Isorhamnetin induces ROS-dependent cycle arrest at G2/M phase and apoptosis in human hepatocarcinoma Hep3B cells

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Abstract. Isorhamnetin is a 3'-O-methylated metabolite of quercetin that is found predominantly in a variety of medicinal plants. Although many previous studies have reported that this flavonol has diverse health-promoting effects, evidence for the underlying molecular mechanism of anti-cancer efficacy is still lacking. In this study, it was examined the anti-proliferative effect of isorhamnetin on human hepatocarcinoma Hep3B cells, and found that isorhamnetin induced cell cycle arrest at G2/M phase and apoptosis. Isorhamnetin-induced G2/M arrest was associated with decreased expression of proliferating cell nuclear antigen as well as cyclin A and cyclin B1. However, isorhamnetin increased expression of p21WAF1/CIP1, a cyclin-dependent kinase (Cdk) inhibitor, and increased p21 complexed with Cdk2 and Cdc2. In addition, isorhamnetin-induced apoptosis was associated with increased expression of Fas/Fas ligand, reduced ratio of Bcl-2/Bax expression, truncation of Bid, cytosolic release of cytochrome c, and activation of caspase-8, -9 and -3. Isorhamnetin also enhanced intracellular levels of reactive oxygen species (ROS), while the addition of N-acetyl cysteine (NAC), a ROS inhibitor, significantly diminished isorhamnetin-induced mitochondrial dysfunction. Furthermore, the interruption of ROS generation using NAC significantly attenuated isorhamnetin-mediated G2/M arrest and apoptosis. Collectively, this is the first report to show that isorhamnetin inhibited the proliferation of human hepatocarcinoma cells by ROS-dependent arrest of the cell cycle at the G2/M phase and induction of apoptosis.

Key words: Isorhamnetin — Hep3B cells — G2/M arrest — Apoptosis — ROS

Introduction

Although new therapies are being developed for the treatment of cancer patients, chemotherapy is still a major approach to cancer treatment. However, some limitations such as adverse side effects, limited efficacy and drug resistance remain to be solved (Fu et al. 2018; Kumar and Jaitak 2019). Therefore, there is an urgent need to develop new therapeutic strategies that minimize these limitations while enhancing therapeutic efficacy. In this regard, there is a growing interest in the importance of compounds derived from natural resources that have traditionally been used for the treatment of various diseases and disorders (Effert et al. 2007; Nobili et al. 2009; Butler et al. 2014). In particular, in many accumulated studies to date, a large number of naturally occurring chemotherapeutic agents have been reported to induce cell cycle arrest and apoptosis in cancer cells, which is not toxic to normal cells (Hanahan and Weinberg 2011; Medema and Macůrek 2012). These agents are also emerging as alternatives to chemopreventive and chemotherapeutic agents as they can specifically regulate various cellular signaling pathways in cancer cells (Schnekenburger et al. 2014; Bolhassani 2015).

Isorhamnetin (3’-methoxy-3,4’,5,7-tetrahydroxyflavone) is a type of flavonol aglycone present in some medicinal plants, including Hippophae rhamnoides L., Ginkgo biloba L. and Oenanthe javanica, which are used as traditional medicines for the treatment of hemorrhage, rheumatism and cardiovascular disease (Zhang and Cui 2005; Gu et al. 2019). This compound is one of the metabolites of quercetin that is structurally similar to kaempferol, also known as 3’-O-methyl quercetin (Castrillo et al. 1986; Rösch et al. 2004; Chen et al. 2013). Isorhamnetin exhibits many biological properties due
to its anti-inflammatory, antioxidant and metabolic properties (Luo et al. 2015; Seo et al. 2016; Yang et al. 2016; Abdollah and Esmat 2017; Qi et al. 2018), and is also considered to have the potential of anti-cancer agents in the results of various cancer cell models. According to previous studies, isorhamnetin has been reported to prevent the proliferation of human leukemia, breast cancer, colon cancer and cervical cancer cells through G2/M phase arrest (Li et al. 2014; Wu et al. 2016; Wei et al. 2018; Zhang et al. 2018), and to induce mitotic block in non-small cell lung carcinoma cells, thus enhancing cisplatin- and carboplatin-induced G2/M arrest (Zhang et al. 2015). On the other hand, isorhamnetin induced S phase arrest in some cancer cells (Wang et al. 2018; Wu et al. 2018), indicating that cell cycle arrest by isorhamnetin depends on the type of cancer cell line.

In addition, isorhamnetin has been shown to induce apoptosis through activation of death receptor (DR)-dependent (extrinsic) and/or mitochondria-dependent (intrinsic) pathways in a variety of cancer cell lines (Lee et al. 2008; Antunes-Ricardo et al. 2014; Li et al. 2015; Luo et al. 2015; Zhang et al. 2015; Huang et al. 2017; Sak et al. 2018). It has also been shown that the anti-cancer effect of isorhamnetin was accompanied by the disturbance of various cellular signaling pathways such as mitogen-activated protein kinase, nuclear factor-kappa B and phosphoinositide 3-kinase/Akt signaling pathway (Hu et al. 2015; Wang et al. 2018; Zhang et al. 2018). Moreover, isorhamnetin showed a cytotoxic effect through reactive oxygen species (ROS)-dependent apoptosis pathway in breast cancer cells (Wu et al. 2018). Although the potential of the growth inhibitory activity of isorhamnetin was raised in human liver cancer cells (Poór et al. 2016; Guo et al. 2017; Zhou et al. 2018), no molecular mechanism has been reported to support its effect. Therefore, this study investigated the anti-cancer efficacy of isorhamnetin in human hepatocarcinoma Hep3B cells focusing on the mechanisms involved in inducing cell cycle arrest and apoptosis.

