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Preclinical report

Cinnamamide, an antitumor agent with low cytotoxicity acting on matrix metalloproteinase

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The antitumor activity of cinnamamide (CNM), an agent acting on matrix metalloproteinase (MMP), was investigated in the present study. CNM displayed low cytotoxicity. By the MTT assay the IC_{50} (50% inhibitory concentration) values of CNM on cell proliferation ranged from 1.29 to 1.94 mM in human oral epidermoid carcinoma KB cells, human hepatoma BEL-7402 cells and human fibrosarcoma HT-1080 cells. Moreover, the IC_{50} for human fetal lung 2BS cells reached 4.33 mM. The administration of CNM in the range of 50–150 mg/kg (i.p. or p.o.) showed moderate antitumor effects in mice. When administered i.p. or p.o., CNM (150 mg/kg) inhibited the growth of transplanted hepatoma 22 by 48.8 or 40.5%, respectively. At the dose of 100 mg/kg, CNM inhibited the growth of colon 26 carcinoma by 39.0% and that of Lewis lung carcinoma by 53.9%. In the Lewis lung carcinoma model, CNM at the dose of 100 mg/kg (i.p.) also reduced the lung metastasis by 59.1%. Gelatine zymography revealed that CNM was able to decrease the level of MMP-2 in conditioned medium of HT-1080 tumor cells in a concentration-dependent manner. These results indicate that CNM is an antitumor agent with low cytotoxicity acting on MMP and may serve as a lead compound in the development of antitumor drugs. [© 2000 Lippincott Williams & Wilkins.]

Key words: Antitumor agent, cinnamamide, matrix metalloproteinase inhibitor.

Introduction

Matrix metalloproteinases (MMP) represent a family of at least 15 secreted and membrane-bound zinc endopeptidases.[1–3] The enzymes can degrade all of the components of the extracellular matrix and the basement membrane. This activity is necessary for local tissue remodeling during tumor growth, tumor-induced angiogenesis, tumor cell intravasation and tissue invasion during metastatic spread. Numerous studies have shown that there is a close association between expression of those endopeptidases such as the 72 kDa type collagenase (MMP-2), and the proliferative, invasive behavior and metastatic potential.[4–7] Animal experiments with MMP inhibitors, such as Batimasta (BB-94) and Marimastat (BB-2516), have demonstrated their antitumor efficacy.[8–11] Due to the unique mode of action, MMP inhibitors may provide a new modality in cancer therapy, particularly useful in preventing post-operative metastatic recurrence.

Natural products have been a good source of antitumor agents. In our laboratory, the nucleoside transport assay was established to direct the discovery of novel antitumor agents of natural origin.[12] Under the direction of the nucleoside transport assay, 6011W-A was isolated from the broth of an Actinomycete strain 6011W collected in Yunnan Province, China. Based on its physicochemical properties and spectral data, 6011W-A was identified to be cinnamamide (CNM; Figure 1). In this paper we report the antitumor activity of CNM and the inhibition of MMP.

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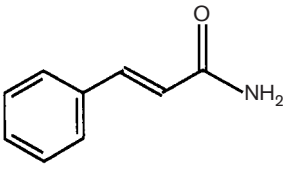

Figure 1. Chemical structure of cinnamamide (CNM).

Materials and methods

Chemicals

CNM was purchased from Beijing Chemical Factory (Beijing, China) and its purity was over 98% as examined by high-performance liquid chromatography.

Cells and cell culture

Human oral epidermoid carcinoma KB cells, human hepatoma BEL-7402 cells, human fibrosarcoma HT-1080 cells and human fetal lung 2BS cells were cultured separately in RPMI 1640 medium supplemented with 10% newborn calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in an incubator containing 5% CO_2.

MTT assay

The growth rate of cultured cells was determined by the MTT assay. Briefly, cells in the mid-log phase were harvested and seeded in 96-well plates (Costar, Cambridge, MA). After 24 h the drug was added and cells were incubated again for 72 h in the presence or absence of the drug. Then the cell population was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), according to the method described by Carmichael *et al.*[13]

Animals

For *in vivo* experiments, BALB/c mice, Kunming (KM) mice and C57BL/6 mice, weighing 18–22 g, were supplied by the Experimental Animal Institute of the Chinese Academy of Medical Sciences (Beijing, China), and kept in a room with controlled temperature and humidity.

