Abstract. Isorhamnetin, a flavonoid isolated from the fruits of herbal medicinal plants, such as Hippophae rhamnoides L., exerts anticancer effects similar to other flavonoids. However, the effect of isorhamnetin on colorectal cancer (CRC) and the underlying molecular mechanism are unclear. This study aimed to determine the effect of isorhamnetin on the proliferation of cells from the human CRC cell lines, HT-29, HCT116 and SW480. It was demonstrated that isorhamnetin suppressed the proliferation of cells from all three cell lines, induced cell cycle arrest at the G2/M phase and suppressed cell proliferation by inhibiting the PI3K-Akt-mTOR pathway. Isorhamnetin also reduced the phosphorylation levels of Akt (ser473), phosph-p70S6 kinase and phosph-4E-BP1 (t37/46) protein, and enhanced the expression of Cyclin B1 protein. Therefore, this compound was revealed to be a selective PI3K-Akt-mTOR pathway inhibitor, and may be a potent anticancer agent for the treatment of CRC, as it restrains the proliferation of CRC cells.

Materials and methods

Reagents. Isorhamnetin was purchased from Chromadex (Irvine, CA, USA), dissolved in dimethylsulfoxide (DMSO) and diluted to 20 mmol/l. Rapamycin and LY294002 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). An MTT kit was purchased from Promoter (Wuhan, Hubei, China). Propidium iodide (PI) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The BCA Protein Assay kit, ECL detection system and horseradish peroxidase-conjugated secondary antibody were obtained from Pierce (Rockford, IL, USA). Antibodies against AKT, phosphorylated (p)-AKT (ser473) and 4E-BP1 (t37/46) protein, were obtained from Cell Signaling Technology, Inc. Antibodies against p70S6k and p-p70S6K were purchased from Epitomics, Inc. (Burlingame, CA, USA); antibodies against p21 and Cyclin B1 were obtained from BD Biosciences (San Jose, CA, USA); and an antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell lines and culture. SW480, HCT116 and HT-29 cell lines were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SW480 and HCT116 cells were cultured in Dulbecco's modified Eagle's medium/high-glucose medium with 10% fetal bovine serum (FBS; Hyclone, Waltham, MA, USA) in a humidified atmosphere (37°C, 5% CO2). HT-29 cells were cultured in 10% FBS/McCoy's 5a under the same conditions.

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For all experiments, the cells were grown to 90% confluency and harvested every 2-3 days.

**MTT assay.** An MTT assay was performed as described previously by Mosmann (13). MTT was dissolved in DMSO to 5 mg/ml. The cells were digested, counted and seeded onto 96-well culture plates at a density of 5x10^3 cells per well. Subsequently, the cells were incubated overnight and the culture medium was replaced with isorhamnetin at concentrations of 0, 10, 20, 40 and 80 µmol/l. After 3 days, 10 µl MTT (5 mg/ml) was added to each well and cells were cultured for another 4 h. The medium was then discarded and 150 µl DMSO was added to the culture wells. After gently agitating for 10 min with a table shaker (Premiere, Changzhou, China) at 40 rpm for 10 min, the samples were placed on a microplate reader (Biotek, Winooski, VT, USA) and the absorbance was detected at 550 nm. Data were calculated and the growth inhibition curve was constructed.

**Western blot analysis.** Cells were seeded onto 6-well plates following treatment with isorhamnetin (0, 20 and 40 µmol/l), and proteins were harvested and collected by NP-40 lysis buffer (Beyotime, Haimen, China). The protein concentrations were determined by a Bicinchoninic Acid Protein Assay kit. Subsequently, 40 µg of each protein sample was added to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Following electrophoresis at 100 V for 2 h, the proteins were transferred to polyvinylidene difluoride membranes at 350 mA for 90 min. The membranes were then blocked with 5% non-fat milk in Tris-buffered saline and Tween 20 (TBST), incubated with primary antibody at 4°C overnight, washed three times with TBST, incubated for 1 h at room temperature and washed again three times. The chemical signal was detected using an enhanced chemiluminescence detection system. The chemical detection instrument used was FluorChem FC2 (Cell Biosciences, Murrieta, CA, USA).

**PI staining.** Cells were seeded onto 6-well plates, treated with isorhamnetin (0, 20 and 40 µmol/l) for 24 h and harvested at the exponential phase. The samples were fixed with 75% ice-cold ethyl alcohol overnight at -20°C. On the following day, the cells were centrifuged at 300 x g for 5 min (Beckman, Brea, CA, USA) and added drop wise with ethyl alcohol. The samples were washed three times with phosphate buffered saline (PBS), resuspended in 100 µl PBS, combined with 10 µl of 500 µg/ml PI and 5 µl of 10 mg/ml RNase A, and then incubated for 2 h in the dark. Following this, 500 µl PBS was added prior to flow cytometry (FCM) analysis.

