Antitumour Activities of Sesquiterpene Lactones from *Inula helenium* and *Inula japonica*

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Eight sesquiterpene lactones were isolated from the roots of *Inula helenium* and flowers of *I. japonica*. Among them, isoalantolactone (**3**) and santamarine (**6**) exhibited significant growth inhibitory activities against gynecologic cancer cell lines, while others weakly inhibited the growth of the cell lines ($IC_{50} \leq 100\,\mu M$). In addition, **3** significantly inhibited the tumour growth of S180 tumour-bearing mice. Compounds **3** and **6** were not toxic to human embryonic lung fibroblast cells *in vitro*. These results demonstrated that the antitumour activities are closely related to the structures of the compounds, that is, an α-exomethylene-γ-lactone ring is necessary for these activities.

Key words: Inula sp., Sesquiterpenes, Antitumour Activity

Introduction

It has been well documented that medicinal plants confer considerable anticancer activity against various tumours (Dai and Mumper, 2010). Plants contain abundant compounds which have consistently been shown to be associated with a lower risk of cancers at almost every site, such as lung, colon, rectum, prostate, cervix, stomach, pancreas, breast, and bladder (Steinmetz and Potter, 1991). Efforts, therefore, are being made to identify naturally occurring anticarcinogens which would prevent, slow, and/or reverse the cancer induction and its subsequent development (Chuang *et al.*, 2000). *Inula helenium* is an important herb traditionally used in the treatment of influenza, fever, tuberculotic enterorrhea, and chronic enterogastritis in China, Japan, and Europe (Okuda, 1986; Olechnowicz-Stepien and Skurska, 1960). Plants of the genus *Inula* have been shown to contain high levels of sesquiterpene lactones (Zhao *et al.*, 2006; Trendafilova *et al.*, 2010), which recently have received considerable attention in the pharmacological community due to their antineoplastic and anti-inflammatory effects (Konishi *et al.*, 2002; Won *et al.*, 2004). Many studies indicated that sesquiterpene lactones exhibit antitumour activities in a variety of malignant cells (Ghantous *et al.*, 2010; Chen *et al.*, 2007). In the present study, we investigated the antiproliferative activity of eight sesquiterpene lactones from *I. helenium* and *I. japonica* against gynecologic cancer cell lines *in vitro*. We also explored the antitumour activity against ascite tumour S180 xenografts in mice *in vivo*.

Results

Isolation of sesquiterpene lactones

The dichloromethane extract of the roots of *I. helenium* contained 3β,9β-diacetoxy-1β,10α-epoxy-11α,13-dihydrocostunolide (**1**) (Milosavljevic *et al.*,

1991; Hatam *et al.*, 1992), 3*β*,9*β*-diacetoxy-11*α*,13-dihydrocostunolide (**2**) (Hatam *et al.*, 1992), isoalantolactone (**3**) (Konishi *et al.*, 2002; Yang *et al.*, 2003), alantolactone (Konishi *et al.*, 2002), 2*α*-hydroxy-11*α*,13-dihydroisoalantolactone (**4**) (Topçu *et al.*, 1993), 11*α*,13-dihydroisoalantolactone (**5**) (Konishi *et al.*, 2002; Yang *et al.*, 2003), and santamarine (**6**) (Romo de Vivar and Jiménez, 1965) (Fig. 1), while britannilactone (**7**) (Zhou *et al.*, 1993; Jeske *et al.*, 1993) and 1-*O*-acetylbritannilactone (**8**) (Zhou *et al.*, 1993; Jeske *et al.*, 1993; Je *et al.*, 2004) were isolated from the ethanol extract of the flowers of *I. japonica*. The spectroscopic data of these compounds were in accord with those in the literature.

Inhibition of growth of HeLa and A549 cells

The cytotoxic effect of compounds **1**–**8** (100 μM) on the gynecologic HeLa cancer cell line was investigated. The MTT (methylthiazolyl tetrazolium) assay revealed that cisplatin, isoalantolactone (**3**), and santamarine (**6**) had a strong dose-dependent antiproliferative effect on HeLa cells; the percentages of growth inhibition by these compounds were 66%, 81%, and 86% , respectively, while the other compounds inhibited growth only weakly (Fig. 2). The IC_{50} values of the proliferation of HeLa cells of cisplatin, isoalantolactone, and santamarine were 20.19, 19.41, and 10.48 μM.

