Isocostunolide, a sesquiterpene lactone, induces mitochondrial membrane depolarization and caspase-dependent apoptosis in human melanoma cells

Chia-Nan Chen a, Hsin-Hsiu Huang a, Chia-Li Wu b, Coney P.C. Lin c, John T.A. Hsu c,d, Hsing-Pang Hsieh c, Shuang-En Chuang a, Gi-Ming Lai a,*

a Division of Cancer Research, National Health Research Institutes (NHRI), 7F, No. 161, Sec. 6, Min-Chuan East Road, Taipei 114, Taiwan, ROC
b Department of Chemistry, Tamkang University, Tamsui 251, Taiwan, ROC
c Division of Biotechnology and Pharmaceutical Research, NHRI, 35, Keyan Road, Zhunan Town, Miaoli County 350, Taiwan, ROC
d Department of chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan, ROC

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Abstract

Isocostunolide is a sesquiterpene lactone isolated from the roots of Inula helenium. Its chemical structure was determined by NMR and FAB-MS spectra. No biological activities of this compound have yet been reported. In this study, we found isocostunolide could effectively induce cytotoxicity in three cancer cell lines (A2058, HT-29, and HepG2), with an IC50 of 3.2, 5.0, and 2.0 µg/mL, respectively. DNA flow cytometric analysis indicated that isocostunolide actively induced apoptosis of cancer cells accompanied by a marked loss of G0/G1 phase cells. To address the mechanism of the apoptotic effect of isocostunolide, we analyzed the induction of apoptosis-related proteins in A2058. The levels of pro-caspase-8, Bid, pro-caspase-3, and poly(ADP-ribose) polymerase (PARP) decreased. However, the level of Fas was increased markedly in a dose-dependent manner. Furthermore, this compound markedly induced a depolarization of mitochondrial membranes to facilitate cytochrome c release into cytosol. The findings suggest that isocostunolide may activate a mitochondria-mediated apoptosis pathway. To address this, we found that isocostunolide-induced loss of mitochondrial membrane potential occurred via modulation of the Bcl-2 family proteins. The production of intracellular reactive oxygen species (ROS) in A2058 was not elicited. In summary, for the first time, we have isolated and characterized isocostunolide from I. helenium. This compound induces apoptosis through a mitochondria-dependent pathway in A2058 cells.

Keywords: Apoptosis; Sesquiterpene lactone; Mitochondrial; ROS; Caspase; Isocostunolide

1. Introduction

Inula helenium (Compositae) is an herb traditionally used as a home remedy in Japan [1], as a diaphoresis in Europe, and in Taiwan and China, as a therapeutic agent for tuberculous enterorhea and chronic enterogastritis [2]. Plants of the genus of Inula have been shown to contain high levels of sesquiterpene lactones [3]. Sesquiterpene lactones recently have received considerable attention in its pharmacological circles due to their anti-neoplastic and anti-inflammatory effects [4–6]. Isocostunolide is a well-known sesquiterpene lactone contained in plants [7]. In this study, this compound was isolated and characterized from the Chinese herb I. helenium. The structure was elucidated

* Corresponding author.
E-mail address: gminlai@nhri.org.tw (G.-M. Lai).

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mainly by NMR spectral evidence and found to be identical to the reported sesquiterpene lactone compound isocostunolide. Although many reports have asserted that sesquiterpene lactones exhibit a broad spectrum of biological activities, including anti-tumor [8], anti-inflammatory [9], anti-fungal [10], and anti-viral [11] activities, the bioactivities of isocostunolide have never been reported. More than 5000 sesquiterpene lactones have been identified from plants [12,13], but their various biological activities are not yet well understood.

