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5-*epi*-Sinuleptolide induces cell cycle arrest and apoptosis through tumor necrosis factor/mitochondria-mediated caspase signaling pathway in human skin cancer cells

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ABSTRACT

Background: Skin cancers are reportedly increasing worldwide. Developing novel anti-skin cancer drugs with minimal side effects is necessary to address this public health issue. Sinuleptolide has been demonstrated to possess anti-cancer cell activities; however, the mechanisms underlying the anti-skin cancer effects of 5-*epi*-sinuleptolide and sinuleptolide remain poorly understood.
Methods: Apoptosis cell, cell-cycle-related regulatory factors, and mitochondria- and death receptor-dependent caspase pathway in 5-*epi*-sinuleptolide-induced cell apoptosis were examined using SCC25 cells.
Results: 5-*epi*-Sinuleptolide inhibited human skin cancer cell growth more than did sinuleptolide. Treatment of SCC25 cells with 5-*epi*-sinuleptolide increased apoptotic body formation, and induced cell-cycle arrest during the G_2/M phase. Notably, 5-*epi*-sinuleptolide up-regulated p53 and p21 expression and inhibited G_2/M phase regulators of cyclin B1 and cyclin-dependent kinase 1 (CDK1) in SCC25 cells. Additionally, 5-*epi*-sinuleptolide induced apoptosis by mitochondria-mediated cytochrome *c* and Bax up-expression, down-regulated Bcl-2, and activated caspase-9 and -3. 5-*epi*-Sinuleptolide also up-regulated tBid, which is associated with up-regulation of tumor necrosis factor-α (TNF-α) and Fas ligand (FasL) and their cognate receptors (*i.e.*, TNF-RI, TNF-R2 and Fas), downstream adaptor TNF-R1-associated death domain (TRADD) and Fas-associated death domain (FADD), and activated caspase-8 in SCC25 cells.
Conclusions: The analytical results indicate that the death receptor- and mitochondria-mediated caspase pathway is critical in 5-*epi*-sinuleptolide-induced apoptosis of skin cancer cells.
General significance: This is the first report suggesting that the apoptosis mediates the anti-tumor effect of 5-*epi*-sinuleptolide. The results of this study might provide useful suggestions for designing of anti-tumor drugs for skin cancer patients.

Abbreviations: 5ES, 5-*epi*-sinuleptolide; 5-Fu, 5-fluorouracil; Apaf-1, apoptotic protease activating factor-1; BCC, basal cell carcinoma; CDK1, cyclin-dependent kinase 1; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; FADD, Fas-associated death domain; FasL, Fas ligand; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMSC, non-melanoma skin cancers; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SCC, squamous cell carcinoma; tBid, truncated form Bid; TNF-R, TNF-receptor; TNF-α, tumor necrosis factor-α; TRADD, TNF-R1-associated death domain

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1. Introduction

The incidence of melanoma and non-melanoma skin cancers (NMSCs), including basal cell carcinoma (BCC) and squamous cell cancer (SCC), is increasing worldwide [1]. More than 1 million skin cancers are diagnosed annually in the United States—80% of which are BCC, 16% are SCC, and 4% are melanomas—making skin cancer by far the most common cancer [2]. Notably, SCC is known to metastasize and grow more rapidly than the more common BCC, and SCC of the skin is less common than BCC, but is more aggressive and has a higher mortality rate [3].

Various modalities for skin cancer treatment have been described, including cryotherapy, curettage and cautery, surgical excision, laser ablation, photodynamic therapy, topical 5-fluorouracil (5-Fu), imiquimod, and dislofenac [4,5]. However, 5-Fu resistance during the treatment course has become common and is an important cause of failure for cancer therapies. Although topical 5-Fu, imiquimod, and dislofenac therapies are effective, successful treatment is inevitably associated with substantial pain, pruritus, burning, erythema, erosion, and scar formation on lesion sites and peripheral normal skin [5]. Therefore, effective chemotherapy agents that have minimal side effects are required to address this public health issue.

Several investigations have demonstrated that death of cancer cells induced by chemotherapeutic drugs occurs by growth arrest and apoptosis [6]. Deregulation of the cell cycle is one of the most frequent alterations during tumor development. Blocking the cell cycle is regarded as an effective strategy for eliminating cancer cells [7]. Apoptosis, a common form of cell death, involves many such factors as intrinsic and extrinsic apoptosis signaling in cells [8]. The intrinsic mitochondria-mediated apoptotic pathway plays a crucial role in regulating cell death, which is often the main target for apoptosis induction by chemotherapeutic drugs. Disruption of mitochondria membrane integrity results in the release of cytochrome *c* from mitochondria into cytosol, which together with the apoptotic protease activating factor-1 (Apaf-1) activates caspase-9 in the presence of deoxyadenosine triphosphate (dATP), and then activates effector caspase-3 and cell death [9]. Additionally, members of the Bcl-2 family, such as Bcl-2 and Bax, can both negatively and positively regulate mitochondrial events of apoptotic cell death by suppressing cytochrome *c* expression [10]. Several studies have shown that chemotherapeutic-agent-induced apoptosis involves cleavage of cytosolic Bid by caspase-8 into truncated form Bid (tBid). Notably, tBid can induce pro-apoptotic Bax, loss of mitochondrial membrane potential, and cytochrome *c* release [11]. The extrinsic apoptotic pathway is mediated by the tumor necrosis factor (TNF) family, including FasL (CD95L) and TNF-α, which interact with TNF-receptor (TNF-R) family molecules Fas (CD95), TNF-R1 and TNF-R2, respectively. The ligand binding of the receptor induces intracellular activation of the caspase system, leading to apoptotic cell death [12].