Materials and Methods

Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and other reagents for cell culture were purchased from WelGENCE Inc. (Daegu, Republic of Korea). Isorhamnetin, dimethylsulfoxide (DMSO) and 4’,6’-diamidino-2-phenylindole (DAPI) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylindodicarbocyanine iodide (JC-1), 5,6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA), N-acetyl-L-cysteine (NAC) and propidium iodide (PI) were obtained from Invitrogen (Carlsbad, CA, USA). The Annexin V-fluorescein isothiocyanate (FITC) staining kit was purchased from BD Biosciences (San Diego, CA, USA). Polyvinylidene difluoride (PVDF) membranes and caspase activity assay kits were obtained from Schleicher & Schuell (Keene, NH, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Bradford Protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). A mitochondrial protein isolation kit was purchased from Active Motif (Carlsbad, CA, USA). Primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences (Westborough, MA, USA). All other chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich Chemical Co.

Cell culture and isorhamnetin treatment

The human hepatocarcinoma Hep3B cells, which are tumor suppressor p53-null type, were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C under a humidified 5% CO2. Isorhamnetin was dissolved in DMSO to a final concentration of 100 mM. Prior to use, the stock solution was diluted to the desired concentration with cell culture medium.

Cell viability assay

Cell viability was determined by MTT assay, as previously described (Park et al. 2014). Briefly, Hep3B cells (1×10^4 cells/per well) were seeded onto 96-well plates in 100 μl medium. After overnight incubation, the cells were exposed to various concentrations of isorhamnetin for 48 h. Thereafter, MTT reagent at 50 μg/ml final concentration was added to each well, and cells were incubated continuously for 2 h at 37°C. After removing the medium, 100 μl DMSO was added to each well to dissolve the formed blue formazan crystals and then measured at 540 nm in a microplate reader (VERSA Max, Molecular Device Co., Sunnyvale, CA, USA). All results were performed in three independent experiments, and the cell viability was expressed as a percentage of the control. The morphological changes of the cells were directly observed and photographed using a phase-contrast microscope (Carl Zeiss, Oberkochen, Germany).

Determination of cell cycle distribution by flow cytometric analysis

PI staining was applied to analyze the DNA content and cell cycle distribution. In brief, cells were exposed to various concentrations of isorhamnetin for 48 h, and the cells...
were harvested and fixed gently in 70% ice-cold ethanol (in phosphate-buffered saline, PBS) at 4°C for 30 min. The cells were re-suspended in PBS containing 40 μg/ml PI, 0.1% Triton X-100 and 100 μg/ml RNase A in a dark room at 37°C for 30 min, and subjected to flow cytometry (BD Biosciences, San Jose, CA, USA), to determine the cell cycle distribution and apoptotic cells (sub-G1 phase).

**Determination of apoptotic cell death by flow cytometric analysis**

The annexin V-FITC staining kit was used to determine and quantify the apoptotic cells, according to the manufacturer’s instruction. In brief, the collected cells were washed with PBS, suspended in the supplied binding buffer, and then stained with FITC-conjugated annexin V and PI at room temperature (RT) for 20 min in the dark. The fluorescent intensities of the cells were detected by flow cytometry, and the annexin V+/PI- and annexin V+/PI+ cell populations were considered indicators of apoptotic cells.

**Protein extraction, co-immunoprecipitation, and Western blot analysis**

After treatment, the cells were harvested, and the whole cellular proteins were prepared using the Bradford protein assay kit, according to the manufacturer’s protocol. For the preparation of mitochondrial and cytosolic proteins from the cells, mitochondrial and cytoplasmic extraction reagents were applied, according to the manufacturer’s instruction. Protein concentration was measured using the Bio-Rad protein assay kit, according to the manufacturer’s instructions. For co-immunoprecipitation assay, the 500 μg of cell lysates from each sample was pre-cleaned with normal rabbit IgG and a protein-A-sepharose bead slurry, and immunoprecipitation was conducted using 1 μg of anti-cyclin-dependent kinase 2 (Cdk2) or cell division cycle 2 (Cdc2, also call Cdk1) antibody and protein-A-sepharose. The protein complex was then prepared according to the previously described method (Park et al. 2014). For Western blot analysis, equal amounts of protein samples or immunoprecipitated proteins were separated by electrophoresis using sodium dodecyl sulphate (SDS)-polyacrylamide gel, and transferred to PVDF membranes. The membranes were blocked with Tris-buffered saline (10 mM Tris-Cl, pH 7.4) containing 0.5% Tween-20 and 5% nonfat dry milk for 1 h at RT, and then probed with the indicated primary antibodies, to react with the blotted membranes at 4°C overnight. Afterwards, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies, developed using an ECL detection kit, and then visualized by Fusion FX Image system (Vilber Lourmat, Torcy, France). All results were obtained in three independent experiments.

**Nuclear staining assay**

The changes of nuclear morphology for assessing apoptosis were assessed by DAPI staining, a cell-permeable nucleic acid dye. Briefly, cells were cultured with or without isorhamnetin for 48 h, and then fixed with 4% paraformaldehyde for 10 min at RT. The cell were rinsed with PBS, and incubated with 1 μg/ml DAPI solution at 37°C for 10 min. Stained cells were visualized and photographed using a fluorescence microscope (Carl Zeiss).

**Caspase activity assay**

The activity of caspases was measured according to the manufacturer’s instructions for the Caspase colorimetric assay kits. Briefly, cells were harvested and lysed in the lysis buffer provided in the kit on ice for 10 min, and then centrifuged at 10,000 × g for 1 min. The supernatants containing equal proteins were incubated with the supplied reaction mixtures, including the fluorogenic peptide substrate (Asp-Glu-Val-Asp specific for caspase-3; Ile-Glu-Thr-Asp for caspase-8; and Leu-Glu-His-Asp specific for caspase-9) labeled with p-nitroaniline (pNA) for 1 h at 37°C in the dark. The amounts of released pNA were measured using a microplate reader by exciting at 405 nm, and emitting at 510 nm.

