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HY253, a novel compound isolated from *Aralia continentalis*, induces apoptosis via cytochrome *c*-mediated intrinsic pathway in HeLa cells

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ABSTRACT

This study was aimed to elucidate the novel structure of HY253 isolated from the roots of *Aralia continentalis* and to evaluate its detailed mechanisms on apoptotic induction in HY253-treated HeLa cells. The structure of HY253 was elucidated based on the interpretation of the NMR spectra, as 7,8a-divinyl-2,4a,4b,5,6,7,8,8a,9,9a-decahydro-1H-fluorene-2,4a,4b,9a-tetraol. The TUNEL assay using flow cytometer revealed an appreciable apoptotic induction in HeLa cells treated with 100 μM of HY253 for 48 h. This apoptotic induction is associated with cytochrome *c* release from mitochondria, via up-regulation of pro-apoptotic Bcl-2 proteins, such as Bax and Bak, which, in turn, resulted in the activation of caspase-8, -9 and -3, and the cleavage of poly(ADP-ribose) polymerase (PARP).

Apoptosis, which plays an important role in tissue homeostasis and the development of organisms, is a major form of cell death characterized by a series of tightly regulated processes that involve in the activation of a cascade of molecular events leading to cell death. An imbalance between cell proliferation and apoptotic cell death results in serious diseases, like cancer. Cells undergoing apoptosis have been found to have an elevated level of cytochrome *c* in the cytosol and a corresponding decrease in the mitochondria.[1] After the release of mitochondrial cytochrome *c*, caspase-3 is activated by proteolytic cleavage into an active heterodimer,[2] thereby becoming responsible for the proteolytic degradation of poly(ADP-ribose) polymerase (PARP), which occurs at the onset of apoptosis.[3,4]

Over the last few decades, much attention has been focused on natural products as potential sources of novel anticancer drugs.[5–12] Thus, the identification of potential chemotherapeutic agents using mechanism-based studies holds great promise for elucidating mechanisms and devising more specific and effective treatments for cancer-related diseases.

Aralia continentalis is a traditional medicinal herb spread widely in northeastern Asia, including China and Korea. Many constituents from its root extracts, including essential oils and diterpene acid, have been isolated as active components for antioxidant, anti-inflammatory, analgesic, sedative, antifungal, anti-thrombotic, and growth inhibition.[13,14]

In the course of screening for a novel antiproliferative compound as an anticancer drug candidate, we isolated HY253 from the roots extract of *A. continentalis*. The current study reports on the isolation and structural elucidation of HY253, and examines its biological properties as an antiproliferative agent in HeLa cells.

To isolate an active compound from the 70% methanol extract of the roots (dry weight: 100 g), the extract was filtrated and concentrated in vacuo and the remnant subsequently extracted with ethyl acetate (EtOAc). The EtOAc fraction, which showed potent antiproliferative activity, was further fractionated by silica-gel column chromatography (DAVISIL® 60–200 μm, Grace Vydac, Hesperia, CA, USA) eluted with hexane, EtOAc, and methanol. The fraction eluted with hexane/EtOAC (8:2) showed the apoptotic induction and further fractionated using the preparative-HPLC (C_{18} column, 250 × 22 mm, Waters, OR, USA) eluted with 57% acetonitrile/water (retention time of HY253: 12 min, flow rate: 20 ml/min). As a result, 5.0 mg of HY253 was purified (total yield: 0.005%) and used for the structure elucidation.

The structure of HY253 was determined using NMR spectroscopy. Seventeen peaks were observed in the ^{13}C NMR spectrum of HY253. Their types were determined by DEPT experiments: seven methylene, six methine, and four quaternary carbons. The correlations between ^{1}H and ^{13}C were decided from the HMQC spectrum. Based on the interpretation of the COSY spectrum, the correlations among 12 ^{1}H peaks at 1.49, 4.10, 5.76, 6.31, 1.34,

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1.43, 4.91, 5.96, 5.20, 5.40, 2.05, 5.81, and 5.00 ppm, and their partial structure could be determined. This result was confirmed by the long-ranged couplings obtained from HMBC. Because the ^{13}C peak at 75.0 ppm was long-range coupled with the ^{1}H peak at 5.76 ppm in the HMBC spectrum, it was determined to be C-4a. Likewise, the backbone structure of HY253 was determined based on the interpretation of the HMBC spectrum. The vinyl group attached to C-7 could be confirmed by the ^{1}H–^{1}H correlation obtained from the COSY spectrum. All NMR data and their complete assignments are shown in the Supplementary materials.[15] As a result, HY253 is a novel compound as 7,8a-divinyl-2,4a,4b,5,6,7,8,8a,9,9a-decahydro-1H-fluorene-2,4a,4b,9a-tetraol (Fig. 1).