Apoptosis, or programmed cell death, has an essential role in controlling cell number in many developmental and physiological settings. Its morphological characteristics include plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation, and formation of apoptotic bodies [14]. Recent reports have claimed that many anti-cancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent tumor promotion and progression. Mitochondria are currently regarded as playing a central role in mediating ‘intrinsic death signals’ and could sever as a novel target for chemotherapies [15,16]. Cytochrome c is a mitochondrial protein that can activate caspases. However, the release of cytochrome c is regulated by the anti- and pro-apoptotic members of the Bcl-2 family. Once in the cytoplasm, it binds Apaf-1 to pro-caspase-9, leading to the activation of caspase-9 and to initiation of the caspase cascade [17]. Many parameters of mitochondrial physiology have been shown to be hallmarks of apoptosis. These include the loss of mitochondrial membrane potential (∆Ψm), the generation of ROS (reactive oxygen species), the termination of oxygen consumption, and the release of cytochrome c [18].

In this report, we investigate the mechanisms of isocostunolide on the induction of the apoptosis effect in human melanoma A2058 cells. We perform experiments to evaluate whether the anti-proliferative effect of isocostunolide in A2058 cells was mediated through apoptosis, and our results also indicate that isocostunolide can indeed promote apoptosis in A2058 cells via mitochondrial membrane depolarization and activation of the caspase cascade.

2. Materials and methods

2.1. Extraction and isolation

The roots of *I. helenium* (0.8 kg, dried weight) were purchased from a medicinal herbs market in Taipei, in May 2004. The herb was extracted with MeOH (3×5.0 L) at 25–27 °C for 2 weeks. After concentration of the combined extracts under reduced pressure, the residue (107.0 g) was suspended in H2O and then extracted with *n*-hexane, EtOAc, CHCl3, and *t*-butanol, respectively. The *n*-hexane extract (52.0 g) was chromatographed over a silica gel column and eluted with an *n*-hexane–EtOAc gradient system (5:0, 4:1, 3:2, 2:3, 1:4, 0:5, then with pure MeOH) to afford seven (F1–F7) fractions. These fractions were assayed on human melanoma A2058 cell proliferation, and the active fractions (F2) were purified again by silica gel column and eluted with *n*-hexane–EtOAc gradient system (19:1, 18:2, 17:3, 16:4, and 15:5). These eluted fractions were screened again for their inhibitory activity on A2058 cells proliferation. The active fractions were purified by HPLC (Phenomenex Luna C-18 silica gel column [10.0×250 mm], eluting with a mixture of MeOH and H2O [80:20, v/v]). Fractions of retention times at 9.5 min containing isocostunolide were collected.

2.2. Cell culture and cell viability assay

Human melanoma cells (A2058), human hepatoma cells (HepG2), and human colon adenocarcinoma cells (HT-29) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS), 1% dilution of the penicillin–streptomycin, and 2 mM glutamine and were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cells (1×106 per dish) were cultured in a 100-mm dish and incubated for 14 h before being treated with various concentrations of isocostunolide (0.625, 1.25, 2.5, 5.0, and 7.5 μg/mL) for 48 h. Isocostunolide was a small compound (MW = 233.1). When these values are converted to micromolar, they are 0, 2.7, 5.4, 10.7, 16.1, 21.5, and 32.2 μM, respectively. Cells were counted, and cell viability was determined by a trypan blue exclusion assay.

2.3. Analysis of the cell cycle

A2058 cells (1×106) in a 100-mm dish were treated with various concentrations of isocostunolide (0.625, 1.25, 2.5, and 5.0 μg/mL) for 48 h or with a fixed concentration of isocostunolide (7.5 μg/mL) for 0, 3, 6, 12, and 24 h. Cells were trypsinized and collected with ice cold PBS. The cells were resuspended in 200 μL PBS and fixed by adding 800 μL of iced 100% ethanol then incubated overnight at −20°C. The cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.1% Triton X-100 in PBS and 1 μg/mL RNase A), and incubated at 4°C for 30 min. Then, 1 mL of PI solution (50 μg/mL) was added, and the mixture was allowed to stand at 4°C for 30 min. Cellular DNA content was then analysed by FACScan cytometry (Becton Dickinson).