**Measurement of ROS production and mitochondrial membrane potential (MMP, ΔΨm)**

The production of ROS was measured using DCF-DA, as described previously (Koh et al. 2018). At the end of the treatment with isorhamnetin for defined periods in the presence or absence of NAC, a well-known antioxidant, cells were incubated with 10 μM DCF-DA in the dark at 37°C for 20 min. Subsequently, cells were analyzed for DCF fluorescence by flow cytometry at 480 nm/520 nm. To measure MMP, JC-1 staining was performed, according to the manufacturer’s instructions. After treatment with isorhamnetin for 48 h in the presence or absence of NAC, cells were exposed to 10 μM JC-1 for 30 min at 37°C, and then analyzed by flow cytometry at 488 nm/575 nm, as previously described (Kim et al. 2019).

**Statistical analysis**

All experiments were performed at least three times. Data were analyzed using GraphPad Prism software (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA), and expressed as the mean ± standard deviation (SD). Differences between groups were assessed using analysis of variance, followed by ANOVA-Tukey’s post hoc test, and p < 0.05 was considered to indicate a statistically significant difference.
Results

Isorhamnetin inhibited cell viability and induced cell cycle arrest at G2/M phase in Hep3B cells

To evaluate the growth inhibitory effect of isorhamnetin on Hep3B cells, cells were treated with different concentrations of isorhamnetin for 48 h, and then the MTT assay was conducted. As shown in Figure 1A, the cell viability was significantly decreased in a concentration-dependent manner in isorhamnetin-treated cells. To investigate the mechanism responsible for the isorhamnetin-induced anti-proliferative effect in Hep3B cells, the cell cycle distribution profile was examined. Flow cytometry data indicated that the percentage of cells arrested at G2/M phase was significantly increased with increasing isorhamnetin treatment concentration, coupled with a decrease in the proportion of cells in G1 and S phases (Fig. 1B and C). In the meanwhile, a significant increase of the cells at sub-G1 phase, which is used as an index of apoptotic cells, was observed in isorhamnetin-treated cells in a concentration-dependent manner (Fig. 1B).

Isorhamnetin regulated the expression of G2/M phase-associated proteins in Hep3B cells

To explore the biochemical event of the isorhamnetin-elicited G2/M arrest in Hep3B cells, the levels of G2/M phase-associated proteins were analyzed. The immunoblotting results as seen in Figure 2A revealed that following isorhamnetin treatment, the levels of cyclin A and cyclin B1 were reduced, while expression of Cdk2 and Cdc2 remained at the control level. Expression of proliferating cell nuclear antigen (PCNA) was also reduced in cells treated with isorhamnetin, in a concentration-dependent manner. However, although Hep3B cells are p53-deficient cells, the expression of Cdk inhibitor p21WAF1/CIP1 was markedly increased in response to isorhamnetin exposure. Additionally, it was performed co-immunoprecipitation to investigate the role of isorhamnetin-induced p21, and found that increased p21 by isorhamnetin treatment was apparently complexed with Cdk2 and Cdc2 (Fig. 2B).

Isorhamnetin-induced G2/M arrest was associated with the induction of apoptosis in Hep3B cells

Under phase-contrast microscope, the phenotypic characteristics of isorhamnetin-treated Hep3B cells showed irregular cell outlines, decrease of cell density, and increase of detached cell (Fig. 3A). Since the increase in the frequency of cells belonging to Sub-G1 and these morphological changes are presumed to be related to the induction of apoptosis, DAPI staining was performed to investigate whether isorhamnetin-induced G2/M arrest was associated with apoptosis induction. As indicated in Figure 3B, morphological changes of the nuclei that were observed in apoptosis-inducing cells such as nuclear fragmentation and chromatin condensation were dominantly found in isorhamnetin-treated cells. To quantify the apoptosis induced by isorhamnetin, annexin V/PI double staining was performed and analyzed. As shown in Figure 3C and D, after treatment with isorhamnetin, the corresponding populations of annexin V-staining positive cells (total apoptotic cells) were significantly increased, as compared to the control, indicating that isorhamnetin-induced G2/M phase arrest in Hep3B cells was associated with the induction of apoptosis.
Isorhamnetin modulated the expression of apoptosis-regulatory factors in Hep3B cells

To examine the pathway of isorhamnetin-induced apoptosis in Hep3B cells, it was assessed whether isorhamnetin activated the caspase signaling pathway and found that isorhamnetin reduced the expression of pro-caspase-8, -9, and -3 (Fig. 3E) and increased their enzymatic activity in a concentration-dependent manner (Fig. 3F). Isorhamnetin also induced cleavage of poly(ADP-ribose) polymerase (PARP), a major substrate protein of activated caspase-3 (Hassan et al. 2014; Kiraz et al. 2016). In addition, the effects of isorhamnetin on the expression of Fas/Fas ligand (FasL) and Bcl-2 family members, which play a critical role in extrinsic and intrinsic apoptotic pathways, respectively (Birkinshaw and Czabotar 2017; Edlich 2018), were determined. As indicated in Figure 3E, both Fas and FasL expression levels were up-regulated in isorhamnetin-stimulated cells. It was also found that isorhamnetin increased the expression of pro-apoptotic Bax, and decreased the expression of anti-apoptotic Bcl-2. In addition, the expression of truncated BH3 interacting-domain death agonist (tBid), a pro-apoptotic protein belonging to the Bcl-2 family proteins, was increased, and the release of cytochrome c from the mitochondria to the cytoplasm was promoted in isorhamnetin-treated cells (Fig. 4A and B).

