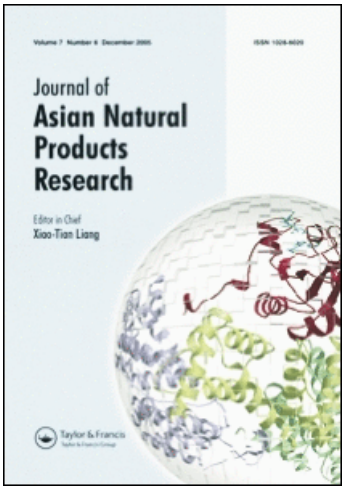

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ANTITUMOR COMPONENTS FROM AN ACTINOMYCETE STRAIN 6011W

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Three bioactive compounds that inhibited nucleoside transport were isolated from the cultured broth of *Streptoverticillium sp. 6011W*. The structures of those compounds were characterized as cinnamamide, N-(tetrahydro-2-oxo-3-thienyl)-acetamide and benzamide, respectively. They all inhibited radiolabeled thymidine transport into Ehrlich carcinoma cells, with IC_{50} values of 30.4, 97.2 and 85.4 μM, respectively. When administered i.p., cinnamamide not only inhibited the growth of transplanted tumors but also reduced the number of lung metastases in mice bearing Lewis lung carcinoma. The results suggest that nucleoside transport inhibition assay is a valuable model to search for antitumor agents of natural origin.

Keywords: Antitumor agent; Cinnamamide; Nucleoside transport inhibitor

INTRODUCTION

Natural products are huge resources of antitumor drugs and lead compounds. Previous studies have shown that plants and microorganisms could produce many anticancer agents used for clinical use, such as adriamycin, mitomycin, vinblastine, and taxol. They bring about antitumor effects through different molecular targets. In our lab nucleoside transport inhibition assay was established to direct the search for novel antitmour agents of natural origin [1]. Nucleoside transport, the first step of salvage pathway

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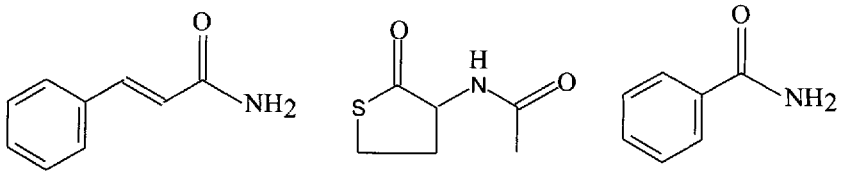
of nucleotide biosynthesis, is one of the attractive targets for cancer chemotherapy [2]. It has demonstrated that nucleoside transport inhibitor could reduce the uptake of exogenous nucleoside and then block nucleotide salvage pathways in cancer cells. Several nucleoside transport inhibitors of natural origin, such as green tea polyphenols [3], antibiotics C3368-A [4] and B [5] are highly active in enhancing the antitumor effects of chemotherapeutic agents and reversing multidrug resistance in tumor cells. A screening program searching for novel nucleoside transport inhibitors from microorganisms has been carried out and a total of 1143 strains were tested. During the screening course, the cultured broth of an actinomycete strain 6011W, which was isolated from a soil sample collected in Yunnan Province, China, showed strong inhibition of nucleoside transport, and three active compounds, 6011W-A, B and C, were isolated from the cultured broth of the strain. The taxonomy, fermentation, isolation and purification, structure elucidation and biological properties of those components were described in this paper.