2.4. Annexin V staining

Double staining for Annexin-V-fluorescein isothiocyanate (FITC) binding and for cellular DNA using PI was performed.
A2058 cells were plated at a density of $3 \times 10^5$ cells/well on six-well plates. After treatment with the isocostunolide (7.5 µg/mL) for 0, 2, 4, and 6 h, cells were trypsinized and collected in ice cold PBS. The cells were washed twice with cold PBS and resuspended in 1 mL binding buffer (BD PharMingen, San Diego, CA). They were then stained with Annexin-V-FITC and PI according to the protocol from BD PharMingen. Briefly, 2 µL of Annexin-V-FITC (50 µg/mL) and 1 µL of PI (500 µg/mL) were added to a 100 µL solution of cells and the solution was incubated for 15 min at room temperature in the dark. Binding buffer was then added, and early and late apoptosis was visualized by constructing a dot-plot using a Becton Dickinson FACScan. Green fluorescence from the Annexin-V-FITC was determined using an FL1 detector having a bandpass filter with specifications 530 ± 15 nm. Red fluorescence from PI was determined using an FL2 detector having a bandpass filter with specifications 585 ± 21 nm. A total of 10,000 events were recorded for each sample.

2.5. Assays of caspase inhibitors

A2058 cells (1 × 10^6) in a 100-mm dish were treated with or without 50 µM of general caspase inhibitor (Z-VAD-FMK) or a specific caspase-3 inhibitor (Z-DEVD-FMK). After 2 h, cells were treated with isocostunolide (3.75 µg/mL) for 24 h. Cellular morphology of A2058 was analysed by phase contrast microscopy.

2.6. Activity of caspase

Cells were collected and washed with PBS and suspended in a buffer containing 25 mM HEPES (pH 7.5), 5 mM MgCl2, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethylsulfonylfluoride, 10 µg/mL pepstatin A and 10 µg/mL leupeptin. After treatment, cell lysates were clarified by centrifugation at 13,200 g for 20 min at 4 °C. Caspase activity in the supernatant was determined by a fluorogenic assay (R&D Caspase-3 Fluorometric Assay System). Briefly, 100 µg of total protein, as determined by 7-amino-4-trifluoromethyl coumarin (AFC) assay (R&D), was incubated with 50 µM substrate DEVD-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) at 37 °C for 1 h. The release of AFC was measured by excitation at 400 nm and emission at 505 nm using a fluorescence spectrophotometer (Hitachi F-4500).

2.7. Measurement of mitochondrial membrane potential ($\Delta \Psi_m$)

Measurement of the mitochondrial membrane potential was assayed by flow cytometry using the dye DiOC6 (Molecular Probes). A2058 cells were plated at a density of $3 \times 10^5$ cells/well on six-well plates. After treatment with isocostunolide (7.5 µg/mL) for 0, 0.5, 1.0, 2.0, 4.0, and 6.0 h or treatment with various concentrations (0.625, 1.25, 2.5, 5.0, and 7.5 µg/mL) for 24 h, cells were trypsinized and collected in ice cold PBS. DiOC6 (100 nM) was loaded into cells suspended in 0.5 mL fresh DMEM and incubated at 37 °C for 15 min. DiOC6 fluorescence was determined at 530 ± 30 nm. Data were obtained and analyzed with the CellQuest software on a Becton Dickinson FACScan.

2.8. Measurement of reactive oxygen species (ROS)

Flow cytometric determination of the ROS production was carried out as described previously. A2058 cells were collected by trypsinization and 200 µL of cell suspension (1 × 10^6 cells/mL) was mixed with 800 µL PBS. Cells were incubated with 10 µM 5-(and-6)-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate, acetyl ester (Molecular Probes) for 15 min, followed by the addition of different concentrations of isocostunolide (0, 2.5, 5.0, and 7.5 µg/mL) or hydrogen peroxide (30 µM). The incubation was continued for 20 min at 37 °C. The oxidative burst (hydrogen peroxide) was detected using a FACScan flow cytometer with excitation and emission settings of 488 and 530 nm, respectively.