Isorhamnetin increased ROS generation and mitochondrial dysfunction in Hep3B cells

To investigate the involvement of ROS on the cytotoxic effect of isorhamnetin in Hep3B cells, conducted flow cytometry analysis was conducted using DCF-DA. Our data indicated that the accumulation of ROS was highest after 1 h of isorhamnetin treatment, and then gradually decreased thereafter, while cells pretreated with NAC, a potent ROS scavenger, showed significantly suppressed ROS levels, compared to those of isorhamnetin alone treated cells (Fig. 4C–E). It was further assessed whether mitochondrial dysfunction was involved in isorhamnetin-induced apoptosis. According to the results of flow cytometry using JC-1 dyes, the formation of JC-1 aggregates in mitochondria was maintained at a relatively high rate in cells not treated with isorhamnetin, while the ratio of JC-1 monomers increased with increasing isorhamnetin treatment concentration, indicating a remarkable depletion of MMP after isorhamnetin treatment (Fig. 4F–H). However, under the condition that NAC existed, it was markedly weakened, indicating that mitochondrial dysfunction by isorhamnetin in Hep3B cells was mediated through ROS-dependent pathway.

ROS acts as an upstream regulator of isorhamnetin-mediated apoptosis and cell cycle blockade in Hep3B cells

The effect of ROS on isorhamnetin-mediated apoptosis and G2/M phase arrest was further investigated to determine the role of ROS in the anti-cancer activity of isorhamnetin in Hep3B cells. As depicted in the results of DAPI staining, artificially blocking the production of ROS using NAC drastically attenuated isorhamnetin-induced apoptosis (Fig. 5A). In addition, NAC pretreatment significantly reversed the isorhamnetin-induced up-regulation of Bax and down-regulation of Bcl-2, and degradation of PARP (Fig. 5B). Consistent with these results, the increased apoptosis by treatment with isorhamnetin was significantly restored by blocking ROS production (Fig. 5C and D). Moreover, blocking of ROS reinstated isorhamnetin-induced decreased expression of cyclin B1 and PCNA, and increased expression of p21 (Fig. 6A), and subsequently protected isorhamnetin-mediated cell cycle arrest at the G2/M stage, which was related to a decrease of sub-G1 phase cells (Fig. 6B and C). In parallel, inhibiting ROS production greatly restored reduced cell viability by isorhamnetin (Fig. 6D), demonstrating that ROS generation was shown to be necessary for the contribution of G2/M arrest and apoptosis by isorhamnetin.

Discussion

Several previous studies have shown that isorhamnetin has anti-cancer effects by inducing cell cycle arrest and apoptosis in various cancer cell lines, under conditions that are not toxic to normal cells (Ramachandran et al. 2012; Hu et
al. 2015). Although the possibility that ROS generation by this compound may act as a major signal for the anti-cancer activity in breast cancer cells has recently been suggested (Wu et al. 2018), the role of ROS is still largely unknown. Also, there have been few studies of molecular mechanisms on the anti-cancer activity of isorhamnetin in human liver cancer cells.

Deregulation of cell cycle control is clearly linked to the development and progression of most tumors, and disruption of this progression is thought to be an important strategy for the inhibition of cancer cell growth (Hanahan and Weinberg 2011; Medema and Macůrek 2012). Therefore, it was first examined whether the growth inhibitory effect of isorhamnetin in Hep3B cells was associated with cell cycle arrest. The results of flow cytometry analysis showed that isorhamnetin caused G2/M phase arrest, similar to the results of previous studies in several human phase arrest, similar to the results of previous studies in several human cancer cell lines (Li et al. 2014; Wu et al. 2016; Wei et al. 2018; Zhang et al. 2018), suggesting that G2/M arrest is one of the mechanisms of isorhamnetin-mediated growth inhibitory effect in Hep3B cells. The progression of the cell cycle in eukaryotic cells is strictly controlled by the interaction of cyclins and Cdns with their inhibitory factors. For example, D-type cyclins bind to and activate Cdk4 and Cdk6, leading to sequential progression from G1 to S phase. On the other hand, cyclin A/Cdk2 and Cdc2 complexes control S and G2 phases, and induction of G2/M transition and processes during mitosis depend on cyclin B/Cdc2 complex (Sánchez-Martínez et al. 2015; Bai et al. 2017). In the present study, isorhamnetin reduced in the level of cyclin A and cyclin B1 protein, without significant

![Figure 3](image-url)
changes in the expression of Cdk2 and Cdc2. Immunoblotting analysis also revealed that the protein level of PCNA, a critical marker of cell proliferation (Wang 2014; Juríková et al. 2016), was markedly inhibited in isorhamnetin-stimulated cells as compared to control cells. However, the level of p21 was markedly induced in p53-deficient Hep3B cells treated with isorhamnetin. Moreover, complexes immunoprecipitated with antibodies Cdk2 and Cdc2 in isorhamnetin-treated cells contained a greater amount of immunologically detectable p21 protein compared to untreated control cells, which may have contributed the reduction of Cdk2 and Cdc2 activity and ultimately leading to G2/M arrest. p21, a typical Cdk inhibitor belonging to the KIP/CIP family, was first reported to be a major inducer of tumor suppressor p53-dependent cell cycle arrest induced by DNA damage, but it could act as a mediator of p53-independent cell arrest in various types of cancer cells (Reinhardt and Schumacher 2012; Karimian et al. 2016). Collectively, our data suggest that isorhamnetin-triggered G2/M arrest was due to decreased expression of cyclin A and cyclin B1, and an increase in p21 levels through p53-independent mechanism.

