Millifolide A, a dimeric ether of degraded sesquiterpene lactones, inhibited the proliferation of human lung cancer cell line A549*

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The inhibitory effect of three degraded sesquiterpene lactones, *iso*-seco-tanapartholide, arteludooicinolide A and millifolide A isolated from *Achillea millefolium* L., on anti-human lung cancer cells was examined using MTT and reporter gene assays. Millifolide A has a significant inhibitory effects on the proliferation of human lung cancer cells probably through inducing cell apoptosis.

Keywords: word; *Achillea millefolium* L.; millifolide A; human lung cells; antiproliferation; apoptosis

1. Introduction

Chemical therapy plays an important role in lung cancer patients particularly with small cell lung cancer, but due to its high toxicity and side effects, the application of cisplatin, taxanes or vincristine in clinical chemotherapy of lung cancer are strongly confined (Li et al. 2010). Yarrow (*Achillea millefolium* L.) as the composite yarrow perennial herbaceous plants in the family Asteraceae, is used as a folk medicine for the treatment of injuries, amenorrhea abdominal pain, rheumatic pain, snake bites, carbuncle furuncle swollen poison *etc*. Traditional Chinese medicine theory believes that this bitter medicine has the function of clearing heat and toxin, promoting blood flow for regulating menstruation (Dong et al. 2015). We have come to the idea that the variety of medicinal activities of yarrow plants are attributed to the sesquiterpene lactones with an *exo*-α,β-unsaturated double bond act as a strong Michael acceptor. In this study, we determined the antiproliferative effects of three sesquiterpene lactones previously isolated by us from this plant (Li et al. 2012) on human lung cancer cells.

2. Results and discussion

Compounds **1-3** were isolated according to our previous report (Li et al. 2012). Fig. 1 shows the inhibitory effect of compounds **1-3** on the antiproliferation of human lung tumor cells. Among them, dimeric ether millifolide A (**3**) showed strong inhibition compared with positive control cisplatin. Cell survival rates of cisplatin and **1** were 29.98% (IC_{50} 19.87 μmol/L) and 17.67% (IC_{50} 3.57 μmol/L) for A549 cells, and 8.25% and 17.67% for QG-90 cells, respectively. The activity of other monomeric compounds **1** and **2** were low. Thus, the size and/or functionalilty of dimeric structure would affect the activity. In addition to the number of *exo*-α,β-unsaturated double bond, dimeric millifolide A (**3**) lost each hydroxy group to form a hydrophobic ether likage getting cell permiability.

(Fig. 1)

Because the induction of apoptosis is associated with cell death, we investigated the apoptic activity of **3** using reporter gene assay. The gene expression results in A549 cells showed that, compared with blank control group, cisplatin and **3** significantly induced pG53-Luc and pGBax-Luc expression. The rate over the blank control by pG53-Luc and pGBax-Luc were 2.99 and 4.73 times for cisplatin, and 1.94 and 5.61 times for **3**, respectively [Fig. S1 (A)]. Western blotting analysis proved that the expression of p53, Bax and Caspase-3 proteins were significantly increased by treatment with **3** in A549 cells [Fig. S1 (B)].

In order to further support the hypothesis that inhibition of the proliferation of A549 cells by millifolide A (**3**) was related to apoptosis, the effect of caspase inhibitor was examined. A cell-permeable caspase inhibitor, Z-VAD-FMK [Z-Val-Ala-Asp(OMe)-CH_2F], was co-incubated with **3** and cisplatin, respectively, and cell survival rate were elucidated using MTT assay [Fig. S1 (C)] (Garn et al. 1994). The caspase

inhibitor recurred the rate of inhibition by **3** from 70.14% to 76.20% at 1 μmol/L, from 30.41% to 48.09% at 10 μmol/L, and 7.37% to 76.20% at 100 μmol/L. Above all, our findings demonstrate that the inhibitory effect of millifolide A (**3**) on human lung tumor cell proliferation may be through inducing cell apoptosis.

Disclosure statement

No potential conflict of interest was reported by the authors

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Note

* This paper is dedicated to Professor Qing-Wen Shi has passed away on 18th Oct 2020.

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Fig. 1. Chemical structures and cell growth inhibitory activity of sesquiterpene lactones **1-3**. (A) Inhibitory effect of **1-3** on growth of A549 and QG-90 cells compared with cisplatin (American Sigma, 034k3693). (B) Details for A549 cell. *$p<0.05$, **$p<0.01$ vs control.

