Isorhamnetin is a flavonoid metabolite of quercetin and isolated from water dropwort (Oenanthe javanica, Umbelliferae). It has been reported that isorhamnetin exerts beneficial effects including antioxidant, anti-inflammatory, and anti-proliferative activities. The present study investigated whether the antioxidant activity of isorhamnetin is correlated with its anti-cancer effects on colorectal cancer cells. Isorhamnetin significantly repressed cobalt chloride (CoCl₂) or hypoxia-induced hypoxia inducible factor-1α (HIF-1α) accumulation in HCT116 and HT29 cells. When compared with quercetin, isorhamnetin showed potent inhibition of HIF-1α. Moreover, it inhibited CoCl₂-induced activity of hypoxia response element reporter gene and HIF-1α-dependent transcription of genes such as glucose transporter 1, lactate dehydrogenase A, carbonic anhydrase-IX, and pyruvate dehydrogenase kinase 1. Isorhamnetin also blocked hydrogen peroxide (H₂O₂)-induced HIF-1α accumulation. The antioxidant effects of isorhamnetin were confirmed by observation of CoCl₂- or H₂O₂-induced reactive oxygen species (ROS) production. Consistently, overexpressed HIF-1α was decreased by isorhamnetin or N-acetyl-L-cysteine in HEK293 cells. In vitro migration and invasion assay further confirmed the inhibitory effects of isorhamnetin on cancer cells. Collectively, these results demonstrate that isorhamnetin inhibits ROS-mediated HIF-1α accumulation, which contributes to its anti-metastatic efficacy.

**Key words** colorectal cancer; hypoxia-inducible factor-1α; isorhamnetin; reactive oxygen species

Isorhamnetin, a 3′-O-methylated metabolite of quercetin (Figs. 1A, B), is a flavonoid-based compound, which is extracted from Oenanthe javanica, Umbelliferae. The beneficial effects of isorhamnetin such as anti-inflammation, antioxidant, and anti-cancer have been reported in various tissues. Isorhamnetin has been shown to relieve edema in carrageenan-induced inflammation. Moreover, it inhibits the expression of nuclear factor-kappa B (NF-κB)-dependent inflammatory genes in macrophages. In addition, isorhamnetin reduces oxidative stress due to free radicals by induction of NF-E2-related factor 2 (Nrf2)-dependent antioxidative genes. Recently, we also reported that the antioxidant activity of isorhamnetin contributes to its anti-inflammatory activity. In cancer cells, isorhamnetin inhibits proliferation by activating the apoptotic signal comprising Bax, caspase-3, and p53. Previous results on the anti-inflammatory and antioxidative effects of isorhamnetin suggest another possibility of suppression of growth and metastasis of cancer independent from the mechanism of inducing cell death. However, studies on the anti-tumour effect of isorhamnetin to date have been limited to the mechanism of suppressing the growth of cancer cells. In addition, studies regarding cancer cell-specific molecular or control mechanism regulated by isorhamnetin are insufficient.

The most important protein that regulates the growth and metastasis of cancer cells is the hypoxia inducible factor-1 (HIF-1). HIF-1α is accumulated at low oxygen environments and acts as a transcription factor for a variety of genes that play key roles in the proliferation and metastasis of cancer cells. At normal oxygen concentration, HIF-1α is hydroxylated by prolyl hydroxylase (PHD) and degraded by the ubiquitin-dependent proteasomal pathway. However, when exposed to a low oxygen concentration, HIF-1α protein stability is increased owing to a decrease in PHD activity, leading to its translocation into the nucleus and activity as a transcription factor in combination with HIF-1β. The proteins under the transcriptional control of HIF-1α are engaged in the regulation of cell proliferation, cell survival, apoptosis, angiogenesis, pH adjustment, and glucose metabolism, which promote proliferation and metastasis of cancer cells. Thus, HIF-1α has been recognized as a major molecular target for the inhibition of cancer.

