and *in vitro*.

Materials and methods The effect of TNP-470 on the growth of Renca cells *in vitro* was measured by angiogenesis assay and cell counting with dye exclusion. In the angiogenesis assay, Renca cells were injected intradermally and the number of blood vessels orientated towards the tumours was counted 3 days after tumour inoculation. To examine the effect of TNP-470 on the subcutaneous tumour growth and lung metastasis of Renca, Renca cells were injected subcutaneously or intravenously in BALB/c mice and they were treated with a subcutaneous injection every 3 days.

Results Dose-dependent growth inhibition *in vitro* was observed with 50% inhibition occurring at 600 ng/ml. Angiogenesis assay revealed that administration of TNP-470 inhibited the angiogenesis induced by Renca in a dose-dependent manner. In the subcutaneous experiment, TNP-470 decreased the growth rate of established subcutaneous tumours rather than reduced the size of the tumour. The administration of TNP-470 in mice with lung metastasis inhibited the development of metastasis of Renca without weight loss or diarrhoea.

Conclusion The present study demonstrated that TNP-470 had an inhibitory effect on tumour-induced angiogenesis and a significant anti-tumour effect on Renca. This suggests that TNP-470 could be useful in the treatment of renal cell carcinoma. Further studies are needed to clarify whether TNP-470 is more effective when combined with other drugs such as interferons.

Keywords Renal cell carcinoma, metastasis, angiogenesis, synthetic analogue of fumagillin

Introduction

Approximately 30% of patients with renal cell carcinoma (RCC) have metastatic disease at the time of initial diagnosis [1]. Most metastatic RCC remains refractory to conventional hormonal treatment, chemotherapy and radiotherapy [2,3]. Recent clinical trials have shown that immunotherapy with α-interferon (α-IFN), interleukin 2 (IL-2), or adoptive immunotherapy with IL-2 and lymphokine-activated killer cells (LAK) or tumour-infiltrating lymphocytes (TIL) produces occasional responses [4–7]. At present, however, there is no effective therapy for metastatic RCC, and virtually all patients not cured by surgical excision of their localized tumour will succumb to their disease. Therefore, new chemotherapeutic agents whose antitumour mechanisms differ from those of currently available drugs are needed to improve the treatment of patients with RCC.

The formation of new blood vessels *in vivo*, termed 'angiogenesis', occurs during a variety of pathological situations, especially in the development of tumours [8,9]. Good correlation between the extent of angiogenesis and the occurrence of metastasis is reported in breast cancer and cutaneous melanoma [10,11]. Targeting the tumour vasculature in the treatment of cancer therefore seems to be an attractive strategy. Ingber *et al.* demonstrated that AGM-1470 (designated TNP-470 in the present study), one of the synthetic analogues of fumagillin, which is a natural product of *Aspergillus fumigatus fresenius*, exhibited potent inhibitory effects on the growth of endothelial cells and solid tumours with relatively few side-effects [12]. However, there is no report that evaluates the effects of fumagillin analogues on RCC. In the present study, we have examined the effects of the angio-inhibitory drug, TNP-470, on murine renal carcinoma (Renca) *in vitro* and *in vivo*.

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Materials and methods

Animals and cells

Male BALB/c mice (aged 5–10 weeks) (Doken Inc., Ibaragi, Japan) were used for *in vivo* experiments. The murine renal cell carcinoma line, designated Renca [13],

was originally established and characterized by Dr G.P. Murphy (Roswell Park Memorial Institute, Buffalo, NY, USA) and supplied by Dr Fujioka (Iwate Medical School, Japan). Renca was maintained by serial transplantation in BALB/c mice. Renca cells were used which were maintained *in vitro* in DMEM (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS) and 2 mM L-glutamine in a 5%-CO_2 incubator.

Reagents

TNP-470 (Takeda Pharmaceutical Co., Osaka, Japan) was stored at −80°C. For *in vitro* experiments, TNP-470 was dissolved in dimethylsulphoxide (DMSO) (Seikagaku Co., Tokyo, Japan) diluted with culture medium to the desired concentrations. For *in vivo* experiments, TNP-470 was dissolved in ethanol and diluted to the desired concentrations with 1% gum arabic (Sigma Chemical Co., St Louis, USA) and normal saline.