In vivo antitumor effects

Antitumor effects *in vivo* (inhibition of primary tumor growth and metastasis) were assessed using colon carcinoma 26 (C26), hepatoma 22 (H22) and Lewis lung carcinoma (LLC) models, which were described previously.[14,15]

In the C26 model, BALB/c mice, eight per group, were s.c. implanted with a tumor fragment approximately 2 mm in diameter. CNM was administrated daily, i.p., beginning 24 h after tumor implantation. Mice were killed 1 day after the last injection, and the tumors were dissected and weighed. In the H22 model, groups of 10 KM mice were s.c. implanted with 1×10^6 cells suspended in saline solution. Drug was administrated daily, i.p. or p.o., beginning 24 or 96 h after tumor implantation. The latter treatment schedule was applied to assess if the inhibition of primary tumor is depended on angiogenesis. Mice were killed 1 day after the last injection, and the tumors were dissected and weighed.

In the LLC model, groups of eight C57BL/6 male mice were s.c. implanted with 0.2 ml suspension of viable LLC tumor tissue and serum-free RPMI 1640 medium in a ratio of 1:4 (w/v). Drug was given i.p., daily or on alternate days, beginning 24 h after tumor implantation and continuing throughout the course of the experiment. Tumor sizes were measured with calipers and tumor volumes were calculated by the formula $a \times b^2/2$, where a is the long axis and b is the short axis perpendicular to a. Mice were killed on day 21. The metastasized nodules on the lung surface were scored using a dissecting microscope after fixation in Bouin's solution.

Zymograph

Identification of MMP secreted by human fibrosarcoma HT-1080 cells was performed by electrophoresis based on the method described by Ata *et al.*[16] HT-1080 cells were seeded in 96-well plates and cultured for 24 h in RPMI 1640 medium containing CNM. The cells were extensively washed with 0.2 M PBS and then further cultured in serum-free RPMI 1640 medium for 24 h. The serum-free culture supernatants were withdrawn and centrifuged at 10 000 r.p.m. and the aliquots of the supernatant were mixed with SDS sample buffer without β-mercaptoethanols. Electrophoresis was carried out at 4°C in 7.5% polyacrylamide gels containing 0.1% gelatine. After being rinsed twice with 2.5% Triton X-100, the gels were incubated for 48 h in 50 mM Tris–HCl (pH 7.4), containing 0.2 M NaCl, 10 mM $CaCl_2$ and 1 μM $ZnCl_2$. Gels were stained overnight in 0.2% (v/v) Coomassie brilliant blue R250 and then destained with 30% methanol/10% acetic acid. The active enzyme bands that appeared as colorless bands on a blue background were quantitated by scanning of absorbance at 560 nm.

Statistical analysis

Significant difference between two values was determined with Student's *t*-test.

Results

Effect of CNM on the growth of cultured tumor cells

In the MTT assay, the IC_{50} values of CNM for cultured human tumor cell lines including epidermoid carcino-

ma KB cells, hepatoma BEL-7402 cells and fibrosarcoma HT-1080 cells were between 1.29 and 1.94 mM, and that for human fetal lung 2BS cells was 4.33 mM (Table 1). These results indicate that CNM shows very low cytotoxicity to cultured cancer cells and even much lower cytotoxicity to fetal lung 2BS cells.

In vivo antitumor effects of CNM

In mice experiments, CNM inhibited the growth of colon carcinoma 26 (Table 2) and hepatoma 22 (Table 3). While the treatment started 96 h after tumor transplantation, CNM still displayed inhibition of tumor growth in the hepatoma 22 model (Table 4). It is suggested that CNM might exert its inhibitory effect at the step of tumor angiogenesis. In mice bearing Lewis lung carcinoma, CNM suppressed the growth of primary tumors as compared with the control (Figure 2). Furthermore, a decrease in the incidence of metastasis and in the number of lung metastases was also observed (Table 5). These results indicate that CNM could inhibit not only the growth of primary tumors but also metastasis.

Table 1. Inhibitory effect of CNM on the growth of cultured human cell lines[a]

Cell line		IC_{50} (mM, mean ± SD)
Human oral epidermoid carcinoma cell	KB	1.62 ± 0.21
Human hepatoma cell	BEL-7402	1.94 ± 0.33
Human fibrosarcoma cell	HT-1080	1.29 ± 0.17
Human normal fetal lung cell	2BS	4.33 ± 1.34

[a]Cells were cultured with CNM for 3 days.