Inhibition of growth of HEC-1, SHIN3, HOC-21, and HAC-2 cells

The MTT assay revealed that cisplatin exhibited the strongest antiproliferative activity against HAC-2 cells (IC_{50} = 8.95 μM) but did not inhibit the proliferation of HEC-1, SHIN3, and HOC-21 cells, respectively (Table I). Isoalantolactone (**3**) was strongly antiproliferative against HEC-1, HOC-21, and HAC-2, but not against SHIN3 cells, while the other compounds did not affect growth.

Inhibition of growth of human embryonic lung fibroblast (HELF) cells

Cisplatin exhibited stronger antiproliferative activity against HELF cells (IC_{50} = 16.89 μM) than isoalantolactone (**3**) and santamarine (**6**). The percentage of growth inhibition of isoalantolactone and santamarine at 100 μM on HELF cells was 47% and 22%, respectively (Fig. 3).

*Effect of isoalantolactone (**3**) on ascites tumours*

Cyclophosphamide (cytoxan) and **3** had comparable antitumour growth activities *in vivo*. Inhibition (see legend to Fig. 2) by **3** at 100 and 10 mg/(kg d) was 64.2% and 43.0%, respectively

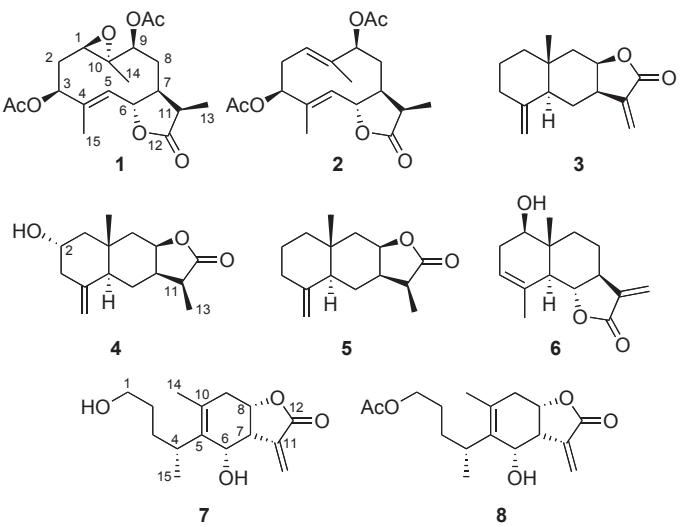

Fig. 1. Chemical structures of sesquiterpene lactones isolated from *Inula* sp.: 3*β*,9*β*-diacetoxy-1*β*,10*α*-epoxy-11*α*,13-dihydrocostunolide (**1**), 3*β*,9*β*-diacetoxy-11*α*,13-dihydrocostunolide (**2**), isoalantolactone (**3**), 2*α*-hydroxy-11*α*,13-dihydroisoalantolactone (**4**), 11*α*,13-dihydroisoalantolactone (**5**), santamarine (**6**), britannilactone (**7**), and 1-*O*-acetylbritannilactone (**8**).

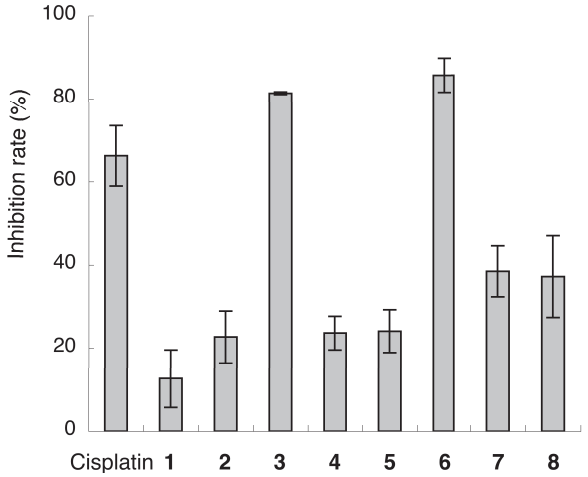

Fig. 2. The effect of the sesquiterpene lactones **1** to **8** on the proliferation of the HeLa tumour cell line. The percentage of growth inhibition by the compounds was calculated by comparing viable cells in the treated group with those in the untreated group. * $P < 0.05$, ** $P < 0.01$ vs. control.