Various natural products that inhibit HIF-1α have been reported. Hispidulin, a flavonoid from natural products, in-
hibits HIF-1α via AMP-activated protein kinase (AMPK) in gallbladder cancer cells. Luteolin can inhibit free radicals in the bronchial epithelial cells, which decreases HIF-1α. In addition, quercetin can suppress HIF-1α-dependent transcriptional activity in hypoxic colon cancer cells.

The anti-cancer activity of isorhamnetin has been identified in lung, liver, and gastric cancers. The mechanisms underlying the anti-cancer activity of isorhamnetin include induction of apoptosis, DNA cleavage, and chromosome condensation. However, isorhamnetin has not been reported to regulate HIF-1α. Therefore, in the present study, we examined whether isorhamnetin inhibits HIF-1α and exerts anti-cancer effects in colorectal cancer cells. In addition, we determined whether the antioxidant effect of isorhamnetin could contribute to the inhibition of migration and invasion of cancer cells.

**MATERIALS AND METHODS**

**Materials** Isorhamnetin was extracted from *Oenanthan javanica* and purified as previously described. Anti-HIF-1α antibody was obtained from BD Biosciences Pharmingen (San Jose, CA, U.S.A.). Anti-HIF-1α antibody was supplied by Proteintech (Chicago, IL, U.S.A.). Antibody against ubiquitin was provided by Cell Signaling Technology (Danvers, MA, U.S.A.). Cycloheximide (CHX), MG132, 5-fluorouracil (5-FU), and leupeptin were purchased from Calbiochem (San Diego, U.S.A.). Cyclohexamidine (CHM), MGI132, 5-fluorouracil (5-FU), and leupeptin were purchased from Calbiochem (San Diego, CA, U.S.A.). Anti-β-actin antibody, chloroquine, cobalt chloride (CoCl₂), 2,7'-dichlorofluorescin diacetate (DCFH-DA), hydrogen peroxide (H₂O₂), N-acetyl-l-cysteine (NAC), quercetin, and other reagents were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.).

**Cell Culture** HCT116 and HT29 cell lines were derived from human colorectal carcinoma epithelium, and were obtained from American Type Culture Collection (ATCC; Manassas, VA, U.S.A.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 µg/ml streptomycin, and cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. After continued incubation for 2–3 d (i.e., 80% confluency) with a change of serum-free medium overnight, cells were used for corresponding experiments. To generate hypoxia (condition of 1% O₂), the cells were exposed to 1% O₂, 5% CO₂, and 94% N₂ at 37°C in a hypoxia incubator (Galaxy 48R, New Brunswick [Eppendorf], Hamburg, Germany).

**Immunoblot Analysis** The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer as previously reported. Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, proteins were transferred onto a nitrocellulose membrane and blocked with 5% skim milk in phosphate buffered saline with 0.05% Tween-20. After blocking, the membrane was incubated with the primary antibody at 4°C overnight, followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were observed using an enhanced chemiluminescence (ECL) chemiluminescence system (GE Healthcare, Buckinghamshire, U.K.). Immunoblotting for β-actin confirmed equal amount of protein samples.

**Hypoxia Response Element (HRE) Reporter Gene Assay** To analyse HIF-1α transcriptional activity, HRE-A549 cells were prepared as previous published. Cells were placed in 12-well plates and transfected with pRL-TK control plasmid DNA (encoding for Renilla luciferase) using the Lipofectamine reagent (Invitrogen, San Diego, CA, U.S.A.) according to the procedure provided by the manufacturer. After 3 h, cells were recovered with minimum essential media supplemented with 1% FBS overnight. The Dual Luciferase Assay System (Promega, Madison, WI, U.S.A.) was used to measure the Renilla and HRE luciferase activities in cell lysates using Luminometer (Promega).