To examine the antiproliferative effect of HY253 in HeLa cells, MTT assay was performed. As shown in Figure 2A, the viability of HY253-treated HeLa cells was significantly decreased in a dose-dependent manner (IC_{50}: 81.5 μM). Based on this IC_{50} value, the final concentration of HY253 for current study was determined as 100 μM.

To investigate the apoptotic induction of HY253 in HeLa cells, nuclear DAPI staining was used to examine any morphologic changes in the nuclei of HeLa cells. As shown in Figure 2B, the nuclear change found in HY253-treated HeLa cells was typical of apoptosis: a fragmented apoptotic body after DAPI staining. Apoptotic bodies were observed with 100 μM of HY253 after incubation for 24 h, and the number of apoptotic cells was increased in a time-dependent manner. Moreover, as shown in Figure 2C, a TUNEL assay also exhibited the induction of apoptosis in HeLa cells treated with 100 μM of HY253 for 24 and 48 h.

Several apoptosis-associated genes or proteins have been shown to play critical roles in regulating apoptosis. These include caspases, Bcl-2 family members, cytochrome *c*, and PARP. To determine whether these proteins are involved in the mediation of HY253-induced cell death in HeLa cells, we examined caspases activation and cleavage of PARP by Western blotting.

As shown in Figure 3, HY253 induced the proteolytic cleavage of inactive procaspase-8, -9, and -3 into active caspase-8, -9, and -3, respectively. One of the substrates for caspase during apoptosis is PARP, an enzyme that appears to be involved in DNA repair and genome surveillance and integrity in response to environmental stress. Therefore, since the proteolytic cleavage of PARP results in a characteristic shift of the protein upon electrophoresis from 116 to 89 kDa, the cleavage of PARP was used as an indicator of caspase activation in response to HY253 treatment, which became obvious after 12 h of HY253 treatment (Fig. 3).

Many lines of evidence demonstrate that Bcl-2-related proteins play an important role in either inhibition or promotion of apoptosis.[16] As shown in Figure 3, our results from Western blot analysis showed that the pro-apoptotic Bcl-2 proteins, such as Bax and Bak, are increased after HY253 treatment for 24 h. Interestingly, we found that the up-regulation of Bax seems to be decreased in HeLa cells at 12 h after HY253 treatment. To rule out the experimental errors, three independent experiments were performed. However, we observed exactly the same results. Such a transitional decrease of Bax protein should be elucidated on transcriptional level in a future using quantitative real-time PCR. Furthermore, anti-apoptotic protein, Bcl-2, seems to be not affected. Accordingly, our current

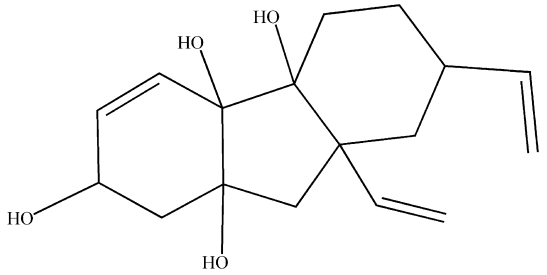

Figure 1. The chemical structure and nomenclature of HY253, 7,8a-divinyl-2,4a,4b,5,6,7,8,8a,9,9a-decahydro-1H-fluorene-2,4a,4b,9a-tetraol.