Cell proliferation studies

Proliferation of cells was characterized by both cell counting with dye exclusion and the colourimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St Louis, USA) assay.

Renca cells were seeded into 6-well tissue culture plates at 1×10^5 cells/well, in triplicate. After culture with TNP-470 or vehicle for 3 days, cells were detached with trypsin, stained with erythrosin and counted with a haemacytometer. A modification of the MTT assay originally described by Mossman [14] was used. Rapidly growing cells were plated at a concentration of 1000 cells in 100 µl medium into 96-well microtitre plates (Costar, Cambridge, MA, USA) using a multichannel pipette. In preliminary experiments, seeding densities were determined, ensuring that cultures did not become confluent before conducting the assay. Cell numbers per microtitre well were proportional to the absorbance of the solubilized formazan (data not shown). After incubation for 24 h, 100 µl of culture medium and culture medium containing various concentrations of TNP-470 or vehicle (DMSO) were dispensed within appropriate wells and the plates were then incubated for 1 or 3 days at 37°C in a 5%-CO_2 incubator prior to the addition of MTT solution. The MTT working solution was prepared as follows: 1 mg MTT/ml phosphate buffered saline (PBS) was filtered with 0.45 µm filter units and stored at 4°C; 50 µg of MTT were then added to each well. After incubation for 4 h at 37°C, 200 µl supernatant were removed using a Transtar-96™ system (Costar, Cambridge, MA, USA), leaving < 30 µl residual medium in each well. MTT formazan crystals were solubilized by adding 150 µl of 100% DMSO to each well. Plates were then agitated on a plate shaker (TAITEC, Saitama, Japan) for 5 min, following which absorbance at 540 nm was immediately measured with a microplate reader Model 450 (Bio-Rad Lab., Richmond, CA, USA). Dose-response curves were plotted, and the 50% inhibitory concentration (IC_{50}) was determined graphically as the dose of drug causing a 50% reduction in absorbance compared with control values. The MTT assay was performed in eight replicates and repeated at least three times.

Angiogenesis assay

The assay for tumour-induced angiogenesis was performed as described by Kreisle and Ershler [15]. BALB/c mice were injected intradermally (i.d.) with 1×10^6 Renca cells at two sites on the abdomen on day 0. Mice were treated with the administration of TNP-470 or vehicle consisting of ethanol and gum arabic in normal saline on days 1 and 2. The animals were killed on day 3 and the skin with tumours was separated from the underlying tissues. Tumour-induced angiogenesis was quantified by counting the number of blood vessels orientated towards the tumour, using a dissecting microscope. The size of the tumour was assessed simultaneously by measuring the diameters of its short and long axes. All counts were made by the same observer.

Experimental tumour models

In the subcutaneous experiment, single cell suspensions of 1×10^6 Renca cells were injected into the backs of 5–7-week-old BALB/c mice. After inoculation of Renca cells, tumour size and mouse weight were measured by the same observer, twice a week, and the estimated tumour volume (V) was calculated by the following formula: $V = \text{Width}^2 \times \text{Length} \times 0.5$. Relative tumour volumes were shown as the relative mean value (V_n/V_0), V_0 being the mean volume at the initiation of treatment and V_n being at any given day. In the metastasis experiment, single cell suspensions of 5×10^4 viable cells were injected in 0.2 ml aliquots into the lateral tail vein of 5–7-week-old BALB/c mice. After treatment with TNP-470, the mice were killed for enumeration of metastatic lung nodules and for weighing the lungs with metastases. The metastases formed discrete white nodules on the blackened surface of the lungs insufflated with a 15% solution of Indian ink when fixed by Fekete's solution [16]. Following fixation for 24 h, the lungs were weighed and the number of lung nodules counted with the aid of a dissecting microscope.

Statistics

Student's 2-tailed *t*-test was used to assess the statistical significance of differences between groups. The differences were considered to be statistically significant when a 2-tailed value of P was <0.05.

