J Korean Soc Appl Biol Chem (2013) 56, 113–116
DOI 10.1007/s13765-012-2234-y

SHORT COMMUNICATION

3-Hydroxyflavanone Induces Apoptosis in HeLa Cells

Soon Young Shin · Young Han Lee

Received: 10 October 2012 / Accepted: 14 November 2012 / Published Online: 28 February 2013

Abstract Flavonoids and their derivatives exhibit many biological properties, including anti-inflammatory and antitumor activities. However, the antitumor action of 3'-hydroxyflavanone (3'-HF) is largely unknown. Antitumor efficacy of 3'-HF was assessed using cervical cancer (HeLa) cells. 3'-HF treatment resulted in a reduction in cell proliferation. A flow cytometric analysis demonstrated that 3'-HF deregulated cell cycle progression and triggered apoptosis. 3'-HF also increased the levels of p53 and p21, but decreased the level of cyclin D1. 3'-HF-induced apoptosis was accompanied by poly(ADP-ribose)polymerase cleavage. Together, these data indicate that 3'-HF possesses antitumor activity, which is mediated via the deregulation of cell cycle progression and induction of apoptosis.

Keywords apoptosis · flavanone · flavonoid · poly(ADP-ribose) polymerase · polyphenol

Flavonoids, polyphenol compounds that are widely distributed in edible fruits and vegetables, can be divided into several classes, including chalcones, flavonols, flavones, flavanones, anthocyanidins, and isoflavonoids. Flavonoids have been shown to possess a broad range of biological properties, including anti-oxidative and anti-inflammatory activities (Pietta, 2000; Yao et al., 2004). Several studies have demonstrated that dietary flavonoids can inhibit the growth of tumor cells (Knekt et al., 1997; Prasad et al., 2010; Araujo et al., 2011). Among flavonoids, flavanones are enriched in most seeds and fruit skin, and some flavanones have been found to have cancer-preventative effects. Recently, we demonstrated that 2-hydroxyflavanone exhibits antitumor activity through the induction of early growth response gene-1 in colon cancer cells (Shin et al., 2012). However, the antitumor potency of 3'-hydroxyflavanone (3'-HF; Fig. 1A) remains to be elucidated. Effectiveness of 3'-HF as a therapeutic agent in cervical cancer was examined and found that 3'-HF inhibited the growth of cervical cancer (HeLa) cells through the induction of apoptosis.

We first examined the effect of 3'-HF on HeLa cell proliferation. 3'-HF was purchased from Indofine Chemical Co. (USA). HeLa cells were obtained from the American Type Culture Collection (USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone, USA). HeLa cells were seeded onto 96-well plates (2×10^3 cells/well) and treated with either vehicle (DMSO) or various concentrations (0, 20, and 40 μM) of 3'-HF for different periods of time (12, 24, and 48 h). Cellular proliferation was assessed using a Cell Counting Kit-8 Assay (Dojindo Molecular Technologies, USA) according to the manufacturer's instructions. A significant, dose-dependent decrease in proliferation was observed in the cells treated with 3'-HF (Fig. 1B).

The cell cycle consists of four distinct phases: G_1 (gap 1), S (synthesis), G_2 (gap 2), and M (mitosis). Two classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), regulate cell cycle progression. Because tumor cells are actively undergoing cell cycle progression, many cancer chemopreventive agents exert their anticancer effects by modulating the expression of cyclins and stimulating caspases, which induce a cell cycle arrest and apoptosis (Deep et al., 2006; Singh and Agarwal, 2006; Shin et al., 2012). To determine whether 3'-HF influences cell cycle progression, cell cycle distribution profiles for 3'-HF-exposed HeLa cells were examined by flow cytometry. HeLa cells were treated with either vehicle (DMSO) or 20 μM 3'-HF for 24 h, fixed in 70% ethanol, washed twice with phosphate-buffered saline, and then stained with 50 μg/mL propidium iodide, as described previously (Shin et al., 2010). The cellular DNA content was analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, USA). The numbers of G1 and G2/M phase cells were reduced in response to 3'-HF treatment (Fig. 2). Notably, the