RESULTS AND DISCUSSION

Structures of 6011W-A, B and C Under the direction of nucleoside transport inhibition assay, 6011W-A, B and C were isolated from the cultured broth of *Streptoverticillium sp. 6011W*. The molecular weights of 6011W-A and C were determined by EI-MS to be 147 and 121, respectively. Their structures were elucidated as cinnamamide and benzamide on the basis of IR, NMR and MS. 6011W-B was obtained as white powder. The molecular weight of B was determined to be 159 by FAB-MS. The ^{13}C-NMR of 6011W-B revealed the presence of six carbon signals, which was attributed to one methyl, two methylene, one methine and two carbonyl carbons. Analysis of ^{1}H-NMR and HMQC revealed the partial structure as CH_2CH_2CH. The EI-MS of 6011W-B gave the ions at *m/z* 131 (M^+–CO), *m/z* 43 (CH_3CO^+), *m/z* 88 (M^+–CH_3CO CO) and *m/z* 56 (M^+–CH_3CO CO S), which indicated the presence of an S atom. HMBC experiments on 6011W-B suggested the long range coupling of H-3 to C-1′ and also the long-range coupling of H-3, H-5 to C-2. Taking into account the above analysis and data, the structure of B was elucidated as N-(tetrahydro-2-oxo-3-thienyl)-acetamide (Fig. 1). The deduction was confirmed on the comparison of the ^{1}H-, ^{13}C-NMR data of 6011W-B with those of N-(tetrahydro-2-oxo-3-thienyl)-acetamide [6].



6011W-A 6011W-B 6011W-C

FIGURE 1 Chemical structures of antitumor components from *Streptoverticillium sp. 6011W.*

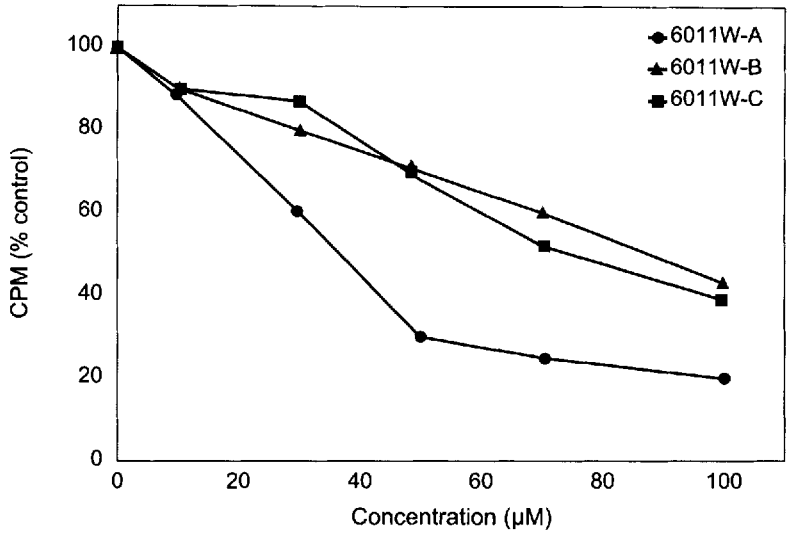


FIGURE 2 Inhibition of nucleoside transport by 6011W-A, B and C into Ehrlich carcinoma cells.

Nucleoside transport inhibition activity As determined by [^{3}H]-thymidine incorporation assay, 6011W-A, B and C could inhibit thymidine transport into mice Ehrlich carcinoma cells (Fig. 2). The IC_{50} values of those compounds were 30.4, 97.2 and 85.4 μM, respectively.

Effects of 6011W-A on the growth of human tumor cells In MTT assay, the IC_{50} value for 6011W-A on the growth of cultured human oral epidermoid carcinoma KB cells was 1.62 mM (Fig. 3).