Results

Effect of TNP-470 on the growth of Renca cells in vitro

Renca cells were cultured with various concentrations of TNP-470 for 1 or 3 days, and their growth was evaluated by both the colourimetric MTT assay and cell counting with dye exclusion. In the colourimetric MTT assay, significant growth inhibition was observed in a dose-dependent manner, with 50% inhibition occurring at 600 ng/ml after 3 days' culture with TNP-470 (Fig. 1). The inhibitory effect of TNP-470 was also confirmed by cell counting with dye exclusion. Renca cells, 1×10^5, were seeded and cultured with vehicle or various concentrations of TNP-470. The mean numbers of cells after 3 days' culture were 18×10^5 cells for vehicle, 16×10^5 cells for 1 ng/ml, 16×10^5 cells for 10 ng/ml, 15×10^5 cells for 100 ng/ml ($P<0.05$ compared with controls treated with vehicle alone), 8×10^5 cells for 1 μg/ml ($P<0.01$), 5×10^5 cells for 10 μg/ml ($P<0.01$), and 0.1×10^4 cells for 100 μg/ml ($P<0.01$). Cytostatic inhibition of Renca growth was observed at concentrations <10 μg/ml, while cytotoxicity (reduction of cell number below the initial plating density) was observed at much higher concentrations (100 μg/ml).

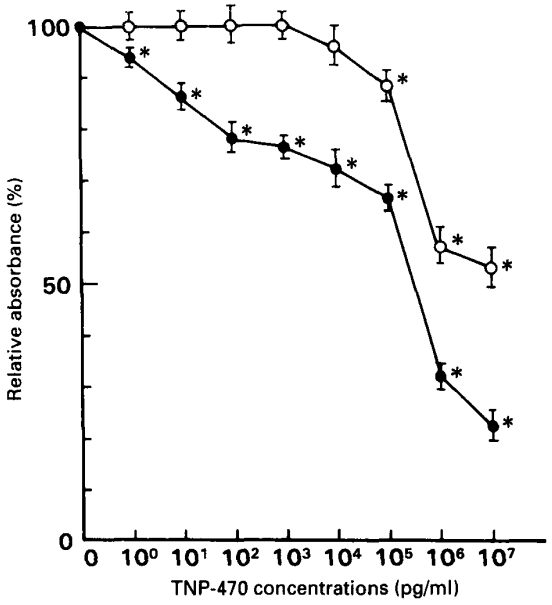

Fig. 1. Effect of TNP-470 on the growth of Renca cells *in vitro* assessed by MTT assay. Renca cells were cultured with indicated concentrations of TNP-470 for 1 day (○) and 3 days (●). The growth of Renca cells was assessed by MTT assay. The results are expressed as a percentage of absorbance of MTT-derived formazan developed by the cells treated with vehicle alone (control). Each point, mean of three experiments performed in eight replicates. Bar, SD. *$P<0.01$ as compared with controls.

Effect of TNP-470 on tumour-induced angiogenesis in vivo

Mice with intradermal tumours were treated with subcutaneous injection of TNP-470 or vehicle ($n=10$ for each group) for 2 days, and angiogenesis was assessed 3 days after tumour inoculation. As shown in Table 1, the number of blood vessels orientated towards the tumour was significantly decreased by treatment with TNP-470 in a dose-dependent manner. Tumour volume in mice treated with TNP-470 was significantly smaller than in vehicle-treated mice. These findings indicate that TNP-470 had an inhibitory effect not only on the angiogenesis induced by Renca but also on intradermal tumour growth. Based upon the results of the angiogenesis assay, doses of 10 mg/kg and 20 mg/kg TNP-470 were used in further studies *in vivo*.

Effect of TNP-470 on the growth of subcutaneous tumours

The antitumour effect of TNP-470 on established subcutaneous tumours was examined. Treatment was initiated when the tumours were at least 250 mm^3 in volume. Tumours of similar size were matched for use in control and experimental groups. Mice were treated with subcutaneous injection of vehicle, 10, or 20 mg/kg TNP-470 ($n=10$ for each group) once every 3 days. TNP-470-treated mice had significantly smaller tumours than the vehicle-treated mice, starting with measurements made on day 15 (Fig. 2). TNP-470 inhibited the growth rate of established subcutaneous tumours in a dose-dependent manner rather than reducing the size of the tumours. Neither weight loss nor diarrhoea was observed.