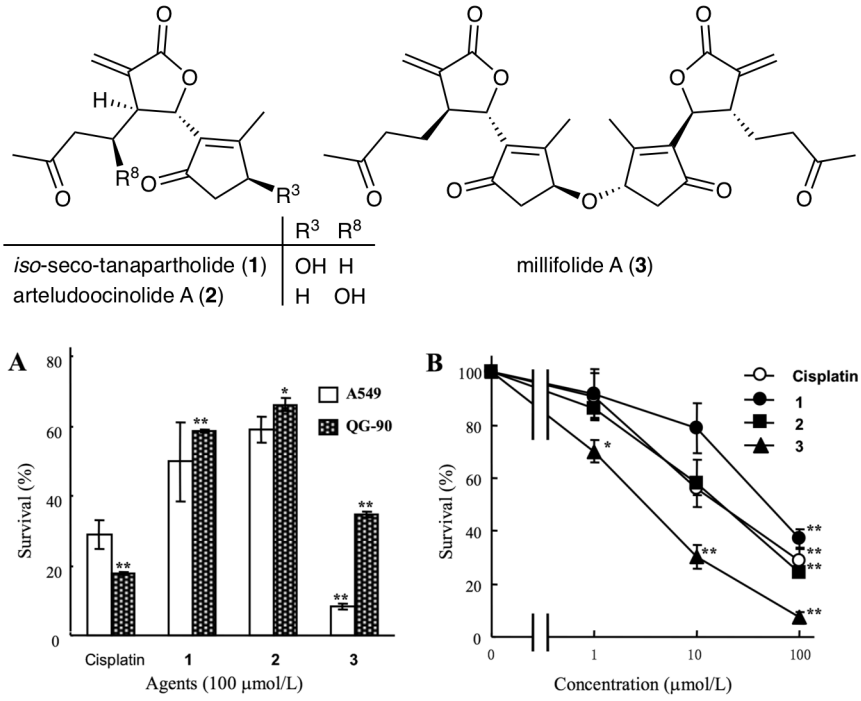
R3 R8
iso-seco-tanapartholide (1) OH H
arteludoocinolide A (2) H OH
millifolide A (3)
A
Survival (%)
A549
QG-90
Cisplatin
1
2
3
Agents (100 μmol/L)
B
Survival (%)
Cisplatin
1
2
3
Concentration (μmol/L)

Fig. 1

Supplementary Material

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Abstract

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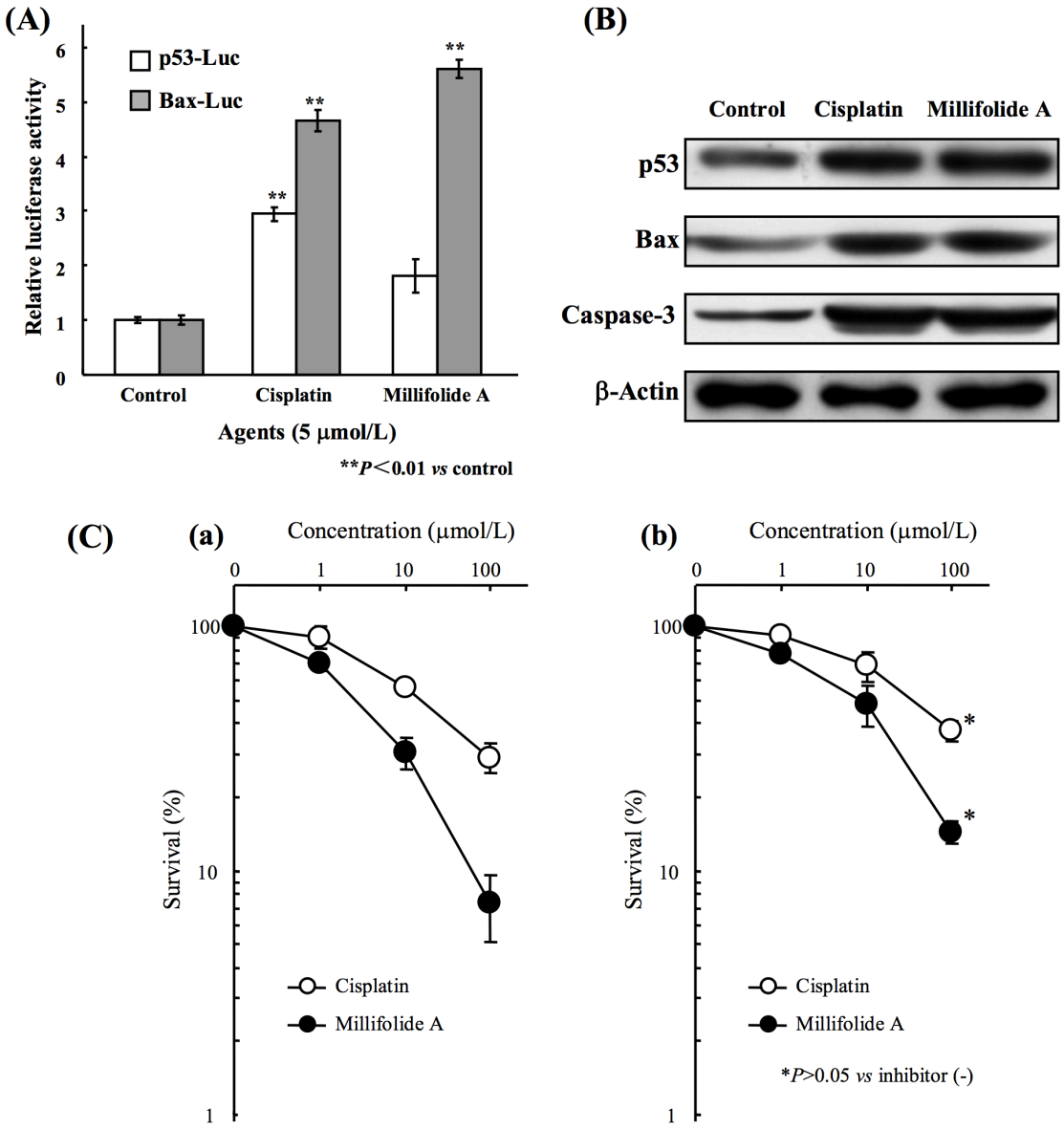