2.9. Western blotting assay

A2058 cells on 100-mm dishes (1.5 × 10^6/dish) were treated with isocostunolide at 1.25, 2.5, 5.0, and 7.5 µg/mL for 48 h. After treatment, cells were collected and resuspended in 100 µL Gold lysis buffer. Equal amounts of
proteins (30 μg), were mixed with 2× sample buffer and resolved by 10% SDS-PAGE for β-actin, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bid, pro-caspase-3, pro-caspase-8, Fas, and cytochrome c detection. Proteins were electrotransferred to an immobilon membrane (PVDF; Millipore Corp.), and equivalent protein loading was verified by staining the membrane with reversible dye amido black (Sigma Chemical Co.). This was followed by overnight blocking with a solution composed of 20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, and 3% BSA. Specific antibodies used were anti-human PARP (1:500 of rabbit polyclonal; Santa Cruz Biotechnology, Inc.), anti-caspase-3, Fas, and anti-caspase-8 antibodies (1:1000 of mouse monoclonal; Pharmingen, Becton Dickinson), anti-Bcl-2 and anti-Bid antibodies (1:1000 of mouse monoclonal; Santa Cruz Biotechnology, Inc.), anti-β-actin antibody (1:3000 of mouse monoclonal; Cashmere Biotech), and cytochrome c antibody (1:1000 of mouse monoclonal; Research Diagnostic, Inc). These proteins were detected by chemiluminescence (ECL, Amersham).

2.10. Cytochrome c release

Mitochondrial and cytosolic fractions were prepared by resuspending cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/mL phenylmethylsulfonylfluoride (PMSF), 8 μg/mL aprotinin, and 2 μg/mL leupeptin, pH 7.4). Cells were passed through a needle 10 times. Unlysed cells and nuclei were pelleted by centrifugation for 10 min at 750×g. The supernatant was then centrifuged at 1,00,000×g for 15 min. This pellet was resuspended in buffer A and represented the mitochondrial fraction. The supernatant was again centrifuged at 1,00,000×g for 1 h. The supernatant from this final centrifugation step represented the cytosolic fraction.

3. Results

3.1. Purification and identification of isocostunolide

Isocostunolide (Fig. 1) was isolated through repeated chromatographies of the MeOH extract of I. helenium under the guidance of the anti-proliferation activity on A2058 cells. Final purification of the active fraction was achieved by HPLC on a reversed-phase C18 column. The total content of the active component isocostunolide was roughly 1.8% of the MeOH extract from I. helenium. The 1H and 13C NMR (in DMSO-d6, Tables 1 and 2) spectra of the purified compound displayed characteristic absorptions of sesquiterpene lactones and subsequently was found identical to the well-known compound, isocostunolide. The molecular formula for isocostunolide was deduced as C15H20O2 on the basis of FAB-MS (positive mode), which showed a quasimolecular ion peak at m/z 233.1 ([M+C]H). Upon comparison of the 13C NMR of isocostunolide with the reported data of costunolide (Fig. 1), the identity of both compounds could be recognized. The carbon signals of isocostunolide were very similar to those of costunolide, as shown in Table 2, except for the 2nd and 10th positions. The bioactivities of isocostunolide have never been reported, and its chemical shifts of proton and carbon (including HMBC, HMQC, NOESY, and COSY) were only partially assigned. Consequently, the stereostructure of isocostunolide was established.

### Table 1

<table>
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<tr>
<th>Position</th>
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<th>HMBC correlations (C #)</th>
<th>NOESY correlations (H #)</th>
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<tr>
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<td>CH2 2.1 (m)</td>
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<tr>
<td>6</td>
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<td>2, 15</td>
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<td>CH 4.74 (d, 4.9)</td>
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<tr>
<td>8</td>
<td>CH 2.65 (s)</td>
<td>7, 10</td>
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<td>9</td>
<td>CH3 1.68 (m)</td>
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<td>10</td>
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<td>8, 9, 14</td>
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<td>CH2 5.67 (d, 3.3)</td>
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<td>14</td>
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<tr>
<td>15</td>
<td>CH3 1.36 (s)</td>
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<td>3, 4, 7, 9</td>
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</table>

Assignments were based on the HMBC and HMQC NMR data.