S. Y. Shin · Y. H. Lee (✉)
Department of Biomedical Science and Technology, SMART-Institute of Advanced Biomedical Science, RCTC, Konkuk University, Seoul 143-701, Republic of Korea
E-mail: yhlee58@konkuk.ac.kr

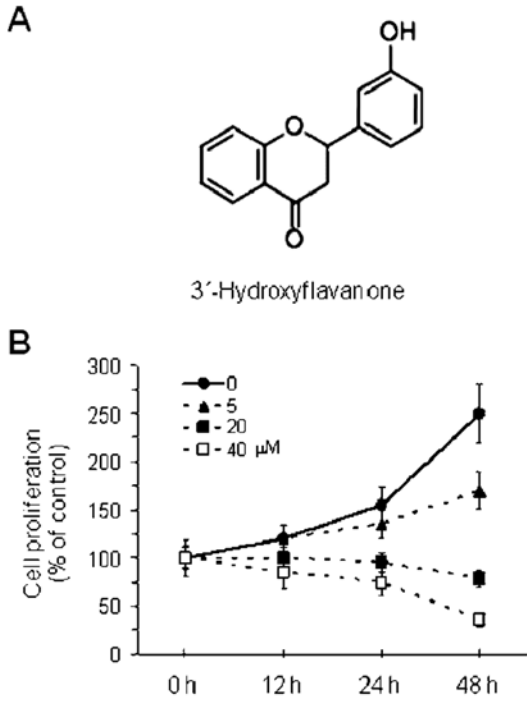

Fig. 1 Effect of 3'-hydroxyflavanone (3'-HF) on the inhibition of cellular proliferation in HeLa cells. (A) Chemical structure of 3'-HF. (B) Cell proliferation assay. Proliferation was measured in HeLa cells treated with different doses (0, 5, 10, or 20 μM) of 3'-HF for varying lengths of time (0–48 h) using a Cell Counting Kit-8 Assay. The data shown represent the mean ± SD for one experiment performed in triplicate.

accumulation of sub-G1 phase cells, a hallmark of apoptosis, was noted at 24 h following 3'-HF treatment, which suggests that 3'-HF deregulates the cell cycle and triggers apoptosis in HeLa cells.

We next examined whether 3'-HF affects the expression of cell cycle or apoptosis regulatory proteins by western blot analysis. Antibodies against glyceraldehyde 3'-phosphate dehydrogenase (GAPDH; 1:500), p53 (1:1000), p21 (1:1000), cyclin D1 (1:500), and proliferating cell nuclear antigen (PCNA; 1:1000) were purchased from Santa Cruz Biotechnology (USA). Antibodies against poly(ADP-ribose)polymerase (PARP; 1:1000), phospho-Erk1/2 (Thr202/Tyr204; 1:1000), phospho-JNK (Thr183/Tyr185; 1:500), and phospho-p38 (Thr180/Tyr182; 1:250) were purchased from Cell Signaling Technology (USA). HeLa cells were treated with 20 μM 3'-HF for 12 or 24 h, followed by separation of cell lysates containing 10–20 μg of protein by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transfer to nitrocellulose filters. The filters were then incubated with antibodies and developed as described previously (Shin et al., 2010). Signals were developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, USA).

Western blot analysis showed increased levels of the tumor suppressor p53 and cyclin-dependent kinase inhibitor 1A (CDKN1A; p21) after 12 h of exposure to 3'-HF (Fig. 3A). In contrast, the level of cyclin D1 began to decrease at 24 h, whereas the PCNA levels did not change in response to 3'-HF treatment. p21 is a well-characterized, potent inhibitor of G1 and G2 CDKs associated with cyclin D1, cyclin E, cyclin A, and CDK2 (Harper et al., 1993; Bunz et al., 1998). p53 induces p21 expression, and enhanced p21 expression mediates a cell cycle arrest in response to DNA damage (el-Deiry et al., 1993; Yang et al., 1995). The upregulation of p21 by antitumor agents is associated with the inhibition of tumor cell proliferation (Yang et al., 1995). Thus, our data suggest that 3'-HF upregulates p53-dependent p21 expression, leading to a cell cycle arrest and the downregulation of cyclin D1.