Figure S1. Induction of apoptosis by millifolide A (**3**) on A549 cells. (A) Luciferase assay (B) Western blot analysis. (C) Effect of a cell-permeable caspase inhibitor, Z-VAD-FMK on growth inhibition by millifolide A (**3**) compared with cisplatin.

Experimental

1. Compounds

Three sesquiterpene lactones, *iso*-seco-tanapartholide (**1**), arteludooicinolide A (**2**) and millifolide A (**3**), were isolated from the flowers of *Achillea millefolium* (supplied by the late Professor Qing-Wen Shi) with purity >99% (analyzed by HPLC and ^{1}H-NMR) (Li et al. 2012).

2. Inhibitory effect of 1-3 on growth of A549 and QG-90 cells

Two human cancer derived cell lines, A549 (non-small lung cancer cells line) and QG-90 (small lung cancer cells line) (provided by the Second Department of Biochemical Medicine in Chiba university, Japan), were respectively cultured in RPMI medium 1640 (USA, GIBCO) containing 10% (v/v) fetal bovine serum and antibiotics (100 μg/mL of streptomycin and 100 U/mL of penicillin G) at 37°C in a humidified atmosphere containing 5% CO_2. Cell viability was monitored by MTT assay, as previously described (Gran et al. 1994). In brief, the logarithmically proliferating cells were plated into 96-well plates (1 x 10^4 cells/well) with the medium containing each lactone and cisplatin at the indicated doses and the solvent control (final concentration of 0.1% DMSO) for 2 d. After the culture, the activity of mitochondrial succinic dehydrogenase was measured by further incubation of the cells with 0.5 mg/mL MTT (American Sigma, M-2128) for 4 h, followed by estimation of absorbance at 570 nm with a reference wavelength of 655 nm.

2. Induction of apoptosis by millifolide A (3) on A549 cells

(A) Luciferase assay (Altmann et al. 2007): A549 cells were seeded into 24-well plates (5 x 10^4 cells per well), and cultured for 24 h. Then luciferase reporter gene plasmid pG13-Luc, or pGBax-Luc (each 500 ng) and control gene plasmid SV-40-Rluc (5 ng, each provided by the Department of Biochemical Medicine, Chiba University, Japan) joined together without serum, being incubated for 3 h. After that, the final concentration of 5 μmol/L cisplatin and millifolide A (**3**) were replaced and cultured continuously for 24 h, respectively. A549 cells were then cracked with 1 x PLB (passive lysis buffer), and each luciferase activity was immediately recorded.

(B) Western blot analysis: A549 cells were cultured with RPMI-1640 medium containing the final concentration of 5 μmol/L of cisplatin and millifolide A (**3**) for 24 h, respectively. Then cells were harvested and washed twice with cold PBS. After ultrasonic treatment, total proteins were extracted and boiled in 100 μL sample buffer for 10 min, the equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (10-12%) and then transferred onto polyvinylidene fluoride membranes (Millipore). Subsequently, the membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with the polyclonal antibodies (Bax, p53), respectively at 4°C over night. After being incubated with the secondary antibody for 20 min, the membrane was washed three times with 1 x TBS-T. Finally, ECL reagent was added and exposed for 10 min. The gray value was analyzed by Image J software and β-actin was used as internal controls.

(C) Effect of a cell-permeable caspase inhibitor, Z-VAD-FMK on growth inhibition by millifolide A (**3**) compared with cisplatin. A549 cells plated in 96-well plates were treated with 3 in the absence (a) or the presence (b) of Z-VAD-FMK (20 μmol/L) for 2 d. Then, cells were further incubated in the medium-containing MTT (Garn et al. 1994) and processed as described in Fig. 1. Cell survival rate (%) = A_{570}(experimental) /A_{570}(control) x 100. Statistical analysis. Results are presented as the mean±SD. T-Test was used to determine the significant difference between two groups, one-way analysis of variance (ANOVA) test with spss 20.0 software was used to determine the significant difference among multi-groups.

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