### Table 2

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<th>Carbon no.</th>
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<th>Costunolide in CDCl3, δC</th>
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</tr>
<tr>
<td>15</td>
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Assignments were based on the HMBC and HMQC NMR data.

α Data taken from Ref. [35].
Fig. 2. Effect of isocostunolide on the viability of cancer cell lines. (a) Cytotoxic activity of isocostunolide in various human cancer cell lines (A2058, HepG2, and HT-29). Cells (1 × 10^6 per dish) were cultured in 100-mm dish and incubated for 14 h before being treated with various concentrations of isocostunolide (0.625, 1.25, 2.5, 5.0, and 7.5 µg/mL) for 48 h. Treated cells were counted and viability was determined by a trypan blue exclusion assay. Results were from three repeats. (b) Phase-contrast micrographs of A2058 cells treated with various concentrations of isocostunolide (7.5 µg/mL) for 48 h. A representative experiment of three repeats was shown. (c) A2058 cells were treated with various concentrations of isocostunolide (2.5–10.0 µg/mL) for 48 h and subsequent cell viability was determined by MTT assay.
3.2. Inhibition of cell growth by isocostunolide

The cytotoxic effects of isocostunolide on three different cancer cell lines were investigated. All cancer cell lines were treated with various concentrations (0.63–7.5 µg/mL) of isocostunolide. After 48 h of treatment, the number of live cells was measured by a trypan blue exclusion assay. As shown in Fig. 2a, all three cancer cell lines (A2058, HT-29, and HepG2) were sensitive to isocostunolide, with an IC50 of 3.2, 5.0 and 2.0 µg/mL, respectively.

3.3. Isocostunolide induces apoptotic cell death in A2058 cells

During cell viability assay, we demonstrated that isocostunolide significantly induces cell death in A2058. Morphological features, such as cell shrinkage and plasma membrane blebbing, were seen in isocostunolide-treated cells. As shown in Fig. 2b, it appears that the cells might have died through the induction of apoptosis. The cytotoxic effect of isocostunolide was further shown to be evaluated dose-dependent by MTT assay as shown in Fig. 2c. To demonstrate whether isocostunolide induced apoptosis in A2058 cells, we used annexin V-staining and flow cytometry (Fig. 3(a)) to evaluate whether isocostunolide-caused cell death occurs via apoptosis. Cells were treated with isocostunolide (7.5 µg/mL) for

Fig. 3. Isocostunolide-induced apoptosis in A2058 cells. (a) Flow cytometric analysis of isocostunolide-treated A2058 cells after fluorescinated anti-Annexin V monoclonal antibody binding (FL1-H) and propidium iodide uptake (FL2-H). Cells were treated with 7.5 µg/mL of isocostunolide for 0, 2, 4, and 6 h and subsequently measured by flow cytometry. The lower right quadrant represents early apoptosis; the upper right quadrant represents late apoptosis. (b) Dose-dependent effect of isocostunolide on cell cycle and sub-G1 phase population. Cells were treated with various concentrations (0.63–5.0 µg/mL) of isocostunolide for 48 h and stained with PI as described in Section 2. Following flow cytometric analysis, the cellular DNA profile was analyzed by Cell Quest software. (c) Time-course effect of isocostunolide on cell cycle and sub-G1 phase population. A2058 cells were treated with concentration of 7.5 µg/mL for 0, 3, 6, 12, and 24 h. Results were from one representative experiment of three repeats.
Fig. 3 (continued)

Isocostunolide (µg/mL)

M1 (Sub-G1): 1.21
M2 (G0/G1): 55.57
M3 (S): 18.76
M4 (G2/M): 24.76

Sub-G1: 2.25
G0/G1: 56.59
S: 20.0
G2/M: 21.42

Sub-G1: 2.58
G0/G1: 51.74
S: 20.53
G2/M: 25.35

Sub-G1: 5.83
G0/G1: 44.27
S: 23.18
G2/M: 26.83

Sub-G1: 16.27
G0/G1: 38.32
S: 25.16
G2/M: 20.59

DNA content
Fig. 3 (continued)