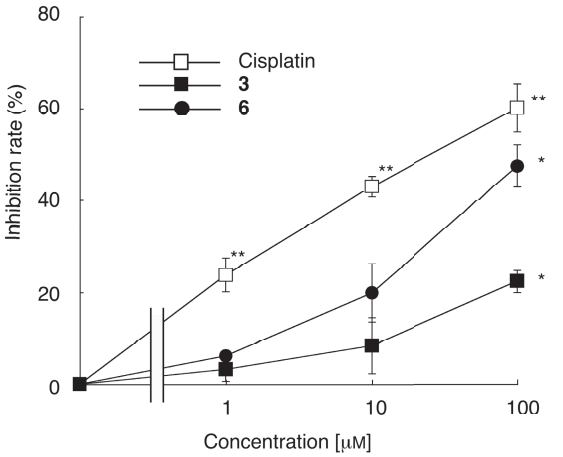

Fig. 3. The effect of cisplatin, isoalantolactone (**3**), and santamarine (**6**) on the proliferation of the HELF cell line. * $P < 0.05$, ** $P < 0.01$ vs. control.

Table I. The effect of eight sesquiterpene lactones on the proliferation of HEC-1, SHIN3, HOC-21, and HAC-2 tumour cell lines.

Compound	IC_{50} [μM]			
	HEC-1	SHIN3	HOC-21	HAC-2
Cisplatin	>100	>100	>100	8.95
3b,9b-Diacetoxy-1b,10α-epoxy-11α,13-dihydrocostunolide (**1**)	>100	>100	>100	>100
3b,9b-Diacetoxy-11α,13-dihydrocostunolide (**2**)	>100	>100	>100	>100
Isoalantolactone (**3**)	32.54	>100	19.65	11.53
2α-Hydroxy-11α,13-dihydroisoalantolactone (**4**)	>100	>100	>100	>100
11α,13-Dihydroisoalantolactone (**5**)	>100	>100	>100	>100
Santamarine (**6**)	>100	12.42	42.62	>100
Britannilactone (**7**)	>100	>100	>100	>100
1-*O*-Acetylbritannilactone (**8**)	>100	>100	>100	>100

(Fig. 4A).The thymus and spleen index, respectively, decreased significantly in the cyclophosphamide group but they were invariable in **3** (Fig. 4B). Furthermore, the data suggested that **3** inhibited tumour growth *in vivo* in a dose-dependent manner.

Discussion

Traditionally, many plants containing high levels of sesquiterpene lactones have been used as folk medicines because of their pharmacological properties. *Inula* species, rich in sesquiterpene lactones, have been widely used as herbal medicines in China, Japan, and Europe to treat a number of diseases, and their pharmacological activities have been confirmed (O'Shea *et al.*, 2009; Cantrell *et al.*, 1999). Some pure compounds have been isolated from *I. helenium* and *I. japonica* to prove their anticancer activity (Konishi *et al.*, 2002; Dorn *et al.*, 2006). In our research, the antitumour activities of eight sesquiterpene lactones, **1**–**8**, extracted from *I. helenium* and *I. japonica* were explored. Our data showed that isoalantolactone (**3**) and santamarine (**6**) significantly inhibited the growth of the human gynecologic cancer cell lines HeLa, HEC-1, SHIN3, HOC-21, and HAC-2, but were not cytotoxic to human embryonic lung fibroblast cells (HELF), while compounds **1**, **2**, **4**, **5**, **7**, and **8** were only weakly growth inhibitory in all cell lines. Furthermore, **3** exhibited *in vivo* antigrowth

