J. Microbiol. Biotechnol. (2015), 25(3), 413–417
http://dx.doi.org/10.4014/jmb.1501.01069

jmb

HY253, a Novel Decahydrofluorene Analog, Induces Apoptosis *via* Intrinsic Pathway and Cell Cycle Arrest in Liver Cancer HepG2 Cells

Ko-woon Choi†, Hyewon Suh†, Seunghun Jang, Dongsik Kim, and Chul-Hoon Lee*

Department of Pharmacy, College of Pharmacy, Hanyang University, Kyeonggido 426-791, Republic of Korea

Received: January 23, 2015
Revised: February 10, 2015
Accepted: February 10, 2015

First published online
February 12, 2015

**Corresponding author*
Phone: +82-31-400-5801;
Fax: +82-31-400-5958;
E-mail: chhlee@hanyang.ac.kr

†These authors contributed equally to this work.

pISSN 1017-7825, eISSN 1738-8872

Recently, we isolated HY253, a novel decahydrofluorene analog with a molecular structure of 7,8a-divinyl-2,4a,4b,5,6,7,8,8a,9,9a-decahydro-1H-fluorene-2,4a,4b,9a-tetraol from the roots of *Aralia continentalis*, which is known as Dokwhal (獨活), a traditional medicinal herb. Moreover, we previously reported its cytotoxic activity on cancer cell proliferation in human lung cancer A549 and cervical cancer HeLa cells. The current study aimed to evaluate its detailed molecular mechanisms in cell cycle arrest and apoptotic induction in human hepatocellular carcinoma HepG2 cells. Flow cytometric analysis of HepG2 cells treated with 60 μM HY253 revealed appreciable cell cycle arrest at the G1 phase *via* inhibition of Rb phosphorylation and down-regulation of cyclin D1. Furthermore, using western blots, we found that up-regulation of cyclin-dependent kinase inhibitors, such as $p21^{CIP1}$ and $p27^{KIP1}$, was associated with this G_1 phase arrest. Moreover, TUNEL assay and immunoblottings revealed apoptotic induction in HepG2 cells treated with 100 μM HY253 for 24 h, which is associated with cytochrome *c* release from mitochondria, *via* down-regulation of anti-apoptotic Bcl-2 protein, which in turn resulted in activation of caspase-9 and -3, and proteolytic cleavage of poly(ADP-ribose) polymerase (PARP). Accordingly, we suggest that HY253 may be a potent chemotherapeutic hit compound for treating human liver cancer cells *via* up-regulation and activation of the *p53* gene.

Keywords: *Aralia continentalis*, apoptosis, $p27^{KIP1}$, $p21^{CIP1}$, cytochrome *c*, HepG2

Uncontrolled induction of programmed cell death and impaired cell cycle progression are the major characteristics of cancer cells, due to an imbalance between cell growth and death. The cyclin-dependent kinase (CDK) belongs to a family of serine/threonine protein kinases that play pivotal roles in cell cycle regulation in cooperation with various endogenous cyclins and CDK inhibitors (CKIs), including $p21^{CIP1}$, $p27^{KIP1}$, and $p16^{INK4a}$ [12, 20].

Apoptosis, which plays a crucial role in maintaining the tissue homeostasis of eukaryotes, is a major form of programmed cell death characterized by a series of tightly programmed processes that involve activation of pro-caspases to effector caspases, leading to cell death [10, 24, 25]. Furthermore, apoptosis induced *via* the intrinsic pathway is executed by the balance of pro- and anti-apoptotic Bcl-2 protein family members through the regulation of cytochrome *c* release from mitochondria [1–3].

Consequently, the key regulators of cell cycle progression and apoptotic induction could be important molecular targets for therapeutic intervention. Therefore, the discovery and identification of potential chemotherapeutic candidate compounds using mechanism-based studies hold great promise for elucidating molecular mechanisms and devising more specific and effective treatments for various human cancers [4, 5, 7–9, 11, 14, 17–19, 23].

Hepatocellular carcinoma (HCC) accounts for 90% of all liver cancers, and HCC is estimated to be the fifth leading cause of cancer death in Korea [6]. Once diagnosed with HCC, only 20–30% of patients are considered eligible for curative intervention, such as surgery or organ transplantation. However, eventually, most patients will receive chemotherapy in the hope of prolonging life.