Because isorhamnetin-mediated cell cycle arrest at G2/M phase has been reported to be related to apoptosis in some cancer cell lines (Li et al. 2014; Zhang et al. 2015; Wu et al. 2016; Wei et al. 2018; Zhang et al. 2018), it was evaluated whether G2/M arrest by isorhamnetin was associated with
apoptosis induction in Hep3B cells. Based on the results of morphological changes and flow cytometry analysis, we found that the cytotoxic effect of isorhamnetin was achieved through the induction of apoptosis associated with G2/M arrest. As is well known, apoptosis, a highly conserved programmed cell death, can be largely divided into extrinsic and intrinsic pathways in mammalian cells (Hassan et al. 2014; Pfeffer and Singh 2018). The extrinsic pathway begins with the activation of caspase-8 by the formation of the death-inducing signaling complex through the binding of death ligands to the cell surface DRs. For example, when FasL, one of the typical death ligands, binds to the corresponding DR, Fas, caspase-8 is sequentially activated (Kantari and Walczak 2011; Pfeffer and Singh 2018). Meanwhile, the intrinsic pathway is characterized by the release of pro-apoptotic proteins, such as cytochrome c, from the mitochondria into the cytoplasm, which is accompanied by increased mitochondrial permeability and the activation of caspase-9. This pathway is tightly regulated by the Bcl-2 protein family that includes pro- and anti-apoptotic proteins, which guard mitochondrial integrity, and control the cytosolic release of cytochrome c through the mitochondrial transition pore (Birkinshaw and Czabotar 2017; Edlich 2018). Caspases-8 and -9, which correspond to the initiator caspases of each pathway, ultimately activate apoptosis through the cleavage of several cellular substrates such as PARP by activating downstream executioner caspases, including caspase-3 (Hassan et al. 2014; Kiraz et al. 2016). Our results indicated that isorhamnetin activated both caspase-8 and -9, and increased the truncation of Bid in Hep3B cells. In addition, consistent with previous studies in non-small cell lung cancer cells and Lewis lung cancer cells (Lee et al. 2008; Ruan et al. 2015), mitochondrial dysfunction was induced, as confirmed by the loss of MMP in isorhamnetin-treated cells. The loss of MMP was accompanied by a down-regulation in the Bcl-2/Bax ratio and promotion of cytochrome c release into the cytoplasm from mitochondria, which are typically observed in the activated intrinsic pathway (Birkinshaw and Czabotar 2017; Edlich 2018). Isorhamnetin treatment also significantly increased the activity of caspase-3, and induced the cleavage of PARP. As is well known in previous studies, caspase-8 activated by the initiation of the extrinsic pathway cleaved and converted Bid to tBid (Billen et al. 2008; Edlich 2018). tBid in turn translocates to the mitochondria to promote the permeability of the mitochondrial outer membrane, leading to the release of cytochrome c, counteracting the cytoprotective activity of Bcl-2 protein, and amplifying the intrinsic pathway (Billen et al. 2008; Kantari and Walczak 2011). Therefore, we speculated that the pro-apoptotic effect of isorhamnetin in Hep3B cells could occur by simultaneously activating the extrinsic and intrinsic pathways through tBid-mediated crosstalk.

Accumulated evidence has shown that many anti-cancer agents promote apoptosis for removal of cancer cells through pro-oxidant properties such as increasing ROS accumulation, or destroying cellular antioxidant systems (Bolhassani 2015; Badrinath and Yoo 2018). Mitochondria are the major

Figure 5. Roles of ROS on isorhamnetin (ISO)-induced apoptosis in Hep3B cells. The cells were either treated with 100 μM ISO for 48 h, or pre-treated with 10 mM N-acetyl cysteine (NAC) for 1 h, before 100 μM ISO treatment, and then collected. A. The DAPI-stained nuclei were pictured under a fluorescence microscope. B. Total cell lysates were prepared. Western blotting was then performed using the indicated antibodies, and an ECL detection system. Actin was used as an internal control. C, D. The percentages of apoptotic cells were determined by expressing the numbers of annexin V+ cells as percentages of all the present cells. The results are presented as the mean ± SD of three independent experiments; *** p < 0.0001 compared to control; **** p < 0.0001 compared to ISO-treated cells. PARP, poly (ADP-ribose) polymerase.
Induction of G2/M arrest and apoptosis by isorhamnetin

Figure 6. Roles of ROS on isorhamnetin (ISO)-induced apoptosis in Hep3B cells. The cells were either treated with 100 μM ISO for 48 h, or pre-treated with 10 mM N-acetyl cysteine (NAC) for 1 h, before 100 μM ISO treatment, and then collected. A. Total cell lysates were prepared. Western blotting was then performed using the indicated antibodies, and an ECL detection system. Actin was used as an internal control. B, C. The cells were collected, and stained with PI solution for flow cytometry analysis. B. Representative profiles. The percentages of apoptotic sub-G1 cells were calculated as the percentage of the number of cells in the sub-G1 population relative to the number of total cells. C. Quantification of the cell population (in percent) in different cell cycle phases of viable cells is shown. Data were expressed as the mean of two independent experiments. D. The cell viability was assessed by MTT assay. Each bar represents the mean ± SD of three independent experiments; *** p < 0.0001 compared to control; ### p < 0.0001 compared to ISO-treated cells). PCNA, proliferating cell nuclear antigen.

Organelles responsible for the production of ROS in the cells, and are also a major target of ROS. Therefore, elevating intracellular levels of ROS production is considered to be one of the ideal mechanisms for killing cancer cells through the activation of intrinsic pathway (Galadari et al. 2017; Moloney and Cotter 2018). Consistent with a previous study in breast cancer cells (Wu et al. 2018), our results show that isorhamnetin treatment markedly increased the levels of ROS production; however, the ROS scavenger, NAC greatly weakened the accumulation of ROS by isorhamnetin. The quenching of ROS generation also markedly diminished the isorhamnetin-induced disruption of MMP to the control level, indicating that ROS act as upstream signaling molecules to enhance isorhamnetin-mediated mitochondrial dysfunction in Hep3B cells. Our results also demonstrate that the presence of NAC markedly attenuated isorhamnetin-induced Bax/Bcl-2 ratio and PARP degradation, and NAC treatment also significantly blocked the apoptosis induced by isorhamnetin. Furthermore, isorhamnetin-induced modulation of cell cycle regulatory proteins such as cyclin B1, PCNA and p21 was reversed in the presence of NAC. Subsequently, NAC pretreatment also significantly protected isorhamnetin-mediated G2/M arrest and viability reduction, confirming that increasing ROS may serve a key contributor to the anti-cancer effects of isorhamnetin.