**RNA Isolation and Real-Time PCR Analysis** Real-time PCR analysis was performed as previously reported. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's protocol and reverse transcribed using oligo(dT)₁₆ primer and RT Premix (Bioneer, Daejeon, Korea). The amplified products were examined using StepOne (Applied Biosystems, Foster City, CA, U.S.A.) with an SYBR Green premix according to the manufacturer's instructions (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Real-time PCR amplifications were performed using the following primer pairs for these human genes: glucose transporter 1 (GLUT1) 5'-CGGGCGCAGGTGTAATCAAA-3' (forward) and 5'-TGGACGATACCCGAGCAATG-3' (reverse); lactate dehydrogenase A (LDH A) 5'-AGCCGCTTACGGATCAACTA-3' (forward) and 5'-CACCCGACATACTTCCATCCA-3' (reverse); carbonic anhydrase-IX (CA-IX) 5'-CCTTGGAGAAATTCGCTGTAGG-3' (forward) and 5'-TGTAGTTGCTCGCTGAGTCG-3' (reverse); pyruvate dehydrogenase kinase 1 (PDHK1) 5'-ACAGGAGGAGGTCCCGGATG-3' (forward) and 5'-CCAAGTTGGAGTTCGATTATGA-3' (reverse).

**Transient Transfection** HEK293 cells were transfected with 3 µg of pCDNA or HIF-1α gene-containing DNA plasmid using Fugene HD transfection reagent (Promega) following the manufacturer's protocol. After 3 h, the medium was changed and the cells recovered for an additional 18 h prior to treatment.

**Immunoprecipitation Assay** Ubiquitin binding to HIF-1α was analysed by immunoprecipitation assay in HCT116 cells, as described earlier. Briefly, cell lysates were prepared and 1.5 mg of protein was incubated with anti-HIF-1α antibody overnight at 4°C. Immune-complexes were immobilized on protein G-agarose beads (Upstate Biotechnology, Lake Placid, NY, U.S.A.) and resolved by 6% SDS-PAGE. A ubiquitin-bound HIF-1α protein was analysed by immunoblotting using an ubiquitin antibody.

**Measurement of Reactive Oxygen Species (ROS) Generation** Measurement of ROS generation was examined by using DCFH-DA in cells according to previously published procedures. Briefly, cells were incubated with 10 μM DCFH-DA dye for 1 h before harvest. The adherent cells were trypsinized and collected. The intensity of DCFH-DA fluorescence was determined using a fluorescence microplate reader (Gemini XPS, Molecular Device, Sunnyvale, CA, U.S.A.) at an excitation/emission wavelength of 485/525 nm. Protein concentration was used to normalize the ROS production, which was calculated relative to the vehicle-treated control according to the following formula: intracellular ROS production (fold)
of control) = [(relative fluorescence unit of treated sample) / (protein concentration of treated sample)] / [(relative fluorescence unit of vehicle-treated control) / (protein concentration of vehicle-treated control)].

**In Vitro Cell Migration and Invasion Assay** In vitro cell migration was analysed using transwell chamber containing a polycarbonate filter with pore size of 8 µm (Costar, Lowell, MA, U.S.A.) according to the manufacturer's instructions. The lower side of chamber was coated with Type I collagen (Corning, Bedford, MA, U.S.A.), and the 24-well plate was supplied with serum-free media containing 0.1% bovine serum albumin (BSA). Cells (2 × 10^5) were seeded into the upper chamber and incubated with or without serum (10% FBS) for 24 h at 37°C. After 24 h, for the migration assay, the cells in the upper surface of chamber (non-migratory cells) were removed and those in the lower surface of chamber (migrated cells) were fixed with ice-cold methanol and stained with 0.5% crystal violet. Images of the migrating cells were captured using a light microscope (magnification, ×100). The numbers of the migrated cells were counted, and expressed as relative fold. In vitro cell invasion assay was conducted in a BioCoat Matrigel Invasion Chamber (Becton-Dickinson, Bedford, MA, U.S.A.) with a membrane coated with matrigel, and other procedures were the same as an in vitro cell migration assay.

**Statistical Analysis** The data are presented as the mean ± standard error (S.E.) from at least three independent experiments. We performed one-way or two-way ANOVA, followed by Bonferroni's correction for multiple comparisons. A p-value less than 0.01 or 0.05 was considered statistically significant.