Previously, we isolated HY253, elucidated as 7,8a-divinyl-2,4a,4b,5,6,7,8,8a,9,9a-decahydro-1H-fluorene-2,4a,4b,9a-tetraol

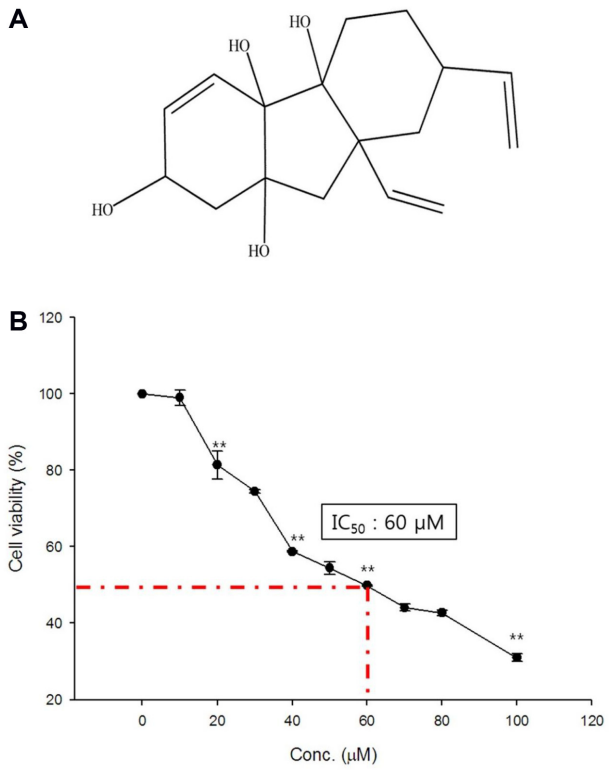

Fig. 1. Antiproliferative effect of HY253 in liver cancer HepG2 cells.

(**A**) 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). (**B**) Cells were seeded in a 96-well plate and incubated with 0–100 μM HY253 for 24 h. Cell viability was determined by the CellTiter 96 AQueous One Solution Cell Proliferation Assay. The results represent the mean ± SD of three independent experiments. The significance was determined by Student's *t*-test (**$p < 0.01$).

(Fig. 1A), and reported its anticancer activities in human lung cancer A549 and cervical cancer HeLa cells [15, 16]. Accordingly, the aim of the current study was to investigate whether HY253 induces apoptosis and cell cycle inhibition in human liver cancer HepG2 cells, and elucidate its underlying molecular mechanisms.

To examine the cytotoxic effect of HY253 in HepG2 cells, the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, MI, U.S.A.) was performed. As shown in Fig. 1B, the cell viability of HY253-treated HepG2 cells was significantly decreased in a dose-dependent manner (IC_{50}: 60 μM). Based on this IC_{50} value and flow cytometric experiments, in the present study, the working concentration of HY253 for cell cycle arrest and apoptotic induction was determined to be 60 and 100 μM, respectively.

To investigate the effects of HY253 on cell cycle progression in HepG2 cells, we measured the DNA content of HepG2 cells treated with 60 μM HY253, using flow cytometric analysis, which revealed an appreciable arrest in the G_1 phase. As shown in Fig. 2, the HepG2 cell population gradually increased from 52.95% at 0 h to 74.56% at 24 h in the G_1 phase after exposure to 60 μM HY253. The apoptotic cell population in the sub-G_1 phase gradually increased from 2.9% at 0 h to 10.28% at 24 h in 60 μM HY253-treated HepG2 cells. However, to elucidate apoptotic induction and the underlying molecular mechanisms, a higher dosage of HY253 should be considered.

To examine the detailed mechanisms of cell cycle arrest at the G_1 phase of HY253-treated HepG2 cells, we assessed western blots to determine the expression patterns of cell cycle-related proteins, such as cyclins and CKIs. We also examined the levels of phosphorylation of retinoblastoma protein (Rb) and p53, the major tumor suppressor proteins, after HY253 treatment [13, 22]. As shown in Fig. 3, we found that HY253-mediated induction of G_1 phase arrest was associated with decreased expression of cyclin D1, and significant up-regulation of CKI, including $p21^{CIP1}$ and $p27^{KIP1}$, which have key roles in regulating the entry of the G_1/S transition checkpoint [21]. In addition, a large decrease in hyperphosphorylated Rb (p-Rb) demonstrated that the