**Bromodeoxyuridine (BrdU) corporation.** Approximately 300 ng/ml BrdU (Sigma-Aldrich) was added to the medium, which was then incubated for 30 min prior to harvesting. Cells were collected and fixed with 75% ice-cold ethyl alcohol overnight at -20°C. On the following day, the samples were washed once and treated with 0.5 ml 2 M HCl for 40 min. Subsequently, 0.5 ml 0.1 M sodium borate (pH 8.5) was added for neutralization, and the samples were incubated with anti-BrdU for 1 h and a secondary antibody for 30 min. After washing three times, PI and RNase were added to the samples for another 30 min of incubation. Finally, all samples were analyzed by FCM.

Figure 1. Isorhamnetin inhibits the proliferation of colon cancer. (A) Chemical structure of isorhamnetin. (B) Curve of inhibition of CRCs and PBMNCs against isorhamnetin (MTT assay). (C) BrdU assay of two CRC lines. (D) Quantitative statistical analysis results of the BrdU assay. PBMNCs, peripheral blood mononuclear cells; CRCs, colorectal cancers; BrdU; bromodeoxyuridine
Statistical analysis. All statistical analyses were conducted using SPSS, version 12.0 (SPSS Inc., Chicago, IL, USA). The values are presented as the mean ± SD. Student's t-test was used to determine the statistical significance of the differences between the treatment group and the control group. The data were statistically analyzed by analysis of variance and P<0.05 was considered to indicate a statistically significant difference.

Results

Isorhamnetin suppresses the proliferation of CRC cells. The effect of isorhamnetin on the proliferation of CRC cells was determined by an MTT assay. The three cell lines were treated with isorhamnetin at five concentrations (0, 10, 20, 40 and 80 µmol/l) for three days. Peripheral blood mononuclear cells (PBMCs) were added to verify the cytotoxicity of isorhamnetin. The results shown in Fig. 1B indicated that the inhibition effect was dose dependent. The IC_{50} values of isorhamnetin were as follows: 56.24±1.25 µmol/l for SW480 cells, 54.87±2.13 µmol/l for HCT116 cells and 43.85±3.45 µmol/l for HT-29 cells. The IC_{50} value of isorhamnetin in PBMCs was 170 µmol/l. This result showed that the IC_{50} value of isorhamnetin in PBMCs was considerably higher than that of the three CRC cell types. Thus, it is possible to eliminate the cytotoxicity of isorhamnetin to non-tumor cells. The effect of isorhamnetin on HCT116 cells has previously been demonstrated (8), therefore the remaining two cell lines were selected for subsequent experiments.

A BrdU assay was directly used to detect cell proliferation, as shown in Fig. 1C and D. The results were similar to those of the MTT assay. At 40 µmol/l isorhamnetin, the average inhibition ratio was 10±4% (P=0.002) in the SW480 cell line and 12±3.6% (P=0.001) in the HT-29 cell line.

Isorhamnetin induces G2/M growth arrest in human CRC cells. PI staining was conducted to further investigate whether isorhamnetin affected the cell cycle. Following treatment with isorhamnetin at three concentrations (0, 20 and 40 µmol/l), the average proportions of the G2/M phase in each group were respectively as follows (Fig. 2): 11.6, 21 and 44.57% in SW480 cells; and 10, 10.9 and 40.83% in HT-29 cells. The G2/M phase of HT-29 cells increased by 9.5% at 20 µmol/l (P=0.003) and 32.8% (P=0.004) at 40 µmol/l. The increase in SW480 cells was 30.8% at 40 µmol/l (P=0.0067), but no obvious change was observed at 20 µmol/l (P=0.65). The result was similar in HCT116 cells (data not shown). These data suggested that isorhamnetin is able to induce cell cycle arrest at the G2/M phase in a dose-dependent manner.

Isorhamnetin inhibits the phosphorylation of Akt in CRC cells. Similar to other flavonoids, isorhamnetin may affect the receptor-tyrosine signaling pathway. Yang et al (14) reported that the phosphorylation of Akt may be suppressed by chrysoeriol, a type of flavonoid. It was hypothesized that isorhamnetin also affects the phosphorylation level of Akt in CRC cells and, therefore, in the present study possible changes were detected by western blot analysis. As shown in Fig. 3, the two cell lines in the dose gradient group were treated with isorhamnetin for 24 h at different concentrations. The results showed that phospho-Akt (ser473) was suppressed at 20 µmol/l isorhamnetin and the change was significant at 40 µmol/l isorhamnetin. All cells in the time gradient group were treated with the same concentration (20 µmol/l) at six time points, 0, 6, 12, 18, 24 and 36 h. It was identified that the phosphorylation level of Akt (ser473) was suppressed after 12 h treatment with isorhamnetin and the change was significant after 24 h. Thus, isorhamnetin downgraded the phosphorylation level of Akt (ser473) in a dose- and time-dependent manner.