DNA content

Fig. 3 (continued)
0, 2, 4, and 6 h, respectively. Our result indicated a time-course dependent increase (0.52–11.47%) in apoptotic cell population following isocostunolide treatment. The induction of apoptosis was further confirmed by the presence of the sub-G1 cell population in flow cytometry. The DNA content of A2058 cells, treated with isocostunolide at various concentrations (0, 0.63, 1.25, 2.5, and 5.0 \( \mu \text{g/mL} \)) for 48 h (Fig. 3b), or treated with a fixed concentration of isocostunolide (7.5 \( \mu \text{g/mL} \)) for 0, 3, 6, 12, and 24 h, was analyzed by flow cytometry as shown in Fig. 3c. The respective percentages of apoptotic cells observed after treatment with 0, 0.63, 1.25, 2.5, and 5.0 \( \mu \text{g/mL} \) of isocostunolide for 48 h were as follows: 1.21, 2.25, 2.58, 5.83 and 16.27%. The time course induction of apoptosis in A2058 cells by isocostunolide at a fixed concentration of 7.5 \( \mu \text{g/mL} \) was also shown (Fig. 3c). These results show that isocostunolide at a concentration of 5.0 \( \mu \text{g/mL} \) for 48 h or treated with isocostunolide at 7.5 \( \mu \text{g/mL} \) for 12 h was sufficient to induce apoptosis in A2058 cells.

### 3.4. Isocostunolide induces apoptosis via activation of caspase-dependent pathway

Initiator caspases (including 8, 9, 10, and 12) are closely coupled to pro-apoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (including 3, 6, and 7), which in turn cleave cytoskeletal and nuclear proteins, such as PARP and lamin A, and finally induce apoptosis. These pro-apoptotic stimuli include FasL, TNF-\( \alpha \), DNA damage, and ER stress. We first evaluated whether caspase-dependent signal pathways were involved in the apoptotic cell death induced by isocostunolide in A2058 cells. Treatment of A2058 cells with 7.5 \( \mu \text{g/mL} \) for 3, 6, 12, and 24 h or treatment with various concentrations of isocostunolide for 24 h resulted in dramatic cleavage of PARP and markedly decreased pro-caspase-3 protein levels (Fig. 4a). Because caspase-8 and Fas play an important role both in the mitochondria dependent and independent pathway in apoptosis, we thus...
further evaluated whether caspase-8 might be activated during the induction of apoptosis by isocostunolide, as shown in Fig. 4b. Our data indicated that isocostunolide 1.25–5.0 μg/mL markedly increased Fas protein expression (Fig. 4c). All these results indicate the PARP, pro-caspase-8, and pro-caspase-3 expression fell markedly under treatment with isocostunolide at a concentration of 5.0–7.5 μg/mL. However, the level of Fas was increased markedly in a dose-dependent manner. To ask whether a particular caspase plays the crucial role in isocostunolide-induced apoptosis, two caspase inhibitors were used to address this question. A2058 cells were pre-treated with general caspase inhibitor (Z-VAD-FMK) or a
specific caspase-3 inhibitor (Z-DEVD-FMK), then treated with isocostunolide (3.75 μg/mL) for 24 h. Our data indicate that not only the general caspase inhibitor markedly inhibits isocostunolide-induced cell death but also caspase-3 inhibitor as shown in Fig. 4d. Activation of caspase-3 has been known to play an important role in the induction of apoptosis by various stimuli. Upon treatment of A2058 cells with 7.5 μg/mL isocostunolide, the activity of caspase-3 increased significantly within 4 h after the start of treatment (Fig. 4e). The results obtained from these experiments suggest that caspase-3 is involved in isocostunolide-induced apoptosis of A2058 cells.