In vivo antitumor effects of 6011W-A In mice bearing Lewis lung carcinoma, the administration (i.p.) of 6011W-A inhibited the tumor growth of

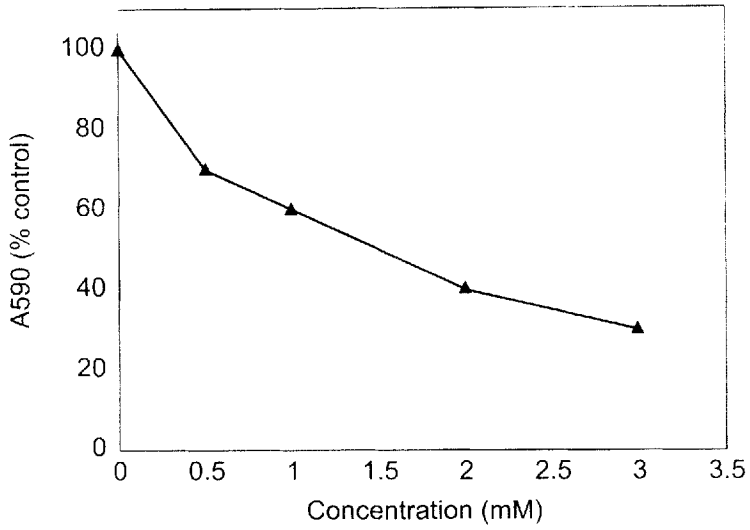


FIGURE 3 Effect of 6011W-A on the growth of human epidermoid carcinoma KB cells.

Lewis lung carcinoma in C57BL/6 mice (Table I). As illustrated in Table II, a decrease in the incidence of metastasis and the number of lung metastases was also observed in the same experiment. These results indicated that 6011W-A could inhibit not only the growth of primary tumor but also pulmonary metastasis.

In the present study, nucleoside transport inhibition assay was used to direct the isolation of antitumor components from microorganism. As the result, cinnamamide, N-(tetrahydro-2-oxo-3-thienyl)-acetamide and benzamide were isolated from the cultured broth of *Streptoverticillium sp. 6011W*. Those compounds could inhibit nucleoside transport in tumor cells. One of them, cinnamamide, has shown moderate antitumor efficacy *in vivo*. The investigation on the effects of 6011W-B and C is now underway. Our previous studies have found that nucleoside transport inhibitors, such as green tea polyphenols [3], antibiotics C3368-A [4] and B [5] as well as emodin [7], may play the role of biochemical modulator potentiating the effects of antitumor drugs. Therefore, it is of interest to discover novel nucleoside transport inhibitors and to determine their possible use in cancer chemotherapy.

All three bioactive components from the actinomycete strain, 6011W-A, B and C, are known compounds. Isolation of 6011W-A and B as products of microbial origin has not been reported so far. Biological activities of benzamide and N-(tetrahydro-2-oxo-3-thienyl)-acetamide were seldom reported, except that several derivatives of benzamide, such as 3-amino-, and

TABLE I Inhibitory effect of cinnamamide on the growth of Lewis lung carcinoma in mice

Group	*Dose* (mg/kg)	*No. of mice (Initial/end)*	*Body weight-change* (g)	*Tumor volume* (mm^3) $\bar{x} \pm SD$	*Inhibition* (%)
Control		8/8	+8.55	7975 ± 1816	
Cyclophosphamide	100, i.p. × 3	8/8	−3.16	3663 ± 816	54.1*
Cinnamamide	50, i.p. × 10	8/8	+1.76	4578 ± 1021	42.6*
Cinnamamide	50, i.p. × 19	8/8	−0.93	2504 ± 1011	68.6*
Cinnamamide	100, i.p. × 10	8/8	+0.42	3745 ± 711	53.9*

*$P < 0.01$ vs control.

TABLE II Effect of cinnamamide on the pulmonary metastasis of Lewis lung carcinoma in mice

Group	*Dose* (mg/kg)	*Incidence of metastases*	*No. of metastases* $x \pm SD$	*Inhibition* (%)
Control		8/8	11.0 ± 5.7	
Cyclophosphamide	100, i.p. × 3	7/8	8.8 ± 5.6	20.5
Cinnamamide	50, i.p. × 10	7/8	10.3 ± 5.7	
Cinnamamide	50, i.p. × 19	5/8	4.8 ± 1.9	56.8*
Cinnamamide	100, i.p. × 10	7/8	4.5 ± 2.6	59.1*

*$P < 0.05$ vs control.