The marine environment, comprising approximately half of the world's biodiversity, is an enormous source of novel and biologically active compounds [13]. Most natural marine products and their derivatives are generated by invertebrates, such as soft corals, sponges, tunicates, mollusks, and bryozoans, and are currently undergoing advanced preclinical evaluations [14]. The *Sinularia* genus is known for its versatile chemical constituents and their biological activity [13,14]. Sinuleptolide, the norditerpene extracted from *Sinularia* sp., inhibited lipopolysaccharide-induced TNF-α and nitric oxide production by murine macrophage-like RAW264.7 cells [15]. A previous study demonstrated the cytotoxic activity of sinuleptolide from *S. leptoclados* against growth of human oral epidermoid carcinoma KB cells and human liver carcinoma Hepa59T/VGH cells (ED_{50} 2.3–2.6 μg/ml) [16,17]. However, the mechanisms and cytotoxic effects underlying the anti-skin cancer effects of 5-*epi*-sinuleptolide and sinuleptolide remain poorly understood. This study is the first to assess the cell growth inhibition activity of 5-*epi*-sinuleptolide and sinuleptolide and examine their effect on cell cycle distribution and apoptosis in human skin cancer cells.

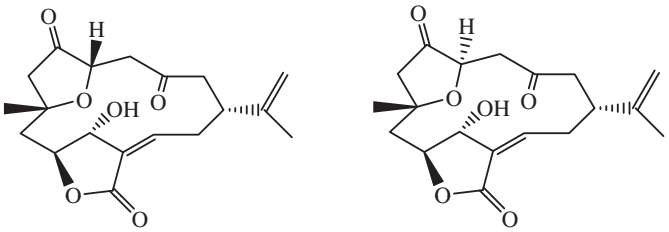
2. Materials and methods

2.1. Reagents and cell lines

5-*epi*-Sinuleptolide and sinuleptolide were isolated from *Sinularia leptoclados* and identified by Jyh-Horng Sheu, National Sun Yat-Sen University, Kaohsiung, Taiwan. Fig. 1 shows chemical structures of 5-*epi*-sinuleptolide and sinuleptolide. ^{1}H NMR, ^{13}C NMR and ESI-MS spectrums of 5-*epi*-sinuleptolide and sinuleptolide have been shown in supplement data 1 and 2. 5-*epi*-Sinuleptolide and sinuleptolide were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. Stock solution was diluted to the desired final concentrations with growth medium immediately before use. The preferential caspase-8 inhibitor z-IETD-FMK, caspase-9 inhibitor z-LEHD-FMK, and caspase-3-like inhibitor z-DEVD-FMK, added to a final concentration of 200 μM, were used 1 h before 5-*epi*-sinuleptolide exposure (R&D Systems, Inc., Palo Alto, CA, USA). Human epidermoid carcinoma A431, human skin basal cell carcinoma BCC, human head and neck squamous cell carcinoma SCC9 (moderate differentiation) and SCC25 (well differentiation), and nontransformed human skin fibroblasts Hs68 cells were purchased from the American Type Culture Collection (Rockville, Maryland). Human premalignant keratinocytic HaCaT cells were kindly supplied by Prof. Hamm-Ming Sheu (National Cheng Kung University Medical College, Tainan, Taiwan). Cells were cultured in medium supplemented with 10% fetal bovine serum (FBS) (Hazelton Product, Denver, PA, USA) and 1% penicillin–streptomycin at 37 °C in 5% CO_2; specifically, A431, HaCaT and Hs68 cells were cultured in Dulbecco's modified Eagle medium (DMEM), and SCC9 and SCC25 cells were cultured in DMEM/F12 medium supplemented with 0.4 μg/ml hydrocortisone (GIBCO, Grand Island, NY).

2.2. Cell viability and morphology change assay

Cells (1×10^5 cells/ml) were seeded in 96-well flat-bottomed plates (Corning, Elmira, NY) for 24 h and then treated with serial concentrations of agents in 100 μl of medium for 72 h. The control groups were treated with DMSO and the final DMSO concentration did not exceed 0.1%. Following treatment, the medium was refreshed. Cell viability was determined by colorimetric tetrazolium MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Promega, Madison, WI) and absorbance was measured at 570 nm (BioTek, Synergy™2). Values are expressed as the percentage of mean cell viability relative to that of the vehicle controls (DMSO). 5-*epi*-Sinuleptolide and sinuleptolide concentrations that inhibited cell growth by 20% (IC_{20}), 50% (IC_{50}) and 80% (IC_{80}) were calculated. All determinations were performed in quaternary and statistically analyzed using the Student *t*-test. For morphological analysis, following incubation various concentrations of 5-*epi*-sinuleptolide for 72 h, the SCC25 cells in each well were washed once with 1× phosphate-



5-*epi*-sinuleptolide sinuleptolide

Fig. 1. Chemical structures of 5-*epi*-sinuleptolide and sinuleptolide.