In summary, our findings demonstrate that isorhamnetin exerts an anti-proliferative effect on human hepatocarcinoma Hep3B cells through the induction of cell cycle arrest at G2/M phase and apoptosis. Isorhamnetin-induced G2/M arrest was attributed to the decrease in cyclin A and cyclin B1 expression and the upregulation of p21. Isorhamnetin also induced apoptosis by activating caspase-8 and -9, which belong to the initiator caspases of the extrinsic and intrinsic pathways, respectively, followed by the activation of effector caspase-3, leading to the degradation of PARP. In addition, isorhamnetin enhanced the truncation of Bim and mitochondrial dysfunction, which was associated with an increase in Bax/Bcl-2 expression ratio and cytochrome c release into the cytoplasm. Moreover, isorhamnetin increased the production of ROS, and the interruption of ROS generation led cells to escape from G2/M arrest and apoptosis. Taken together, these results suggest that the
production of ROS by isorhamnetin plays a critical role in the induction of G2/M arrest and apoptosis through simultaneous initiation of both extrinsic and intrinsic pathways in HepG3 cells.

Conflict of interest. The author has no conflict of interest to declare.

References

Abdallah HM, Esmat A (2017): Antioxidant and anti-inflammatory activities of the major phenolics from Zygocephillum simplex L. J. Ethnopharmacol. 205, S1–S6 https://doi.org/10.1016/j.jep.2017.04.022

Antunes-Ricardo M, Moreno-García BE, Gutiérrez-Uribe JA, Aráiz-Hernández D, Alvarez MM, Serna-Saldivar SO (2014): Induction of apoptosis in colon cancer cells treated with isorhamnetin glycosides from Opuntia ficusindica pads. Plant Foods Hum. Nutr. 69, 331-336 https://doi.org/10.1007/s1130-014-0438-5

Badrinath N, Yoo SY (2018): Mitochondria in cancer: in the aspects of tumorigenesis and targeted therapy. Carcinogenesis 39, 1419-1430 https://doi.org/10.1093/carcin/bgy148

Bai J, Li Y, Zhang G (2017): Cell cycle regulation and anticancer drug discovery. Cancer Biol. Med. 14, 348–362 https://doi.org/10.20892/j.issn.2095-3941.2017.0033

Billen LP, Kokoski CL, Lovell JF, Leber B, Andrews DW (2008): Bcl-XL inhibits membrane permeabilization by competing with Bax. PLoS Biol. 6, e147 https://doi.org/10.1371/journal.pbio.0060147

Birkinshaw RW, Czabotar PE (2017): The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation. Semin. Cell Dev. Biol. 72, 152-162 https://doi.org/10.1016/j.semcdb.2017.04.001

Bolhassani A (2016): Cancer chemoprevention by natural carotenoids as an efficient strategy. Anticancer Agents Med. Chem. 15, 1026-1231 https://doi.org/10.2174/1871520615666150302125707

Butler MS, Robertson AA, Cooper MA (2014): Natural product and natural product derived drugs in clinical trials. Nat. Prod. Rep. 31, 1612-1661 https://doi.org/10.1039/C4NP00064A

Castrillo JL, Vanden Berghe D, Carrasco L (1986): 3-Methylquercetin is a potent and selective inhibitor of poliovirus RNA synthesis. Virology 152, 219–227 https://doi.org/10.1016/0042-6822(86)90386-7

Chen C, Xu XM, Chen Y, Yu MY, Wen FY, Zhang H (2013): Identification, quantification and antioxidant activity of acetylated flavonol glycosides from sea buckthorn (Hippophae rhamnoides ssp. sinensis). Food Chem. 141, 1573-1579 https://doi.org/10.1016/j.foodchem.2013.03.092

Edlich F (2018): BCL-2 proteins and apoptosis: Recent insights and unknowns. Biochem. Biophys. Res. Commun. 500, 26-34 https://doi.org/10.1016/j.bbrc.2017.06.190

Effert Th, Fu YJ, Zu YG, Schwarz G, Konkimalla VS, Wink M (2007): Molecular target-guided tumor therapy with natural products derived from traditional Chinese medicine. Curr. Med. Chem. 14, 2024-2032 https://doi.org/10.2174/0929867077818368441

Fu B, Wang N, Tan HY, Li S, Cheung F, Feng Y (2018): Multi-component herbal products in the prevention and treatment of chemotherapy-associated toxicity and side effects: A review on experimental and clinical evidences. Front. Pharmacol. 9, 1394 https://doi.org/10.3389/fphar.2018.01394

Galadari S, Rahman A, Pallichankandy S, Thayyullathil F (2017): Reactive oxygen species and cancer paradox: To promote or to suppress? Free Radic. Biol. Med. 104, 144-164 https://doi.org/10.1016/j.freeradbiomed.2017.01.004

Gu Q, Duan G, Yu X (2019): Bioconversion of flavonoid glycosides from Hippophae rhamnoides leaves into flavonoid aglycones by Eurotium amstelodami. Microorganisms 7, E122 https://doi.org/10.3390/microorganisms7050122

Guo R, Guo X, Li T, Fu X, Liu RH (2017): Comparative assessment of phytochemical profiles, antioxidant and anti proliferative activities of Sea buckthorn (Hippophae rhamnoides L.) berries. Food Chem. 221, 997–1003 https://doi.org/10.1016/j.foodchem.2016.11.063

Hanahan D, Weinberg RA (2011): Hallmarks of cancer: the next generation. Cell 144, 646-674. https://doi.org/10.1016/j.cell.2011.02.013

Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N (2014): Apoptosis and molecular targeting therapy in cancer. Biomed. Res. Int. 2014, 150845 https://doi.org/10.1155/2014/150845

Hu S, Huang L, Meng L, Sun H, Zhang W, Xu Y (2015): Isorhamnetin inhibits cell proliferation and induces apoptosis in breast cancer via Akt and mitogen activated protein kinase kinase signaling pathways. Mol. Med. Rep. 12, 6745-6751 https://doi.org/10.3892/mmr.2015.4269

Huang SP, Ho TM, Yang CW, Chang YJ, Chen JF, Shaw NS, Horng JC, Hsu SL, Liao MY, Wu LC, Ho JA (2017): Chemopreventive potential of ethanolic extracts of luobuma leaves (Apoecynum venetum L.) in androgen insensitive prostate cancer. Nutrients 9, E948 https://doi.org/10.3390/nu9090948

Juríková M, Danielík L, Polák Š, Varga I (2016): Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer. Acta. Histochem. 118, 544-552 https://doi.org/10.1016/j.acthis.2016.05.002

Kantari C, Walczak H (2011): Caspase-8 and bid: caught in the act between death receptors and mitochondria. Biochim. Biophys. Acta 1813, 558-563 https://doi.org/10.1016/j.bbamcr.2011.01.026

Karimian A, Ahmadi Y, Yousef B (2016): Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. DNA Repair (Amst) 42, 63-71 https://doi.org/10.1016/j.dnarep.2016.04.008

Kim DY, Kim JH, Lee JC, Won MH, Yang SR, Kim HC, Wie MB (2019): Zinc oxide nanoparticles exhibit both cyclooxygenase- and lipoxygenase-mediated apoptosis in human bone marrow-derived mesenchymal stem cells. Toxicol. Res. 35, 83-91 https://doi.org/10.5487/TR.2019.35.1.083
Induction of G2/M arrest and apoptosis by isorhamnetin

Kiraz Y, Adan A, Kartal Yandim M, Baran Y (2016): Major apoptotic mechanisms and genes involved in apoptosis. Tumour Biol. 37, 8471-8486. https://doi.org/10.1007/s13277-016-5035-9

Koh EM, Lee EK, Song CH, Song J, Chung HY, Chae CH, Jung KJ (2018): Ferulate, an active component of wheat germ, ameliorates oxidative stress-induced PTK/PTP imbalance and PP2A inactivation. Toxicol. Res. 34, 333-341. https://doi.org/10.5487/TR.2018.34.4.333

Kumar A, Jaitak V (2019): Natural products as multidrug resistance modulators in cancer. Eur. J. Med. Chem. 176, 268-291. https://doi.org/10.1016/j.ejmech.2019.05.027

Lee HJ, Lee HJ, Lee EO, Ko SG, Bae HS, Kim CH, Ahn KS, Lu J, Kim SH (2008): Mitochondria-cytochrome c-caspase-9 cascade mediates isorhamnetin-induced apoptosis. Cancer Lett. 270, 342-353. https://doi.org/10.1016/j.canlet.2008.05.040

Li C, Yang X, Chen C, Cai S, Hu J (2014): Isorhamnetin suppresses colon cancer cell growth through the PI3K Akt mTOR pathway. Mol. Med. Rep. 9, 935-940. https://doi.org/10.3892/mmr.2014.1886

Li Q, Ren FQ, Yang CL, Zhou LM, Liu YY, Xiao J, Zhu L, Wang ZG (2015): Anti-proliferation effects of isorhamnetin on lung cancer cells in vitro and in vivo. Asian Pac. J. Cancer Prev. 16, 3035-3042. https://doi.org/10.7314/APCP.2015.16.7.3035

Luo Y, Sun G, Dong X, Wang M, Qin M, Yu Y, Sun X (2015): Isorhamnetin suppresses atherosclerosis by inhibiting macrophage apoptosis via PI3K/AKT activation and HO-1 induction. PLoS One 10, e0120259. https://doi.org/10.1371/journal.pone.0120259

Medema RH, Macérek L (2012): Checkpoint control and cancer. Oncogene 31, 2601-2613. https://doi.org/10.1038/onc.2011.451

Moloney JN, Cotter TG (2018): ROS signalling in the biology of cancer. Semin. Cell Dev. Biol. 80, 50-64. https://doi.org/10.1016/j.secmdb.2017.05.023

Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E, Capoccioli S (2009): Natural compounds for cancer treatment and prevention. Pharmacol. Res. 59, 365-378. https://doi.org/10.1016/j.phrs.2009.01.017

Park C, Jeong NY, Kim GY, Han MH, Chung IM, Kim WJ, Yoo YH, Choi YH (2014): MDM2-lactone B induces apoptosis and G1 arrest of the cell cycle in human melanoma U937 cells through downregulation of pRB phosphorylation and induction of the cyclin-dependent kinase inhibitor p21Waf1/Cip1. Oncol. Rep. 31, 1653-1660. https://doi.org/10.3892/or.2014.3008

Pfeffer CM, Singh ATK (2018): Apoptosis: A target for anticancer therapy. Int. J. Mol. Sci. 19, 448. https://doi.org/10.3390/ijms19020448

Poor M, Zrínyi Z, Kőszegi T (2016): Structure related effects of flavonoid aglycones on cell cycle progression of HepG2 cells: Metabolic activation of fisetin and quercetin by catechol-O-methyltransferase (COMT). Biomed. Pharmacother. 83, 998-1005. https://doi.org/10.1016/j.biopha.2016.08.009

Qi F, Sun JH, Yan JQ, Li CM, Lv XC (2018): Anti-inflammatory effects of isorhamnetin on LPS-stimulated human gingival fibroblasts by activating Nrf2 signaling pathway. Microb. Pathog. 120, 37-41. https://doi.org/10.1016/j.micpath.2018.04.049