Apoptosis is tightly regulated by caspases, which are cysteine-dependent aspartate-specific proteases. PARP is a chromatin-associated enzyme that uses NAD as a substrate to catalyze the

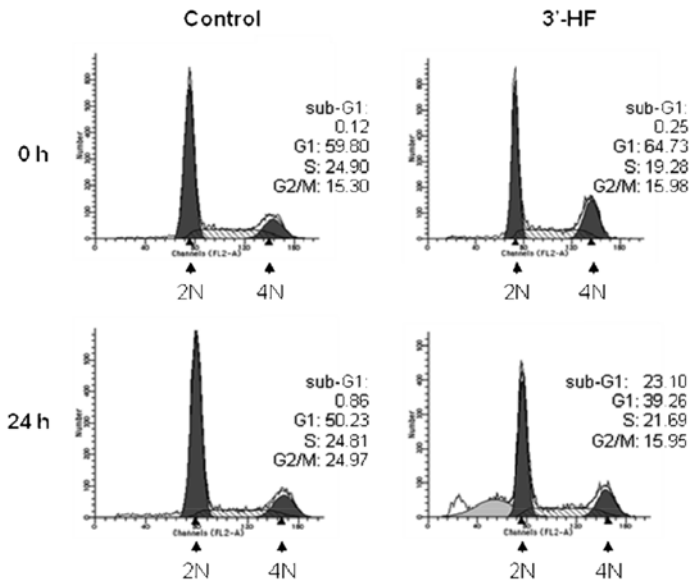

Fig. 2 Effect of 3'-HF on cell cycle progression. HeLa cells were treated with 20 μM 3'-HF for 24 h, after which the cells were harvested, fixed with ethanol, and stained with propidium iodide. The cellular DNA contents were determined by flow cytometry for detecting the cell cycle distribution. 2N, diploid; 4N, tetraploid. The results shown are representative of three independent experiments.

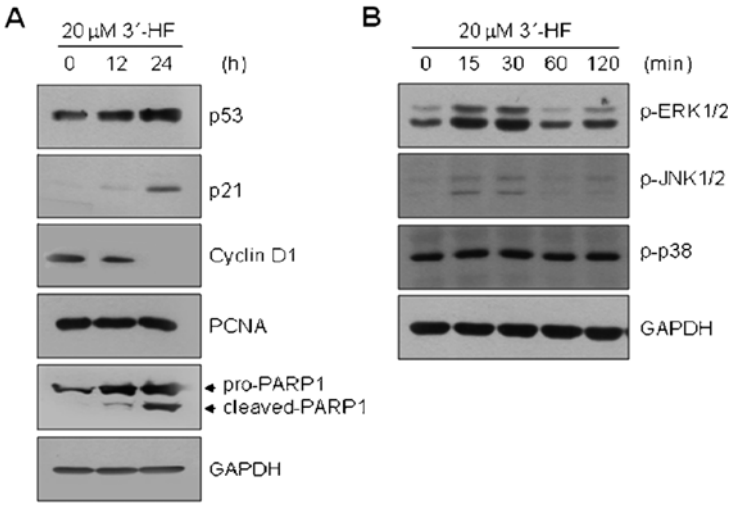

Fig. 3 Effect of 3'-HF on the expression of cell cycle-related proteins and activation of MAPKs. HeLa cells were treated with 20 μM 3'-HF for various lengths of time, and then whole cell lysates were prepared and subjected to western blotting using antibodies against cell cycle-related proteins (A) and phosphor-specific MAPKs (B). Anti-GAPDH antibodies were used as an internal control to show equal protein loading. The results shown are representative of three independent experiments.

covalent transfer of ADP-ribose to nuclear proteins. The processing of native PARP (113 kDa) to its 89- and 24-kDa forms is catalyzed by effector caspases, including caspase-3 and -7, during apoptosis (Lazebnik et al., 1994). PARP plays an important role in the apoptotic program (Yu et al., 2006). To test whether 3'-HF induces the activation of effector caspases, the levels of cleaved PARPs were measured. 3'-HF treatment caused the accumulation of the typical cleaved forms of PARP (Fig. 3A). The transcription factor p53 regulates multiple target genes involved in apoptosis, including PUMA, NOXA, and Bax, in response to diverse genotoxic stresses, thereby contributing to the sequential activation of caspases-9 and -3/-7 (Vousden and Lane, 2007). The activation of caspases-3 and -7 leads to the cleavage and inactivation of many vital proteins, including lamin and PARP, resulting in apoptotic cell death (Riedl and Salvesen, 2007). Thus, our results demonstrate that 3'-HF possesses antitumor activity mediated via the deregulation of cell cycle progression and apoptosis through the induction of p53 and p21.