**RESULTS**

**Inhibition of HIF-1α Accumulation by Isorhamnetin**

In an effort to identify isorhamnetin as an HIF-1α inhibitor, we investigated whether isorhamnetin reduces HIF-1α accumulation in two different colorectal cancer cells. First, to demonstrate the effects of isorhamnetin on HIF-1α protein level, HCT116 and HT29 cells were cultured under hypoxia (1% O2) or normoxia (21% O2). A dramatic increase of HIF-1α protein was detected in HCT116 (Fig. 2A, left) and HT29 cells (Fig. 2A, right) cultured under hypoxic conditions. However, isorhamnetin (30 µM) significantly reduced hypoxia-induced HIF-1α accumulation in HCT116 (Fig. 2A, left) and HT29 cells (Fig. 2A, right). Quercetin (30 µM) also inhibited HIF-1α accumulation, but the inhibitory efficacy was lower than that of isorhamnetin (Fig. 2A). To confirm isorhamnetin as an HIF-1α inhibitor, CoCl2 (a hypoxia mimetic agent) was used. Treatment with CoCl2 markedly increased HIF-1α accumulation in HCT116 (Fig. 2B, left) and HT29 cells (Fig. 2B, right). Similar to the above results, isorhamnetin significantly suppressed the accumulation of HIF-1α by CoCl2 in HCT116 (Fig. 2B, left) and HT29 cells (Fig. 2B, right). However, the inhibitory effect of quercetin on HIF-1α was detected only in HT29 cells (Fig. 2B, right). Total levels of HIF-1α were not changed by isorhamnetin or quercetin treatment (Fig. 2B). Next, we examined the concentration-dependent effects of isorhamnetin on HIF-1α. At 30 or 60 µM, isorhamnetin significantly reduced HIF-1α accumulation in HCT116 (Fig. 2C, left) and HT29 cells (Fig. 2C, right), respectively. At 3–60 µM, isorhamnetin did not alter cell viability (data not shown). These results indicate that isorhamnetin inhibits HIF-1α accumulation in colorectal cancer cells, and its efficacy is higher than that of quercetin.

**Repression of HIF-1α-Target Gene Expression by Isorhamnetin**

To examine whether isorhamnetin affects HIF-1α-dependent gene transcription, mRNA levels of HIF-1α target genes were observed. Hypoxia increased the mRNA expression level of GLUT1, LDH A, CA-IX, and PDK1 in HCT116 cells (Fig. 3A). However, the increased mRNA levels of HIF-1α-target genes were significantly reduced by isorhamnetin (Fig. 3A). Next, to determine the effects of isorhamnetin on HRE-dependent gene transcription, HRE-luciferase activity assay was conducted in cells stably transfected with an HRE reporter construct. The activity of HRE luciferase was enhanced by CoCl2 treatment for 24 h, but was effectively decreased by pre-treatment with isorhamnetin (Fig. 3B). These results indicate that isorhamnetin attenuates HIF-1α-dependent gene regulation in colorectal cancer cells.

**Effect of Isorhamnetin on HIF-1α Protein Degradation**

Series of studies have been shown that protein degradation is important to regulate HIF-1α.6) To explore the underlying mechanism of HIF-1α inhibition by isorhamnetin, inhibitors of proteasome or lysosome were used. First, to assess whether isorhamnetin promotes ubiquitination-dependent proteasomal degradation of HIF-1α, ubiquitination of HIF-1α was detected by immunoprecipitation assay. Polyubiquitinated HIF-1α was clearly detected by MGI32 with CoCl2 treatment (Fig. 4A, lane 4). However, simultaneous isorhamnetin treatment with CoCl2 had no effect on ubiquitination of HIF-1α protein (Fig. 4A). In addition, isorhamnetin did not facilitate the ubiquitination of HIF-1α under MGI32 (Fig. 4B). Moreover, isorhamnetin clearly reduced HIF-1α protein levels even in the presence of MGI32 (Fig. 4B). To determine whether lysosomal degradation was involved in the action of isorhamnetin, two lysosomal inhibitors (chloroquine, leupeptin) were used. Treatment with chloroquine or leupeptin resulted in a remarkable accumulation of HIF-1α, but isorhamnetin lowered the levels of HIF-1α protein (Fig. 4C). These results indicate that effect of isorhamnetin on HIF-1α protein degradation could not explain its mechanism of HIF-1α inhibition.