Ramachandran L, Manu KA, Shanmugam MK, Li F, Siven KS, Vali S, Kapoor S, Abbasi T, Surana R, Smoot DT, Ashktorab H, Tan P, Ahn KS, Yap CW, Kumar AP, Sethi G (2012): Isorhamnetin inhibits proliferation and invasion and induces apoptosis through the modulation of peroxisome proliferator-activated receptor γ activation pathway in gastric cancer. J. Biol. Chem. 287, 38028-38040. https://doi.org/10.1074/jbc.M112.388702

Reinhardt HC, Schumacher B (2012): The p53 network: cellular and systemic DNA damage responses in aging and cancer. Trends Genet. 28, 128-136. https://doi.org/10.1016/j.tig.2011.12.002

Rösch D, Krumbein A, Mülle K, Kroh LW (2004): Structural investigations of flavonol glycosides from sea buckthorn (Hippophae rhamnoides) pomace by NMR spectroscopy and HPLC-ESI-MS(n). J. Agric. Food Chem. 52, 4039-4046. https://doi.org/10.1021/jf0306791

Ruan Y, Hu K, Chen H (2015): Autophagy inhibition enhances isorhamnetin induced mitochondria dependent apoptosis in non small cell lung cancer cells. Mol. Med. Rep. 12, 5796-5806. https://doi.org/10.3892/mmr.2015.4148

Sak K, Lust H, Kase M, Jaal J (2018): Cytotoxic action of methylequercetins in human lung adenocarcinoma cells. Oncol. Lett. 15, 1973-1978. https://doi.org/10.3892/ol.2017.7466

Sánchez-Martínez C, Gelbert LM, Lallena MJ, de Dios A (2015): Cyclin dependent kinase (CDK) inhibitors as anticancer drugs. Bioorg. Med. Chem. Lett. 25, 3420-3435. https://doi.org/10.1016/j.bmcl.2015.05.100

Schnekenburger M, DiCato M, Diedrich M (2014): Plant-derived epigenetic modulators for cancer treatment and prevention. Biotechnol. Adv. 32, 1123-1132. https://doi.org/10.1016/j.biotechadv.2014.03.009

Seo S, Seo K, Ki SH, Shin SM (2016): Isorhamnetin inhibits reactive oxygen species-dependent hypoxia inducible factor (HIF)-1α accumulation. Biol. Pharm. Bull. 39, 1830-1838. https://doi.org/10.1248/bpb.b16-00414

Wang JL, Quan Q, Ji R, Guo XY, Zhang JM, Li X, Liu YG (2018): Isorhamnetin suppresses PANC-1 pancreatic cancer cell proliferation through S phase arrest. Biomed. Pharmacother. 108, 925-933. https://doi.org/10.1016/j.biopha.2018.09.105

Wang SC (2014): PCNA: a silent housekeeper or a potential therapeutic target? Trends Pharmacol. Sci. 35, 178-186. https://doi.org/10.1016/j.tips.2014.02.004

Wei J, Su H, Bi Y, Li J, Feng L, Sheng W (2018): Anti-proliferative effect of isorhamnetin on HeLa cells through inducing G2/M cell cycle arrest. Exp. Ther. Med. 15, 3917-3923. https://doi.org/10.3892/etm.2018.8592

Wu Q, Kroon PA, Shao H, Needs PW, Yang X (2018): Differential effects of quercetin and two of its derivatives, isorhamnetin and isorhamnetin-3-glucuronide, in inhibiting the proliferation of human breast-cancer MCF-7 cells. J. Agric. Food Chem. 66, 7181-7189. https://doi.org/10.1021/acs.jafc.8b02420
Choi

Wu X, Chen X, Dan J, Cao Y, Gao S, Guo Z, Zerbe P, Chai Y, Diao Y, Zhang L (2016): Characterization of anti-leukemia components from Indigo naturalis using comprehensive two-dimensional K562/cell membrane chromatography and in silico target identification. Sci. Rep. 6, 25491
https://doi.org/10.1038/srep30103

Yang JH, Kim SC, Kim KM, Jang CH, Cho SS, Kim SJ, Ku SK, Cho IJ, Ki SH (2016): Isorhamnetin attenuates liver fibrosis by inhibiting TGF-β/Smad signaling and relieving oxidative stress. Eur. J. Pharmacol. 783, 92-102
https://doi.org/10.1016/j.ejphar.2016.04.042

Zhang BY, Wang YM, Gong H, Zhao H, Lv XY, Yuan GH, Han SR (2015): Isorhamnetin flavonoid synergistically enhances the anticancer activity and apoptosis induction by cisplatin and carboplatin in non-small cell lung carcinoma (NSCLC). Int. J. Clin. Exp. Pathol. 8, 25-37

Zhang HW, Hu JJ, Fu RQ, Liu X, Zhang YH, Li J, Liu L, Li YN, Deng Q, Luo QS, Ouyang Q, Gao N (2018): Flavonoids inhibit cell proliferation and induce apoptosis and autophagy through downregulation of PI3Ky mediated PI3K/AKT/mTOR/p70S6K/ULK signaling pathway in human breast cancer cells. Sci. Rep. 8, 11255
https://doi.org/10.1038/s41598-018-29308-7

Zhang Q, Cui H (2005): Simultaneous determination of quercetin, kaempferol, and isorhamnetin in phytopharmaceuticals of Hippophae rhamnoides L. by high-performance liquid chromatography with chemiluminescence detection. J. Sep. Sci. 28, 1171-1178
https://doi.org/10.1002/jssc.200500055

Zhou JL, Fang XY, Wang JQ, Zhao LG, Li Y, Tang F, Yue YD (2018): Structures and bioactivities of seven flavonoids from Osmanthus fragrans ‘Jinqui’ essential oil extraction residues. Nat. Prod. Res. 32, 588-591
https://doi.org/10.1080/14786419.2017.1318387

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