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Haplophytin-A induces caspase-8-mediated apoptosis via the formation of death-inducing signaling complex in human promyelocytic leukemia HL-60 cells

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ABSTRACT

Haplophytin-A (10-methoxy-2,2-dimethyl-2,6-dihydro-pyrano[3,2-c]quinolin-5-one), a novel quinoline alkaloid, was isolated from the *Haplophyllum acutifolium*. In this study, we investigated the effect of haplophytin-A on the apoptotic activity and the molecular mechanism of action in human promyelocytic leukemia HL-60 cells. Treatment with haplophytin-A (50 μM) induced classical features of apoptosis, such as, DNA fragmentation, DNA ladder formation, and the externalization of annexin-V-targeted phosphatidylserine residues in HL-60 cells. In addition, haplophytin-A triggered the activations of caspase-8, -9, and -3, and the cleavage of poly (ADP-ribose) polymerase (PARP) in HL-60 cells. In addition, haplophytin-A caused the loss of mitochondrial membrane potential ($\Delta\Psi_m$) and the release of cytochrome *c* and Smac/DIABLO to the cytosol, and modulated the expression levels of Bcl-2 family proteins. We further demonstrated that knockdown of caspase-8 using its siRNA inhibited the mitochondrial translocation of tBid, the activations of caspase-9 and caspase-3, and subsequent DNA fragmentation by haplophytin-A. Furthermore, haplophytin-A-induced the formation of death-inducing signaling complex (DISC) and then activated caspase-8 in HL-60 cells. During haplophytin-A-induced apoptosis, caspase-8-stimulated tBid provide a link between the death receptor-mediated extrinsic pathway and the mitochondria- mediated intrinsic pathway. Taken together, these results suggest that the novel compound haplophytin-A play therapeutical role for leukemia via the potent apoptotic activity through the extrinsic pathway, involving the intrinsic pathway.

1. Introduction

Leukemia is one of the most common malignant diseases, and because chemotherapy frequently causes resistance and is associated with side effects, effective therapeutic agents are urgently needed for the treatment of leukemia [1,2]. Acute myeloid leukemia (AML) is the most common form of acute leukemia that affects adults, and its incidence increases with age. Furthermore, overall performance status and chemotherapeutic tolerance are usually poor in elderly patients [3]. Over the last decade AML treatments have not been substantially improved [4], and thus, alternative therapeutic strategies with lower toxicities are urgently required [5]. The final goal of most anti-cancer drugs is tumor cells eradication by apoptosis [6,7], and it has been known that apoptosis is regulated by extrinsic and intrinsic pathways [8,9]. The extrinsic pathway is initiated via the stimulation of death receptors located on cell membranes; these receptors include, Fas, death receptor 4/5 (DR4/5), and tumor necrosis factor receptor 1 (TNFR1) [10]. On the other hand, the intrinsic pathway regulates the apoptosis via a convergence of signals at mitochondria. The intrinsic pathway involves the alteration of mitochondrial membrane potentials, the release of cytochrome *c* into the cytosol, and the activation of procapase-9, which is followed by the activations of effector caspases, such as, caspase-3 and -7 [11].

A large number of the chemotherapeutics currently used are derived from bioactive natural products, and this source continues to contribute hugely to drug discovery [12,13]. In particular, camptothecin, a quinoline alkaloid, which is used for colorectal and ovarian cancer was first isolated from *Camptotheca acuminate* [14].

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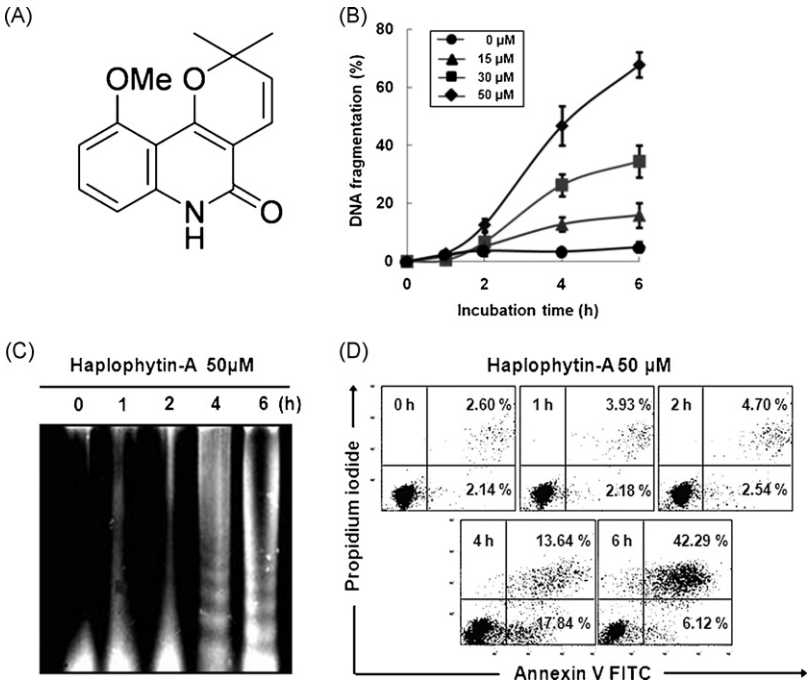