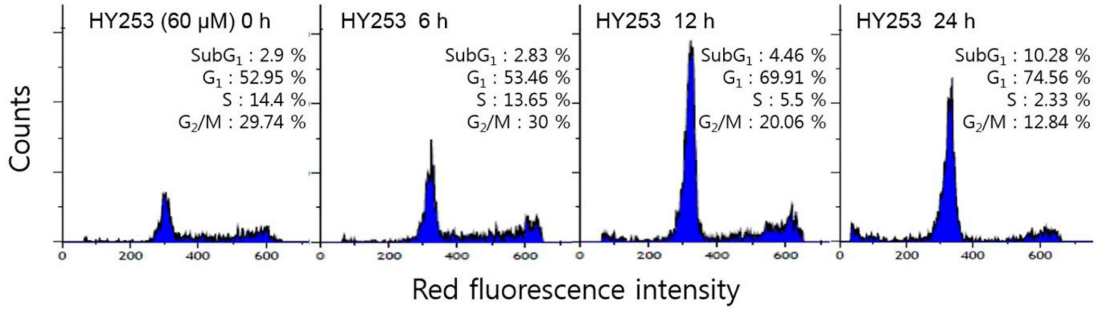

Fig. 2. Quantitative histogram of flow cytometric analysis of HepG2 cells treated with 60 μM HY253.

Cells were treated with 60 μM HY253 for the indicated time (6, 12, and 24 h), and then stained with propidium iodide, and the nuclei were analyzed for their DNA content by flow cytometry.

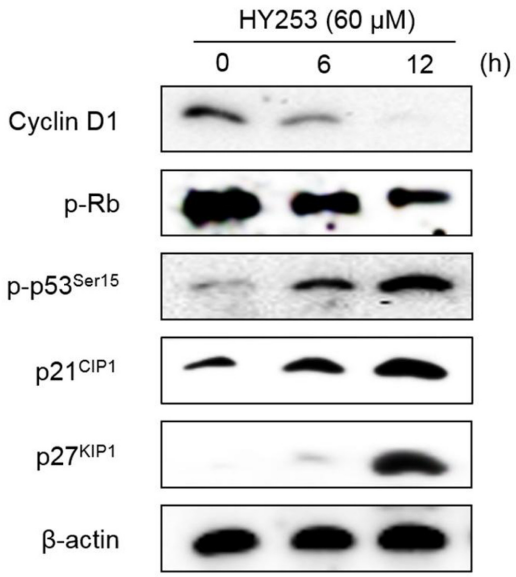

Fig. 3. Western blot analysis of HepG2 cells treated with 60 μM HY253 on cell cycle regulatory proteins.
β-Actin was used as the loading control.

hypophosphorylated form is increased through HY253-mediated inhibition of Rb phosphorylation in 60 μM HY253-treated HepG2 cells. When it is hypophosphorylated, Rb functionally becomes active, which in turn results in cell cycle arrest at the G_1 phase due to association with E2F. In addition, p53 activation through phosphorylation (p-p53) at Ser15 could explain the up-regulation of $p21^{CIP1}$, one of the downstream target genes of p53, in a time-dependent manner in HepG2 cells treated with 60 μM HY253.

As mentioned previously, the effective sample dosage for further experiments on apoptotic induction was determined to be 100 μM, by flow cytometric analysis, due to marginal apoptotic induction in 60 μM HY253-treated HepG2 cells (data not shown). As shown in Fig. 4A, the apoptotic event was obviously identified as fragmented apoptotic bodies in DAPI-stained HepG2 cells treated with 100 μM HY253 for 24 h. Moreover, to examine DNA fragmentation in the nuclei of HepG2 cells, a TUNEL assay was performed. As shown in Fig. 4B, apoptotic induction was identified in 100 μM HY253-treated HepG2 cells for 24 h.