3.5. Mitochondria-dependent pathway was activated during isocostunolide-induced apoptosis

Cytosolic Bid is cleaved by caspase-8 and myristoylated before translation into mitochondria during mitochondria-dependent pathway of apoptosis. To determine if this pathway is activated by isocostunolide treatment, we next examined the Bid protein level after isocostunolide (7.5 μg/mL) treatment with various concentrations (1.25, 2.5, 5.0, and 7.5 μg/mL) for 24 h (Fig. 5a) or treatment with 3, 6, 12, and 24 h (Fig. 5b) in A2058 cells. The results indicate that Bid was increased transiently at 6 h after treatment with isocostunolide and cleaved markedly later on 12–24 h. Another Bcl-2 family protein, Bcl-2 was significantly suppressed protein expression by treated with isocostunolide (7.5 μg/mL) in a time-course manner (Fig. 5c). Our data also indicated that cytochrome c was markedly released from mitochondria into the cytoplasm in a dose-dependent manner (Fig. 5d). Taken together, these results suggest convincingly that the isocostunolide-induced apoptosis of A2058 cells may be through the mitochondria-dependent pathway, as evidenced by the activation of caspase-8, Bid, and caspase-3.

3.6. Mitochondrial membrane depolarization by isocostunolide

To further demonstrate the induction of apoptosis by isocostunolide, the mitochondrial membrane depolarization was examined by using DiOC_6. The A2058 cells were treated with isocostunolide at a concentration (7.5 μg/mL) for 0.5, 1.0, 2.0, 4.0, and 6.0 h (Fig. 6a). Our results indicate that isocostunolide induced mitochondrial membrane depolarization in a time course-dependent manner (14.1–55.8%). Fig. 6b shows the dose-dependency of the induction of mitochondrial membrane depolarization effects in A2058 cells treatment with isocostunolide concentrations of 0.63–7.5 μg/mL. These results clearly show that isocostunolide is an efficient inducer of A2058 cells mitochondrial membrane depolarization.

Fig. 5. Induction of apoptosis via mitochondria-dependent pathway and activation of Bid, and induction of cytochrome c release from mitochondria into cytosol. A2058 cells were treated with various concentrations of isocostunolide (1.25–7.5 μg/mL) for 48 h, or treated with isocostunolide (7.5 μg/mL) for 3, 6, 12, and 24 h. Cell lysates were prepared and subjected to SDS-PAGE and immunoblotting using respective specific antibodies. Dose-dependent changes of Bid (a), time course-dependent change of Bid (b), time course-dependent change of Bcl-2 (c), dose-dependent release of cytochrome c from mitochondria (d).
3.7. Mitochondrial membrane depolarization by isocostunolide treatment was not accompanied by the production of intracellular reactive oxygen species (ROS)

Many reports have implicated that intracellular ROS are involved in the signal transduction pathways of apoptosis. In this study, we evaluated whether the induction of mitochondrial membrane depolarization induced by isocostunolide occurred via modulated production ROS. We measured the production of ROS after treatment with various concentrations of isocostunolide (0, 2.5, 5.0, and 7.5 μg/mL) or of hydrogen peroxide (30 μM, as a positive control) for 15 min. The oxidative burst was detected by a FACScan flow cytometry as shown in Fig. 7. Our results showed that the production of ROS increased with increasing concentrations of isocostunolide and hydrogen peroxide.

![Graph showing the production of ROS](image)

Fig. 6. Analysis of mitochondrial membrane depolarization in A2058 cells treated with isocostunolide in a time course- and dose-dependent manner. (a) Percentage of cells (of 10,000 analyzed) with depolarized ΔΨm was measured after treatment with isocostunolide (7.5 μg/mL) for 0.5, 1.0, 2.0, 4.0, and 6.0 h. (b) The same conditions prevailed for measuring the membrane depolarization assays in a dose-dependent manner. A2058 cells were treated with various concentrations of isocostunolide (0.63–7.5 μg/mL) for 24 h. The fluorescence dye (DiOC6) in the flow cytometric analysis of the mitochondrial membrane depolarization was used. Detail procedures were as described in Section 2. Results were from the representative experiment of three repeats.
results indicate that isocostunolide did not elicit the production of intracellular ROS during membrane depolarization.