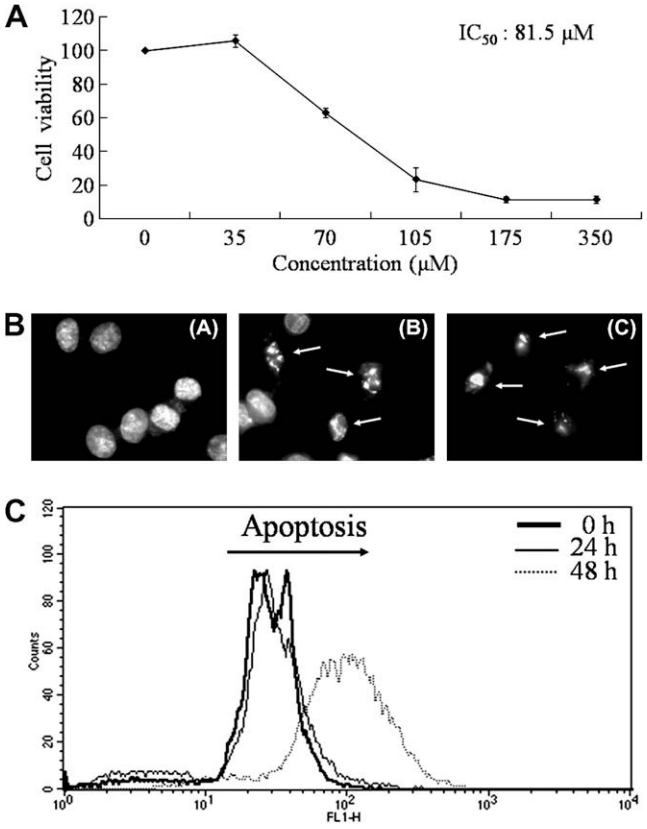

Figure 2. (A) Effect of HY253 on proliferation of HeLa cells in a dose-dependent manner, as determined by MTT assay for 48 h. (B) Morphologic changes induced by HY253 in HeLa cells. Fluorescence microscopic examination of untreated cells (A) or those treated with 100 μM of HY253 for (B) 24 h and (C) 48 h followed by DAPI staining. Arrows indicate apoptotic bodies of nuclear fragmentation. (C) Induction of apoptosis by HY253 in HeLa cells, as determined by TUNEL assay. The cells were treated with HY253 for 24 and 48 h, then stained with d-UTP FITC and PI in the dark and analyzed using a flow cytometer.

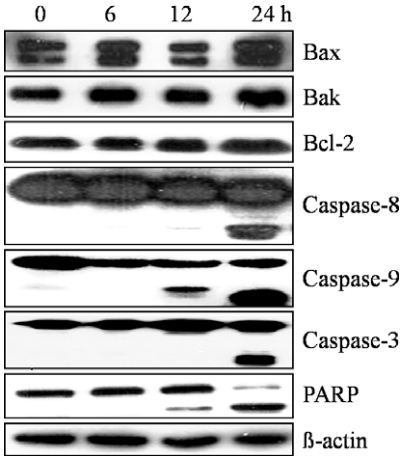

Figure 3. Effects of HY253 on activation of caspase-8, -9 and -3, and on cleavage of PARP. Cells pretreated with 100 μM of HY253 for different lengths of time were washed with PBS, lysed, and a Western blot analysis performed. β-Actin was used as the internal control.

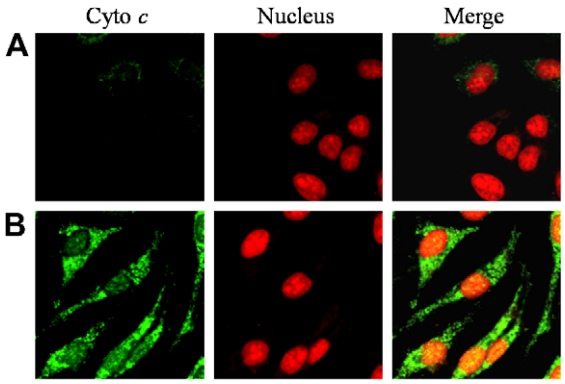

Figure 4. Effect of HY253 on the cytochrome *c* release from mitochondria. (A) HeLa cells with confocal medium and (B) cells treated with 100 μM of HY253 for 24 h were fixed and labeled for cytochrome *c* (green) and nucleus (red). Images were obtained using confocal laser scanning microscopy. Cyto *c*: cytochrome *c*.

results show that HY253 induces apoptosis in HeLa cells via intrinsic pathway.

To investigate the mitochondria-mediated intrinsic pathway involved in HY253-induced apoptosis, we analyzed the cytochrome *c* release from mitochondria using confocal microscopy. As shown in Figure 4, cytochrome *c* (green) release was observed significantly in HeLa cells treated with 100 μM of HY253 for 24 h. However, cytochrome *c* release in non-treated control cells was not observed. Therefore, we conclude that cytochrome *c* is released from mitochondria to cytosol in HY253-treated HeLa cells.

In conclusion, the current study showed that HY253, a novel antiproliferative compound isolated from the roots of *A. continentalis*, induces apoptosis in HeLa cells. This apoptotic induction in HY253-treated HeLa cells is associated with cytochrome *c* release from mitochondria, via up-regulation of pro-apoptotic Bcl-2 proteins, such as Bax and Bak, which, in turn, resulted in the activation of caspase-8, -9 and -3, and the cleavage of PARP.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.009.

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