Fig. 1. Effects of haplophytin-A on the apoptosis of HL-60 cells. (A) The chemical structure of haplophytin-A isolated from *Haplophyllum acutifolium*. (B) Cells were treated with various concentrations (15, 30, 50 μM) of haplophytin-A for the indicated times. Extents (%) of DNA fragmentation were determined by fluorometric method using DAPI. (C) Cells were treated with or without 50 μM haplophytin-A for the indicated times. Fragmentation of genomic DNA was extracted and resolved on 2% agarose gels. Apoptotic DNA fragmentation was visualized by ethidium bromide staining. (D) Cells treated with or without 50 μM haplophytin-A for the indicated times were co-stained with PI and FITC-conjugated Annexin V, which specifically detects the translocation of phosphatidylserine (PS). Cells were then examined by flow cytometry.

Furthermore, it has been suggested that quinoline alkaloids inhibit cell proliferation by inducing reactive oxygen species (ROS) and DNA damage, and by disrupting mitochondrial function [15–17]. In a previous study, we isolated haplophytin-A (10-methoxy-2,2-dimethyl-2,6-dihydro-pyrano[3,2-c]quinolin-5-one, Fig. 1A), a novel quinoline alkaloid, from the methanol soluble fraction of *Haplophyllum acutifolium* [18]. No previous study has been performed to investigate the biological activity of haplophytin-A. Here, we describe the apoptotic activity of haplophytin-A in human leukemia cells, and investigate the mechanism involved.

2. Materials and methods

2.1. Materials

Haplophytin-A (Fig. 1A) used for this study was isolated from *H. acutifolium* and structural identities were determined spectroscopically (^{1}H and ^{13}NMR, IR, HR-MS, HMBC) as described previously [18] and were >97% pure by LC–MS. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies (Carlsbad, CA, USA). 4′,6-Diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), RNase, propidium iodide (PI), phenylmethylsulfonylfluoride (PMSF), dithiothreitol were purchased from Invitrogen (Carlsbad, CA, USA). Ethidium bromide (EtBr), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Antibodies for caspase-8, X-linked inhibitor of apoptosis protein (XIAP) and cytochrome *c* were purchased from BD Biosciences (San Diago, CA, USA). Antibodies for caspase-3, Bcl-2, Bax, Bid, poly (ADP-ribose) polymerase (PARP), Fas, Fas L, Fas-associated death domain protein (FADD), second mitochondria-derived activator of caspases/direct IAP binding protein with low PI (Smac/DIABLO), β-actin, α-tubulin and voltage dependent anion selective channel (VDAC) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for caspsae-9 was purchased from Cell Signaling Technology (Beverly, MA, USA) and the peroxidase conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Caspase inhibitors (z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk, and z-VAD-fmk) were purchased from Calbiochem (Bad Soden, Germany). Small interfering RNA (siRNA) and transfection reagents for caspase-8 were purchased from Qiagen (Valencia, CA, USA).

2.2. Cell culture and MTT assay

HL-60 (human promyelocytic leukemia), U937 (human histiocytic lymphoma), KB (human mouth carcinoma), A-549 (human lung adenocarcinoma), HCT-15 (human colon adenocarcinoma) and SK-OV-3 (human ovary adenocarcinoma) were obtained from the Korean Cell Line Bank (KCLB, Seoul, South Korea). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) at 37 °C in an atmosphere of 5% CO_2.

The cell cytotoxicity was estimated by the quantification of the MTT reduction to a blue formazan product by cellular dehydrogenases as previously described [19]. Briefly, the cells (5×10^4) were seeded in each well containing 100 μl of the RPMI medium supplemented with 10% FBS in a 96-well plate. After 24 h, various concentrations of haplophytin-A were added. After 48 h, 50 μl of MTT (5 mg/ml stock solution) was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 100 μl DMSO. The OD was measured at 540 nm.

2.3. Quantification of DNA fragmentation

DNA fragmentation was quantified using DAPI staining and analysis of DNA fragmentation by agarose gel electrophoresis was performed as described previously [20]. In brief, cells were lysed