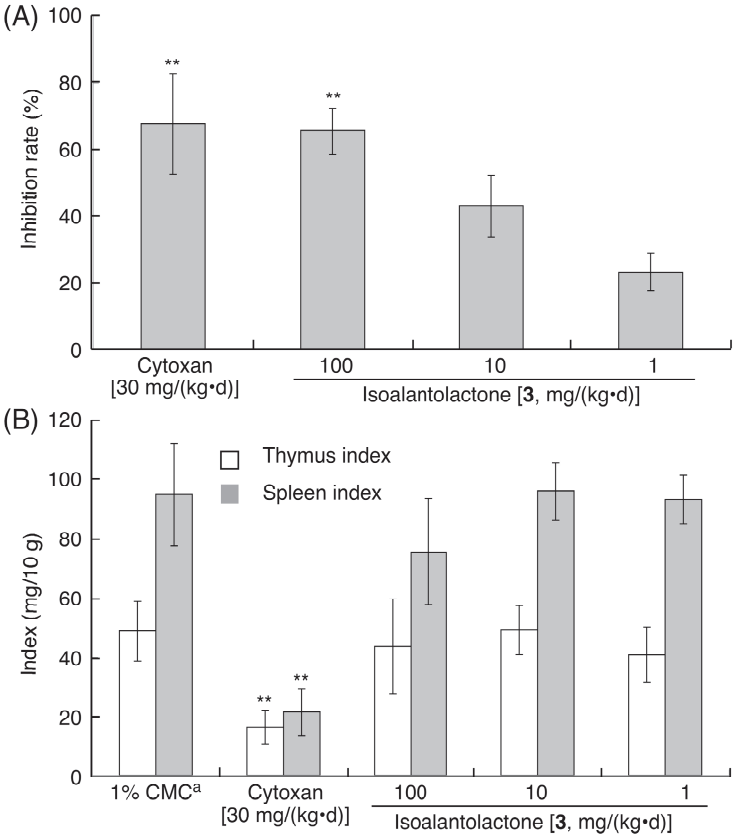

Fig. 4. The antitumour activity of isoalantolactone (**3**) on S180 cells in mice. (A) Inhibition rate. (B) Index. ** $P < 0.01$ vs. control. [a]CMC, carboxymethyl cellulose.

activity against ascites tumours in a dose-dependent manner.

Of the eight sesquiterpene lactones isolated from *Inula* species, **1** and **2** belong to germacrane sesquiterpenes with a 10-membered ring, **3** – **6** belong to eudesmane sesquiterpenes with a *trans*-decalin (6/6-membered) ring, while **7** and **8** are britannilane sesquiterpenes with a 6-membered ring. Among the four eudesmanes, the exomethylene group at the γ-lactone ring of **3** and **6** is saturated in **4** and **5**.

The experimental results indicate that the antigrowth activities against the human lung tumour cell lines are closely related to the carbon skeletons and the α-exomethylene group at the γ-lactone ring. Saturation of this α-exomethylene group or cleavage of the 6/6-membered ring leads to the loss or decrease of the antigrowth activity. This is in good agreement with Konishi's results where the antitumour activity of alantolactone derivatives significantly decreased with C11,13-saturation (Konishi *et al.*, 2002).

In summary, isoalantolactone (**3**) and santamarine (**6**), two sesquiterpene lactones isolated from *Inula helenium*, showed potent anticancer activities against various human lung cancer cells *in vitro* and xenograft ascites tumours *in vivo*. These findings indicate that **3** and **6** have significant therapeutic potential and might serve as powerful novel antitumour lead compounds.