3-methoxybenzamide, are inhibitors of adenosine diphosphate-ribosyl transferase and could enhance the antitumor effect of bleomycin [8].

As shown in this study, cinnamamide exhibited only slightly inhibitory effect on the growth cultured human cancer cells, but it could inhibit not only the growth of primary tumor but also metastasis in mice bearing Lewis lung carcinoma. Thus cinnamamide may exert antitumor effect by inhibiting cancer progression behavior, such as invasion, metastasis and angiogenesis, rather than killing tumor cells. In our studies, cinnamamide does cause a dose-dependent inhibition of type-IV collagenolytic activity as measured by zymography. Type-IV collagenase plays important roles in tumor growth, metastatic process and angiogenesis [9]. It has been reported that many cinnamoyl analogs show antitumor activity *in vitro* [10,11]. So far, no report on the antitumor effect of cinnamamide *in vivo* has yet been reported. Our study has demonstrated that cinnamamide is active against tumors *in vivo*. Collectively, cinnamamide with a relative simple chemical structure might provide a lead compound in developing novel class of antitumor and antimetastatic drugs.

EXPERIMENTAL SECTION

General experimental procedures [^{3}H]-thymidine was purchased from the Institute of Atomic Energy, Chinese Academy of Sciences (Beijing, China).

The radioactivity was counted in a Beckman LS1810 liquid scintillation system. IR was recorded on a Shimadzu IR-435 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were measured on Bruker AM-500 or JMN-GX400 instruments with TMS as internal standard. EI-MS and FAB-MS were taken on Zab-2F and Zabspec mass spectrometer. Silica gel (10–40 μ, Qingdao) and Sephadex LH-20 (Pharmacia), RpC18 silica gel (100–200 mesh, Ouya) were used as normal and reversed phases. For animal experiments, Kunming (KM) mice and C57BL/6 mice, weighing 18–22 g, were supplied by Experimental Animal Institute of Chinese Academy of Medical Sciences (Beijing, China) and kept in a room with controlled temperature and humidity.

Taxonomy The producing organism, strain 6011W, was isolated from a soil sample collected in Yunnan Province, China. Based on the microbial characteristics of strain 6011W, it is identified as the genus *Streptoverticillium*.

Fermentation Well grown agar slant of *Streptoverticillium sp. 6011W* was used to inoculate in a 500 ml Erlenmeyer flask containing 100 ml of medium consisting of glycerol 1%, soluble starch 1.5%, KCl 1.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ (pH 7.0). The fermentation was carried out at 27°C for 3 days on a rotary shaker. Eighty ml of vegetative inoculum were then transferred into a 5000 ml Erlenmeyer flask containing 1000 ml of the same medium as described above, then incubated on reciprocal shaker at 27°C for 5 days. A large scale fermentation was carried out in a 40 l fermentator. The seed culture prepared by the above procedure was transferred into the fermentator containing 40 l of the same medium. The fermentator was run at 27°C for 5 days.

Isolation and purification The broth filtrate was adsorbed on a column of GDX 101 resin and the active fractions were eluted with 80% acetone The eluent was combined with acetone extract of the mycelial cake and then concentrated *in vacuo* to dryness. The oily residue was applied to a vacuum liquid column of silica gel. A cyclohexane gradient in acetate was used to develop the chromatograph and gave three active eluents. The isolation of the first fraction was accomplished by preparative silica TLC with an eluent as cyclohexane–acetate (4 : 1), followed by a column chromatograph of Sephadex LH-20 with $CHCl_3$- MeOH (1 : 1) as solvent. 6011W-A (200 mg) was obtained as a colorless needle. Fraction 2 was chromatographed on a Sephadex LH-20 column eluted with 90% MeOH and then on a MPLC of reversed RpC18 column with an eluent with 60% MeOH, which gave 6011W-B 4 mg. Fraction 3 was purified on a Sephadex LH-20 column and the eluent of MeOH gave 6011W-C 44 mg.