Effect of CNM on the level of MMP-2

The above results demonstrate that CNM shows activity to inhibit tumor growth as well as tumor metastasis. The effect of CNM on the level of MMP was examined in zymography to study the mechanism of the antitumor actions of CNM. When the conditioned medium of HT-1080 cells was analyzed by gelatine zymography, a proteolytic enzyme of apparent weight

Table 2. Inhibitory effect of CNM on the growth of colon carcinoma 26 in mice[a]

	No. of mice (initial/end)	Body weight change (g)	Tumor weight) (g, mean ± SD)	Inhibition (%)
Control	8/8	+2.64	2.41 ± 0.40	
CNM, 40 mg/kg	8/8	+0.94	1.67 ± 0.53	30.7[b]
CNM, 100 mg/kg	8/8	+0.46	1.47 ± 0.26	39.0[b]

[a]Colon carcinoma 26 was implanted s.c. on day 0. CNM was given 24 h after transplantation, i.p., daily for 10 days.
[b]$p < 0.01$ versus control.

Table 3. Inhibitory effect of CNM on hepatoma 22 in mice[a]

	No. of mice (initial/end)	Body weight change (g)	Tumor weight (g, mean ± SD)	Inhibition (%)
Control	10/10	+10.85	3.01 ± 1.01	
CNM, 75 mg/kg, i.p.	10/10	+8.08	1.76 ± 0.67	41.5[b]
CNM, 75 mg/kg, p.o.	10/10	+8.19	2.29 ± 0.97	23.9[b]
CNM, 150 mg/kg, i.p.	10/10	+3.38	1.54 ± 0.38	48.8[b]
CNM, 150 mg/kg, p.o.	10/10	+6.69	1.79 ± 0.58	40.5[b]

[a]Hepatoma 22 was implanted s.c. on day 0. CNM was given 24 h after transplantation, i.p. or p.o., daily for 10 days.
[b]$p < 0.01$ versus control.

Table 4. Inhibitory effect of CNM on established hepatoma 22 in mice[a]

	No. of mice (initial/end)	Body weight change (g)	Tumor weight (g, mean ± SD)	Inhibition (%)
Control	10/10	+14.31	2.92 ± 1.21	
CNM, 75 mg/kg	10/10	+8.66	1.63 ± 0.56	42.2[b]
CNM, 150 mg/kg	10/10	+7.11	1.47 ± 1.26	50.3[b]

[a]Hepatoma 22 was implanted s.c. on day 0. CNM was given 96 h after transplantation, i.p., daily for 7 days.
[b]$p < 0.01$ versus control.

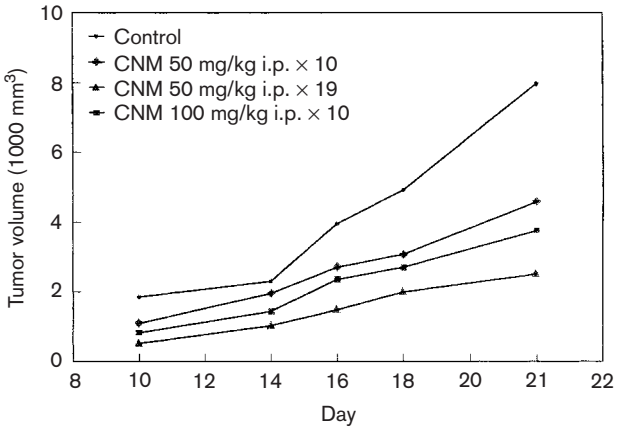

Figure 2. Effect of CNM on the growth of Lewis lung carcinoma in mice. Lewis lung carcinoma was implanted s.c. on day 0. CNM was given 24 h after transplantation, i.p., daily or on alternate days. There were eight mice in each group and no animal died during the experiment.

Table 5. Effect of CNM on the pulmonary metastases of Lewis lung carcinoma in mice[a]

	No. of mice (initial/end)	Incidence of metastases	No. of metastases (mean ± SD)	Inhibition (%)
Control	8/8	8/8	11.0 ± 5.7	
CNM, 50 mg/kg, i.p. × 10	8/8	7/8	10.3 ± 5.7	
CNM, 50 mg/kg, i.p. × 19	8/8	5/8	4.8 ± 1.9	56.8[b]
CNM, 100 mg/kg, i.p. × 10	8/8	5/8	4.5 ± 3.6	59.1[b]

[a]Lews lung carcinoma was implanted s.c. on day 0. CNM was given 24 h after transplantation, i.p., daily or on alternate days.
[b]$p < 0.01$ versus control.

72 kDa (MMP-2) was detected as a clear band (Figure 3). Densitometric analysis revealed that degradation of gelatine substance by MMP-2 was inhibited by CNM in a concentration-dependent manner. It demonstrates that CNM reduced the level of MMP-2 in tumor cells.