Effect of TNP-470 on metastasis of Renca cells

The effect of TNP-470 on the development of metastasis was then examined. Renca cells were injected intravenously in BALB/c mice on day 0, and the animals were treated with subcutaneous injection of vehicle, 10, or 20 mg/kg TNP-470 ($n=8$ for each group) once every 3 days from day 1 to day 28. The results shown in Table 2 demonstrate that TNP-470 inhibited lung metastasis of Renca cells significantly in a dose-dependent manner. There was no significant difference in lung

Table 1. Effect of TNP-470 on angiogenesis induced by Renca

Treatment		*Tumour volume (mm³)*	*No. of blood vessels*
Vehicle	($n=10$)	17.2±4.3*	21.3±2.3
5 mg/kg TNP-470	($n=10$)	14.5±6.1	16.9±1.8†
10 mg/kg TNP-470	($n=10$)	10.7±5.3†	15.9±2.0†
20 mg/kg TNP-470	($n=10$)	10.6±6.1‡	14.8±3.5†

*Mean±SD. †$P<0.01$ as compared with controls treated with vehicle alone. ‡$P<0.05$ as compared with controls treated with vehicle alone.

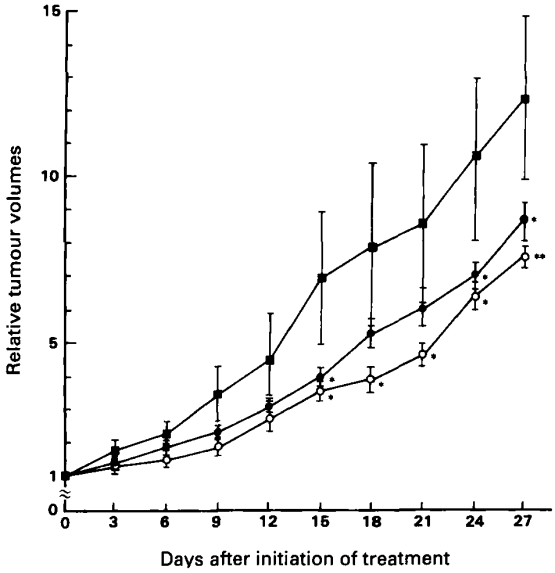

Fig. 2. Effect of TNP-470 on the established subcutaneous tumours of Renca. Renca cells (1×10^6) were injected subcutaneously into the backs of BALB/c mice and tumour size was measured. Tumours of similar size were matched for use in control and experimental groups ($n=10$ for each group). Treatment was initiated on day 0 when tumours were at least 250 mm³ in volume. Mice were treated with subcutaneous injection of vehicle (■) or TNP-470 (●, 10 mg/kg; ○, 20 mg/kg) once every 3 days. Each point, relative tumour volume (see Materials and Methods). Bar, SD. *$P<0.05$ as compared with controls. **$P<0.01$ as compared with controls.

weights between vehicle-treated mice and TNP-470-treated mice. At no time was weight loss or diarrhoea observed in the TNP-470-treated mice.

Discussion

Most RCCs have typical features of malignant hypervascularity. Several reports have demonstrated that the more hypovascular papillary RCC is less aggressive than the more vascular RCC [17]. In addition, RCCs metastasize frequently to the lung, bone and liver, probably via haematogenous spread, and these metastases remain refractory to conventional therapy [2,3]. In view of these biological and clinical characteristics, angio-inhibition seems to be a reasonable approach in the treatment of metastatic RCC. In the present study, the antitumour effect of the angio-inhibitory drug, TNP-470, on Renca cells was evaluated *in vitro* and *in vivo*, and the IC_{50} of TNP-470 after 3 days' culture was demonstrated to be 600 ng/ml. Furthermore, *in vivo* studies revealed that administration of TNP-470 inhibited angiogenesis induced by Renca, inhibited the growth of established subcutaneous tumours, and the development of lung metastases in a dose-dependent manner without weight loss or diarrhoea.