Mitogen-activated protein kinase (MAPK) signaling pathways, including p42/44 MAPK (ERK), JNK1/2, and p38 MAPK, can mediate DNA damage responses and apoptosis (Dent et al., 2003). To investigate whether 3'-HF modulates MAPK signaling, serum-starved HeLa cells were treated with 20 μM of 3'-HF for various lengths of time, and the MAPK phosphorylation status was determined by western blot analysis. The levels of phosphorylated ERK1/2 and JNK1/2 increased within 30 min and were maintained for up to 30 min, whereas that of p38 MAPK was not altered by 3'-HF (Fig. 3B). Activation of JNK is generally activated by stress signals and involved in the induction of apoptosis, whereas ERK MAPK is associated cell proliferation and survival (Xia et al., 1995). Previously, we have reported the role of ERK1/2 MAPK in sodium arsenite-induced apoptosis through the induction of Elk-1-mediated p21 and Bax expression (Shin et al., 2011). Several studies have demonstrated that ERK signaling mediates up-regulation of p21 expression to growth arrest (Datto et al., 1995; Pumiglia and Decker, 1997; Beier et al., 1999; de Siervi et al., 2004; Facchinetti et al., 2004; Ciccarelli et al., 2005; Tu et al., 2007) and the induction of apoptosis through activation of p53 (Persons et al., 2000; Wu, 2004) in various cell types. At present, although defining the roles of different MAPK pathways is difficult, the ERK and JNK MAPK pathways are likely involved in mediating the antitumor activity of 3'-HF. The exact molecular targets of 3'-HF are currently unknown. However, it has been shown that some flavanones and flavones, such as apigenin (4',5,7-trihydroxyflavone), can be catalyzed by peroxidase in the presence of glutathione (GSH), which results in producing intracellular reactive oxygen species (ROS) (Wang et al., 1999; Miyoshi et al., 2007). Because the generation of ROS may contribute to p53-mediated apoptotic cell death (Martindale and Holbrook, 2002), it is possible that 3'-HF induces p53-mediated cell cycle arrest and apoptosis through generation of ROS. Further study remains to be clarified whether this event is actually involved in the 3'-HF-induced apoptosis.

In summary, we found that 3'-HF induced accumulation of the tumor suppressors p53 and p21, deregulated cell cycle progression, and stimulated ERK1/2 and JNK1/2 MAPK signaling, which triggered apoptosis in HeLa cells. These findings provide new insight into the molecular mechanisms responsible for the antitumor effects of hydroxylated flavanone derivatives.

Acknowledgments This paper resulted from the Konkuk University research support program.

References

Araujo JR, Goncalves P, and Martel F (2011) Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutr Res* **31**, 77–87.

Beier F, Taylor AC, and LuValle P (1999) The Raf-1/MEK/ERK pathway regulates the expression of the p21(Cip1/Waf1) gene in chondrocytes. *J Biol Chem* **274**, 30273–9.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP et al. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**, 1497–501.

Ciccarelli C, Marampon F, Scoglio A, Mauro A, Giacinti C, De Cesaris P et al. (2005) p21WAF1 expression induced by MEK/ERK pathway activation or inhibition correlates with growth arrest, myogenic differentiation and onco-phenotype reversal in rhabdomyosarcoma cells. *Mol Cancer* **4**, 41.

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, and Wang XF (1995) Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci USA* **92**, 5545–9.

de Siervi A, Marinissen M, Diggs J, Wang XF, Pages G, and Senderowicz A (2004) Transcriptional activation of p21(waf1/cip1) by alkylphospholipids: role of the mitogen-activated protein kinase pathway in the transactivation of the human p21(waf1/cip1) promoter by Sp1. *Cancer Res* **64**, 743–50.

Deep G, Singh RP, Agarwal C, Kroll DJ, and Agarwal R (2006) Silymarin and silibinin cause G1 and G2-M cell cycle arrest via distinct circuitries in human prostate cancer PC3 cells: a comparison of flavanone silibinin with flavanolignan mixture silymarin. *Oncogene* **25**, 1053–69.