4. Discussion

In this study, we evaluated the inhibitory effect of isocostunolide on the proliferation of embryonic fibroblast and cancer cells. We show that isocostunolide, a sesquiterpene lactone isolated from *I. helenium*, exerted a more marked cytotoxic effect at 2.5–5.0 μg/mL or less against three different cancer cell lines (A2058, HT-29, and HepG2) than embryonic fibroblast cells (data not shown). Our result suggests that isocostunolide’s anti-proliferative effect is mediated through apoptosis that involves caspase activation. No biological activities of this compound have ever been reported. Traditionally, many plants contain high levels of sesquiterpene lactones have been used as folk medicines because of their exerted many pharmacologic properties. For example, its ingredient parthenolide was reported to be capable of inhibiting DNA
binding of transcription factors NF-κB and STAT-3 and to reduce MAP kinase activity and the reactive oxygen species (ROS) generation [19–21]. Artemisinin, a sesquiterpene lactone with an endoperoxide group, has been used as an anti-malarial drug [22]. Costunolide, a naturally occurring sesquiterpene lactone, was reported to strongly inhibit human breast cancer MCF-7 cell growth [23]. Another report demonstrated that costunolide suppresses gene expression of hepatitis B virus surface antigen in human hepatoma cells [11].

Two major apoptotic pathways have been identified: (1) ‘intrinsic or mitochondrial’ and (2) ‘extrinsic or death receptor-related’ [24,25]. The intrinsic pathway involves the cell oxidative stress that triggers the mitochondria-dependent pathway, resulting in induction of cytochrome c release from mitochondria into the cytosol and activation of caspase-9. The extrinsic pathway is triggered by the binding of ligands such as FasL and TNF with their receptors and the recruitment of adaptor proteins such as FADD, followed by activation of caspase-8. In this study, we show that isocostunolide-induced apoptosis in A2058 cells might have up-regulating Fas expression and activation of both caspase-8 and Bid. These results suggest that isocostunolide-induced apoptosis in A2058 cells might occur via the cooperative effects of both an ‘extrinsic’ and an ‘intrinsic’ pathway.

Mitochondria are important in energy production, cellular calcium homeostasis, generation of ROS and capacity to release apoptogenic proteins (members of the Bcl-2 family) [26,27]. Several factors can stimulate mitochondria-mediated apoptosis, these factors include DNA damaging agents, UV, activation of tumor suppressors, and chemotherapeutic agents [28–30]. The Bcl-2 family of proteins, as the pivotal regulators of the mitochondrial apoptotic pathway can either induce or inhibit the change of mitochondrial membrane potential and the release of cytochrome c from mitochondria to cytosol [31,32]. Our results indicate that isocostunolide can decrease mitochondrial membrane potential, and this decrease may be modulated via activation of Bid and down-regulation of Bcl-2 protein expression.

ROS have been implicated as second messengers in multiple signaling pathways [33]. In our study, we have observed no increasing the level of ROS due to isocostunolide exposure in A2058 cells. Many natural products, such as tea polyphenol EGCg [34], which can induce apoptosis of cancer cells via production of ROS and induction of mitochondrial membrane depolarization. However, in our studies, isocostunolide did not seem to induce a mitochondrial membrane depolarization effect through the generation of ROS. Taken together, our data suggest that isocostunolide initiates...
cell death through caspase- and mitochondria-dependent pathways in A2058 cells. Modulation of Bid or Bcl-2 lead to changes in the pores of mitochondrial membrane to facilitate the release of cytochrome c from mitochondria to cytosol, where it triggers the formation of apoptosome, a multimeric molecular complex containing Apaf-1 (apoptotic protease activating factor-1), ATP, and cytochrome c. The apoptosome in mammalian cells initiates the recruitment and activates caspase-9, which in turn targets and activates caspase-3, and finally triggering the apoptotic signaling cascade.

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References