Experimental

Material

The roots of *Inula helenium* (3 kg dry weight) and dried flowers of *I. japonica* (10 kg) were purchased at the Anguo medicinal herbs market, Hebei province, China, in April 2008. Male Kunming mice (10-week-old, 18–22 g) were purchased from the Hebei Medical University, Hebei, China,

and were fed a standard pellet diet and drinking water *ad libitum*. The gynecologic cancer cell lines HeLa, HEC-1, SHIN3, HOC-21, and HAC-2 were obtained from the Department of Environmental Biochemistry, Graduate School of Medicine, Chiba University, Chiba, Japan. Human embryonic lung fibroblast (HELF) cells were obtained from the Biology Laboratory, Hebei Medical University, Hebei, China. Methylthiazolyl tetrazolium (MTT) and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Extraction of sesquiterpene lactones and structure determination

The dichloromethane extract of the roots of *Inula helenium* was applied to a silica gel column eluted with CH_2Cl_2/acetone step gradients (30:1 to 1:1) to yield 78 major fractions designated Fr_{DA-1} to Fr_{DA-78}. Fr_{DA-1}–Fr_{DA-5} were submitted to preparative TLC [petroleum ether/Me_2CO (3:1, v/v)] to isolate compounds **1**–**6** and alantolactone. The dichloromethane-soluble fraction of the 95% ethanol extract of the flowers of *Inula japonica* was applied to silica gel column chromatography successively for preliminary fractionation, and elution was monitored by TLC. Similar fractions were combined into several sub-fractions, which were subjected to silica gel column chromatography, Sephadex LH-20 gel column chromatography, and preparative TLC for further separation and purification to obtain the pure compounds **7** and **8**. The structures of these compounds were established on the basis of 1D NMR and 2D NMR analyses and comparison with data of authentic samples.

Growth inhibition assay

The human gynecologic cancer cell lines HeLa, HEC-1, SHIN3, HOC-21, HAC-2, and HELF cells were cultured in RPMI 1640 medium (Applichem, Darmstadt, Germany) containing 10% fetal bovine serum (FBS; Cellgro, Manassas, VA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere with 5% CO_2 at 37 °C. The viability of cells treated with various chemicals was determined by the MTT reduction assay performed in triplicate. Cells (10^4) were incubated in 100 μl culture medium/well in 96-well plates for 12 h. Then the culture medium was exchanged by fresh medium containing various concentrations of the compounds (1 μM, 10 μM, 100 μM) with the positive control group containing cisplatin (Sigma Chemical Co.). After treatment with the compounds for 48 h, 10 μl of 5 mg/ml MTT was added into each well, respectively, for another 4 h. Finally, 150 μl of stop solution [10 ml of 10% sodium dodecylsulfate (SDS), 6 μl of 12 M HCl] were added into each well, and the plate was placed in an incubator with 5% CO_2 at 37 °C for 12 h. Absorbance at 570 nm was measured with a microplate reader using wells without cells as blanks (reference wavelength 490 nm). Cell survival was calculated from the absorbance and presented as percentage of the surviving cells. The growth inhibition was calculated using the following formula: growth inhibition (%) = $(1 - T/C) \cdot 100\%$, where T and C are the mean absorbance values of the treated and control groups, respectively.

*Effects of isoalantolactone (**3**) on ascites tumours*

Ascite tumour cells (S180) aspirated from the peritoneal cavity of mice were washed with saline, and 10^7 tumour cells were implanted into the right mediodorsal flask. One day later, animals were divided into five groups (ten mice per group). Animals in group I were kept with water (0.02 ml/g); animals in group II received intraperitoneal administration of cyclophosphamide [Sigma; C0768; 30 mg/(kg d)]; animals in groups III–V were given intragastric administration of isoalantolactone at concentrations of 1, 10, 100 mg/(kg d), respectively. Daily administration of cyclophosphamide and isoalantolactone was carried out from day 1 to day 10. Animals were observed for the development of ascite tumours. At the termination of the experiment all animals were sacrificed, and tumours, thymuses, and spleens were dissected and weighed. Tumour inhibition was calculated using the following formula: tumour inhibition (in % of control) = $(C-T)/C \cdot 100\%$, where T and C are the mean tumour weights of treated and control mice, respectively. Spleen indexes were calculated by the formula: spleen index = mean spleen weight (mg)/mean body weight (g) · 100%.

Statistical analysis

Statistical analysis was performed by ANOVA with Bonferroni for multiple comparisons. The data are given as mean ± SD. A value of $P > 0.05$ was considered statistically significant.

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