Many experimental studies have shown that angiogenesis is essential for the growth and metastasis of solid tumours [8,9]. If a tumour grows beyond the point where simple diffusion cannot nourish it (a few millimetres in diameter, about 10^6 cells), further expansion of the tumour cell population requires the induction of new capillary blood vessels. The induction of angiogenesis is mediated by specific angiogenic factors released by the tumours [9] and often by macrophages attracted to it [18]. One of the factors that stimulates angiogenesis is basic fibroblast growth factor (bFGF) [19]. Mydlo *et al.* isolated an angiogenic heparin-binding growth factor homologous to bFGF from RCC [20]. Chodak *et al.*

Table 2. Inhibition of development of lung metastasis of Renca by TNP-470

Treatment		*No. of lung nodules*	*Lung weight (mg)*
Vehicle	($n=8$)	74.2±31.8*	619.2±61.8
10 mg/kg TNP-470	($n=8$)	13.5±13.0†	618.8±90.7
20 mg/kg TNP-470	($n=8$)	12.8±16.5†	548.2±28.5‡

*Mean±SD. †$P<0.01$ as compared with controls treated with vehicle alone. ‡$P<0.05$ as compared with controls treated with vehicle alone.

showed the presence of FGF-related protein in RCC tissues, and demonstrated that patients with RCC had increased levels of bFGF-like activity in their urine [21]. Furthermore, Fujimoto *et al.* detected bFGF mRNA in RCC tissues by Northern blot analysis, and showed that serum bFGF levels were elevated in patients with RCC [22]. The present authors have already confirmed that bFGF was detected immunohistochemically in Renca, which is a hypervascular tumour (data not shown). These findings suggest that bFGF as an angiogenic mediator plays an important role in determining the vascularity seen in RCC.

Although the mechanisms underlying the antitumour effects of TNP-470 are not understood completely, reports suggest that TNP-470 exerts its antitumour effects primarily by acting on the tumour vasculature rather than the tumour cells [12]: (i) doses of TNP-470 had to be at least 10 times higher for comparable inhibition of the human tumour cell lines *in vitro*; (ii) M5076 tumour cell lines, which were very sensitive *in vivo*, were found to be refractory to TNP-470 *in vitro*; (iii) TNP-470 had no significant effect on ascitic (less dependent on neovascularization) leukaemic cell growth; (iv) TNP-470 inhibited angiogenesis induced by bFGF in the rat sponge implantation assay [23], or by human choriocarcinoma cell lines in a dose-dependent manner [24]. In the present study, TNP-470 inhibited not only the growth and metastasis of Renca but also angiogenesis induced by Renca where bFGF was detected immunohistochemically. However, the IC_{50} of Renca cells was 600 ng/ml, which is extremely high when compared with that of endothelial cells (IC_{50}: 10–20 pg/ml) [12]. These findings also suggest that TNP-470 acts primarily on the tumour vasculature probably induced by angiogenic mediators, including bFGF released from Renca, resulting in growth inhibition of Renca.

The *in vivo* antitumour effects of TNP-470 varied according to the different assays: the inhibitory effects seen in the angiogenesis assay were significant following treatment with low-dose TNP-470 (5 mg/kg) within a short period (3 days), while the effects on the established subcutaneous tumours were seen 15 days after initiating treatment with 20 mg/kg TNP-470. The difference in these inhibitory effects seems to be related to the difference in the phase of tumour formation when the treatment was initiated: in the angiogenesis assay, treatment was initiated 1 day after tumour cell inoculation, while treatment began when the tumours were over 250 mm^3 in the assay with established subcutaneous tumours. Saiki *et al.* [25] examined the effects of an angioinhibitory drug, synthetic polymeric peptide (poly (RGD)), on the angiogenesis and lung metastasis of metastatic B16-BL6 melanoma, and showed that its antitumour effect was more significant when treatment was initiated earlier after tumour inoculation (when the tumour formation was in the early phase). In their report, treatment was initiated at various times following B16-BL6 cell inoculation. Single administration on day 1 or day 7 after tumour inoculation caused significant inhibition of lung metastasis, while that on day 14 failed to inhibit lung metastasis. These findings suggest that the antitumour effect of TNP-470 is more significant when treatment begins soon after tumour inoculation, and may explain the differences in the *in vivo* inhibitory effects of TNP-470 seen in the various assays.

In conclusion, the present study showed that TNP-470 had a significant antitumour effect on Renca without weight loss or diarrhoea, suggesting that TNP-470 could be useful in the treatment of RCC. As the administration of TNP-470 alone did not result in a complete response under the conditions employed in this study, further research is required to establish whether TNP-470 is more effective when used in combination with other drugs such as IFNs.

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