Dent P, Yacoub A, Fisher PB, Hagan MP, and Grant S (2003) MAPK pathways in radiation responses. *Oncogene* **22**, 5885–96.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM et al. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**, 817–25.

Facchinetti MM, De Siervi A, Toskos D, and Senderowicz AM (2004) UCN-01-induced cell cycle arrest requires the transcriptional induction of p21(waf1/cip1) by activation of mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway. *Cancer Res* **64**, 3629–37.

Harper JW, Adami GR, Wei N, Keyomarsi K, and Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**, 805–16.

Knekt P, Jarvinen R, Seppanen R, Hellovaara M, Teppo L, Pukkala E et al. (1997) Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *Am J Epidemiol* **146**, 223–30.

Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, and Earnshaw WC (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**, 346–7.

Martindale JL and Holbrook NJ (2002) Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol* **192**, 1–15.

Miyoshi N, Naniwa K, Yamada T, Osawa T, and Nakamura Y (2007) Dietary flavonoid apigenin is a potential inducer of intracellular oxidative stress: the role in the interruptive apoptotic signal. *Arch Biochem Biophys* **466**, 274–82.

Persons DL, Yazlovitskaya EM, and Pelling JC (2000) Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. *J Biol Chem* **275**, 35778–85.

Pietta PG (2000) Flavonoids as antioxidants. *J Nat Prod* **63**, 1035–42.

Prasad S, Phromnoi K, Yadav VR, Chaturvedi MM, and Aggarwal BB (2010) Targeting inflammatory pathways by flavonoids for prevention and treatment of cancer. *Planta Med* **76**, 1044–63.

Pumiglia KM and Decker SJ (1997) Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. *Proc Natl Acad Sci USA* **94**, 448–52.

Riedl SJ and Salvesen GS (2007) The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol* **8**, 405–13.

Shin SY, Kim CG, Kim SH, Kim YS, Lim Y, and Lee YH (2010) Chlorpromazine activates p21Waf1/Cip1 gene transcription via early growth response-1 (Egr-1) in C6 glioma cells. *Exp Mol Med* **42**, 395–405.

Shin SY, Kim CG, Lim Y, and Lee YH (2011) The ETS family transcription factor ELK-1 regulates induction of the cell cycle-regulatory gene p21(Waf1/Cip1) and the BAX gene in sodium arsenite-exposed human keratinocyte HaCaT cells. *J Biol Chem* **286**, 26860–72.

Shin SY, Kim JH, Lee JH, Lim Y, and Lee YH (2012) 2'-Hydroxyflavanone induces apoptosis through Egr-1 involving expression of Bax, p21, and NAG-1 in colon cancer cells. *Mol Nutr Food Res* **56**, 761–74.

Singh RP and Agarwal R (2006) Natural flavonoids targeting deregulated cell cycle progression in cancer cells. *Curr Drug Targets* **7**, 345–54.

Tu Y, Wu W, Wu T, Cao Z, Wilkins R, Toh BH et al. (2007) Antiproliferative autoantigen CDA1 transcriptionally up-regulates p21(Waf1/Cip1) by activating p53 and MEK/ERK1/2 MAPK pathways. *J Biol Chem* **282**, 11722–31.

Vousden KH and Lane DP (2007) p53 in health and disease. *Nat Rev Mol Cell Biol* **8**, 275–83.

Wang IK, Lin-Shiau SY, and Lin JK (1999) Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. *Eur J Cancer* **35**, 1517–25.

Wu GS (2004) The functional interactions between the p53 and MAPK signaling pathways. *Cancer Biol Ther* **3**, 156–61.

Xia Z, Dickens M, Raingeaud J, Davis RJ, and Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326–31.

Yang ZY, Perkins ND, Ohno T, Nabel EG, and Nabel GJ (1995) The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo. *Nat Med* **1**, 1052–6.

Yao LH, Jiang YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R et al. (2004) Flavonoids in food and their health benefits. *Plant Foods Hum Nutr* **59**, 113–22.

Yu SW, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM et al. (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. *Proc Natl Acad Sci USA* **103**, 18314–9.