Discussion

It has been reported that some cinnamoyl analogs may show antitumor activity *in vitro*.[17] Pretreatment of tumor cells *in vitro* with U-77,863 (*O*-methyl cinnamamide) could inhibit their ability of invasion.[18] To our knowledge, no report on the *in vivo* antitumor effect of CNM has yet been published. As shown in this report, CNM exhibited an inhibitory effect not only on the growth of solid primary tumor but also tumor metastasis in mice. Cell culture experiment results show that IC_{50} values of CNM for cultured cancer cells ranged between 1.29 and 1.94 mM, a level approximately several thousand times higher that of widely used anticancer drugs such as methotrexate, doxorubicin and vincristine. Apparently, CNM is an active compound with low cytotoxicity. The antitumor effect of CNM *in vivo* does not seem to be related to a direct cytotoxic effect on tumor cells. Treatment of established tumor by CNM at a late dosing schedule still exerted inhibition on the growth of primary tumor. This suggests that the effect of CNM may be related to the inhibition of angiogenesis in tumor tissue.[19] The antitumor action of CNM is similar to that obtained in other studies with synthetic MMP inhibitors. It is speculated that CNM exerts its antitumor effects, at least in part, on the level of MMP-2 of tumor cells. CNM may inhibit the breakdown of the extracellular matrix and the basement membrane by reducing the level of MMP, and thus the aggressive behavior of tumor cells is retarded. Further study on the mechanism of antitumor activity of CNM is underway.

The antitumor effect of CNM is moderate as compared with that of the antitumor drugs used in chemotherapy. However, the results described here are interesting. Neither weight loss or nor any other obvious sign of toxicity was observed in the *in vivo* experiments. Moreover, CNM has a relatively unique

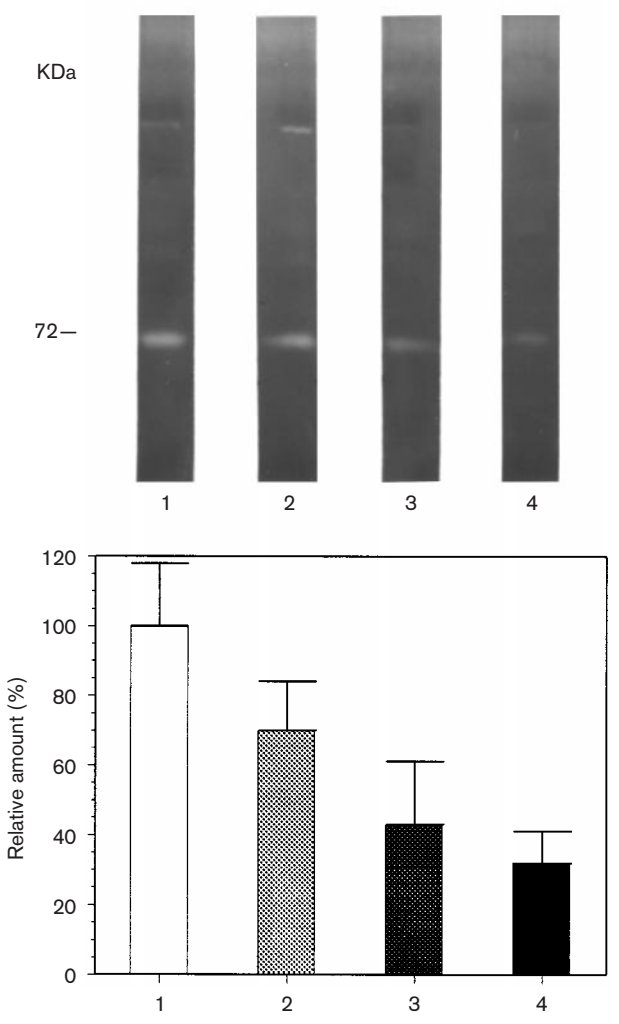

Figure 3. Effect of CNM on the level of MMP-2 in HT-1080 cells. HT-1080 cells were cultured for 24 h in serum-free RPMI 1640 medium with or without CNM: medium (lane 1); CNM, 200 μM (lane 2); CNM, 800 μM (lane 3) and CNM, 2 mM (lane 4). The conditioned media were subjected to electrophoresis in a gelatine-embedded SDS–polyacrylamide gel. After electrophoresis, strips of the gel were incubated and then stained with Coomassie brilliant blue. The locations of gelatinolytic enzymes were detected as clear bands (upper panel) and the gelatinolytic levels were densitometrically quantified (lower panel).

structure compared with that of other MMP inhibitors, which have a collagen-mimicking hydroxamate structure.[3] CNM has a simple structure containing only one phenyl ring and an acrylamido side chain. It is feasible to modify the structure of CNM to design more effective compounds. Those features of CNM suggest that it might provide a lead compound for developing a novel class of antitumor and antimetastatic drugs.

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