6011W-A Colorless needle; IR (KBr) ν_{max} 3380, 3170, 1680, 1610, 1400, 970, 690 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 7.65 (d, 1H, J = 15.6 Hz), 7.51 (m, 2H), 7.36 (m, 3H), 6.47 (d, 1H, J = 15.6 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ ppm: 168.9, 142.1, 134.4, 129.8, 128.7, 127.7, 119.5; EI-MS m/z (%): 147 (M^+), 131 (M^+-NH_2), 103 (M^+-NH_2-CO), 77 ($M^+-NH_2-CO-C_2H_2$).

6011W-B White powder; IR (KBr) ν_{max} 3267, 1699, 1651, 1548, 1375, 910 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ ppm: 4.57 (dd, 1H, J = 7.0, 11.5 Hz), 3.34 (dt, 1H, J = 5.5, 11.5 Hz), 3.21 (ddd, 1H, J = 1.5, 7.0, 11.5 Hz), 2.50 (dddd, 1H, J = 1.5, 5.5, 7.5, 11.5 Hz), 2.03 (dq, 1H, J = 7.0, 11.5 Hz), 1.91 (s, 3H); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ ppm: 206.9, 173.5, 59.9, 31.7, 27.9, 22.3; EI-MS m/z (%): 131 (M^+-CO), 88 (M^+-CH_3CO-CO), 56 (M^+-CH_3 CO–CO–S), 43 (CH_3CO^+); FAB-MS m/z (%): 182 $[M+Na]^+$, 160 $[M+1]^+$.

6011W-C Colorless needle; IR (KBr) ν_{max} 3010, 2920, 2840, 1710, 1460, 1410, 1380, 830, 620 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ ppm: 6.24 (d, 2H, J = 7.0 Hz), 5.90 (t, 1H, J = 7.0 Hz), 5.81 (t, 1H, J = 7.0 Hz); EI-MS m/z (%): 121 (M^+), 105 (M^+-NH_2), 77 (M^+-NH_2-CO).

Nucleoside transport inhibition activity For the nucleoside transport assay [3], Ehrlich carcinoma cells drawn from ascites-bearing KM mice were washed with 0.85% NaCl and suspended in serum-free RPMI-1640 medium, at 7×10^6 cells/0.9 ml. After the addition of 0.1 ml drug solution, the mixture was pre-incubated at 37°C for 5 min. Then, [^{3}H]-thymidine (1 μCi/tube) was added and the tube was kept at 37°C for 30s. Immediately after ice-cold 0.85% NaCl was poured into tube, the content of the tube was filtered on a glass-filter disc. After the addition of 0.1 ml of 0.1 M NaOH and subsequent drying, the radioactivity on disc was determined by a Beckman LS1202 liquid scintillation system.

MTT assay The growth rate of cultured cells was determined by MTT assay [12]. Briefly, cells in the mid-log phase were harvested and grown in 96-well plate. After 24 h, drug was added and cells were incubated again for 72 h in the presence or absence of the drug, then the cell number and viability were estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

In vivo antitumor and antimetastatic effects Antitumor effects *in vivo* (inhibition of primary tumor growth and metastasis) were assessed using Lewis lung carcinoma models, which were described previously [13]. Groups of 8 C57BL/6 male mice were subcutaneously implanted with 0.2 ml ground mixture of viable tumor tissue and serum-free RPMI-1640 medium in a

ratio 1 : 4 (w/v). Drug was given i.p. daily or on alternate days started 24 h after tumor implantation and continuing throughout the course of the experiment. Tumor sizes were measured with calipers and volumes were calculated by the formula $a \times b^2/2$ where a is the long axis and b is the short axis of the tumor. Mice were killed on day 21. The metastasized colonies in the lung surface were scored using a dissecting microscope after fixation in Bouin's solution.

Acknowledgments

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