Isorhamnetin affects the phosphorylation of p70S6K and 4E-BP1 protein. Isorhamnetin degraded the phosphorylation level of Akt; thus, on the following day it...
was determined whether isorhamnetin affected the other Akt-related proteins, particularly those downstream of Akt. Certain PI3K-AKT-mTOR-related proteins were detected and it was identified that the phosphorylation levels of the two predominant mTOR target proteins, p70S6K and 4E-BP1, were inhibited following treatment with isorhamnetin. The results are shown in Fig. 4. In contrast to other PI3K inhibitors, isorhamnetin did not induce feedback activation of Akt, as is the case with rapamycin. The inhibition effect of isorhamnetin on p70S6K and 4E-BP1 was similar to (although marginally lower than) that of LY294002. Thus, it was inferred that isorhamnetin inhibited the PI3K-Akt-mTOR pathway. Moreover, the phosphorylation levels of GSK3β protein were not changed (data not shown).

**Isorhamnetin increases the level of Cyclin B1 proteins.** To determine whether the G2/M arrest induced by isorhamnetin in CRC cells was due to the changes in cell cycle-related proteins, western blot analysis was performed to assess the expression of these proteins, including Cyclin B1, Cyclin D1 and p21. As shown in Fig. 5, the expression levels of Cyclin D1 were not changed in HT-29 cells but increased in SW480 cells; those of p21 were not changed in HT-29 cells but decreased in SW480 cells; and the expression levels of Cyclin B1 increased in the two cell types. The Cyclin B1-CDK1 complex was correlated with the G2-mitosis phase, i.e., the increase in Cyclin B1 expression levels prolonged the G2/M phase. Therefore, the
isorhamnetin-induced cell cycle arrest was associated with the increase in Cyclin B1 expression levels. Cyclin D1 and p21 expression levels did not change similarly in the two types of cells; thus, it was not possible to confirm the effect of isorhamnetin on these two cell cycle proteins.

Discussion

The PI3K-Akt-mTOR signaling pathway is important throughout the life of a cell, including during proliferation, glucose metabolism and for survival (15,16). In normal cells, this pathway benefits growth and metabolism, and its function is controlled by other regulatory proteins. One negative regulatory protein is phosphatase and tensin homolog (PTEN), which is known to be an antioncogene. The PTEN gene is usually inactivated due to mutation, deletion or epigenetic silencing. By contrast, the PI3K-Akt-mTOR pathway is frequently activated in tumorigenesis. Thus, this signaling pathway is widely recognized as a key regulator of cancer cells and is an important target of anticancer drugs (17,18). Numerous small-molecule inhibitors that inhibit the aberrant PI3K-Akt-mTOR pathway have been developed (19,20). However, a number of unidentified inhibitors may be toxic.

Rapamycin was the first mTORC1 inhibitor approved by the Food and Drug Administration (21). The number of studies in this field are increasing, but no satisfactory inhibitor that may be used to cure cancer has been identified. The possible reasons for this include incomplete inhibition and secondary activation. Rapamycin inhibits mTORC1 but simultaneously triggers a negative feedback mechanism that activates an upstream bypass (22). This bypass persistently activates the p-S6K1 protein, which is downstream of mTORC1 and leads to treatment failure.

Flavonoids are a group of natural plant secondary metabolites or yellow pigments, with a structure similar to that of flavones. They are most commonly known for their antioxidant activity in vitro. Flavonoids are able to inhibit tumor invasion, and their antiproliferative effects are associated with their structure (23). However, the potential mechanisms underlying their anticancer activity are unclear. Isohamnetin is a dietary flavonoid found in several fruits, such as apples, pears and blackberries. It is also a predominant plasma metabolite of quercetin. The expression pattern of cell cycle-related proteins was analyzed further. The cell cycle checkpoint is a monitoring mechanism that ensures the faithful replication of cells (27). Cyclin B1 is produced at the late S phase and degraded at the meta-mitosis phase. The Cyclin B1-CDK1 complex aids in G2/M transition, resulting in entry of the cells into mitosis. The G2/M transition phase is an important checkpoint of the cell cycle and a target for the inhibition of cell proliferation. A previous study has demonstrated that Cyclin B1 is enriched when cells are arrested at the G2/M checkpoint (32). When SW480 and HT-29 cells were treated with 40 µmol/l isorhamnetin, the expression of Cyclin B1 increased compared with that in the controls. Cyclin D1, which controls the G1/S checkpoint, did not change when the cells were treated with chrysoeriol. These results suggested that isorhamnetin predominantly induced G2/M cell cycle arrest, which may be attributed to the increase in Cyclin B1 expression levels.

All of the aforementioned biological activities were exerted in vitro by isorhamnetin at small concentrations; however, the values may not coincide with in vivo results. In conclusion, cancer is a proliferative disease characterized by an imbalance between oncogenes and antioncogenes. Cancer cells often grow rapidly, escape from the cell-cycle checkpoints and cause abnormal activation of certain signaling pathways. In the present study, isorhamnetin significantly inhibited the proliferation of CRC cells, delayed the G2/M cell cycle phase and inhibited the PI3K-Akt-mTOR pathway.
Thus, isorhamnetin may potentially serve as an agent for CRC therapy as evidenced by preliminary data. However, additional studies are required to explain the role of isorhamnetin as a PI3K-Akt-mTOR pathway inhibitor.

References