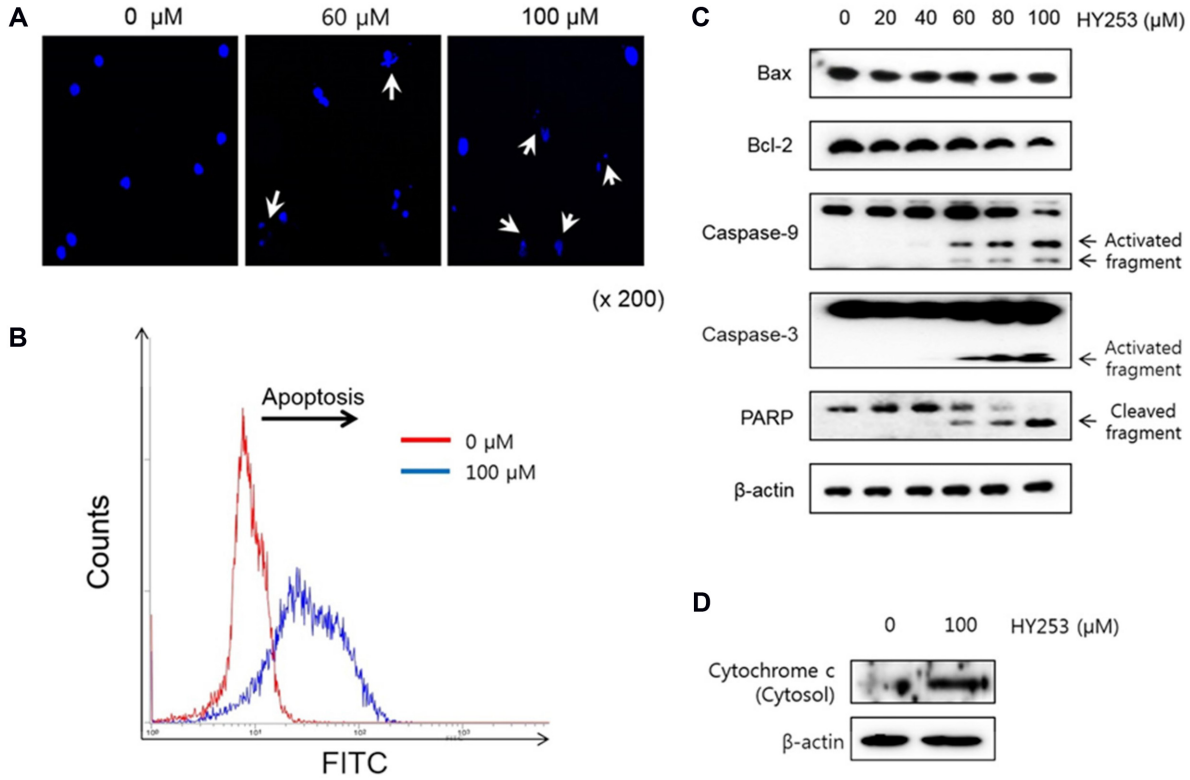

Fig. 4. Apoptotic induction by HY253 in HepG2 cells.
(**A**) Fluorescence microscopic examination of DAPI-stained untreated cells (left) or cells treated with 60 and 100 μM HY253 for 24 h. White arrows indicate the typical apoptotic bodies. (**B**) TUNEL assay. Cells were treated with 100 μM HY253 for 24 h, then stained with d-UTP FITC and propidium iodide in the dark, and analyzed using a flow cytometer. (**C**) Western blot analysis of HepG2 cells (treated with 100 μM HY253) on apoptosis-related proteins. (**D**) Effects of HY253 on cytochrome *c* release from mitochondria. β-Actin was used as the loading control.

Furthermore, to determine whether apoptosis-related regulators are involved in the mediation of HY253-induced cell death in HepG2 cells, we examined caspase activation and cleavage of poly(ADP-ribose) polymerase (PARP), one of the major targets of active caspase-3, by western blot analysis. As shown in Fig. 4C, HY253 induced cell death, which was associated with down-regulation of anti-apoptotic Bcl-2 protein, which in turn resulted in activation of caspase-9 and -3, and proteolytic cleavage of PARP, an enzyme that appears to be involved in DNA repair and genome surveillance.

In addition, to examine the mitochondria-mediated intrinsic pathway involved in HY253-mediated apoptosis, we analyzed cytochrome *c* release from mitochondria to the cytosol using western blots. As shown in Fig. 4D, significant cytochrome *c* release was observed, due to an increase in the Bax/Bcl-2 ratio in HepG2 cells treated with 100 μM HY253 for 24 h.

In conclusion, the current study showed that HY253, a novel compound isolated from the roots of *A. continentalis*, induces G_1 phase arrest in HepG2 cells. This cell cycle arrest was mediated by the decreased expression of cyclin D1 and phosphorylation of p53 at Ser-15, as well as the increased expression of CKIs such as $p27^{KIP1}$ and $p21^{CIP1}$. These results indicate that HY253 could be a hit compound for a novel anticancer agent.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Korean government (MSIP) (2013R1A1A2011531).

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