**Antioxidant Effect of Isorhamnetin on HIF-1α Accumulation**

ROS are produced under hypoxic condition and contribute to the increase in HIF-1α.8) In addition, we previously reported that isorhamnetin has antioxidant activity.4) Hence, we further examined whether the antioxidant effect of isorhamnetin is involved in HIF-1α inhibition. First, ROS production was detected in HCT116 cells. Concomitant treatment with isorhamnetin blocked the CoCl2- or H2O2-induced ROS production (Figs. 5A, B). Moreover, treatment with isorhamnetin potently inhibited H2O2-induced HIF-1α accumulation (Fig. 5C). The lysate from hypoxia-exposed cell was loaded as a positive control for HIF-1α detection (Fig. 5C). Immunoblot analysis confirmed that isorhamnetin reduced the overexpressed HIF-1α level in HEK293 cells. Furthermore, NAC (an antioxidant) treatment inhibited HIF-1α, confirming that blockade of ROS production was implicated in the process of HIF-1α regulation (Fig. 5D). These data suggest that the antioxidant effects of isorhamnetin might be responsible for HIF-1α inhibition.

**Inhibition of in Vitro Cancer Cell Migration and Invasion by Isorhamnetin**

To investigate the functional role of HIF-1α inhibition by isorhamnetin, in vitro cancer cell
Fig. 2. Inhibition of Hypoxia Inducible Factor-1α (HIF-1α) Accumulation by Isorhamnetin

(A) Immunoblot analysis. HCT116 (left) or HT29 (right) cells were treated with isorhamnetin (30 µM) or quercetin (30 µM) for 1 h, and continuously incubated under the condition of normoxia (21% O₂) or hypoxia (1% O₂) for 3 h. Lysates from these cells were used to determine the protein level via immunoblotting. The arrow denotes non-specific bands. The blots shown are representative of at least three different replicates; **p < 0.01 when compared to cells under normoxia; *p < 0.05 or †p < 0.01 when compared to cells under hypoxia.

(B) Immunoblot analysis. HCT116 (left) or HT29 (right) cells treated with isorhamnetin (30 µM) or quercetin (30 µM) for 1 h, and continuously exposed to CoCl₂ (100 µM) for 6 h (left) or 3 h (right). Lysates from these cells were used to determine protein level via immunoblotting. The blots shown are representative of at least three different replicates; **p < 0.01 when compared to the vehicle-treated cells; †p < 0.01 when compared to the CoCl₂-treated cells; N.S., not significant.

(C) Immunoblot analysis. HCT116 (left) or HT29 (right) cells treated with isorhamnetin (3–60 µM) for 1 h, and continuously exposed to CoCl₂ (100 µM) for 6 h (left) or 3 h (right). Lysates from these cells were used to determine protein level via immunoblotting. The blots shown are representative of at least three different replicates; **p < 0.01 when compared to the vehicle-treated cells; †p < 0.05 or ††p < 0.01 when compared to the CoCl₂-treated cells.
Fig. 3. Repression of HIF-1α-Target Gene Expression by Isorhamnetin

(A) Real-time PCR assays. HCT116 cells were treated with isorhamnetin (IsoR, 30 µM) for 1 h, and continuously incubated under the condition of normoxia (21% O₂) or hypoxia (1% O₂) for 3 h. The results shown are the mean±S.E. of at least three different replicates; **p<0.01 when compared to cells under normoxia; ##p<0.01 when compared to cells under hypoxia. (B) HRE reporter gene assay. HRE-A549 cells were treated with isorhamnetin (30 µM) for 1 h, and continuously exposed to CoCl₂ (100 µM) for 24 h. Luciferase activity was measured in the cell lysates. The results shown are the mean±S.E. of at least three replicates; **p<0.01 when compared to the vehicle-treated cells; #p<0.05 when compared to the CoCl₂-treated cells.

Fig. 4. The Effect of Isorhamnetin on HIF-1α Protein Degradation

(A) Immunoprecipitation assay. HCT116 cells that had been transfected with the plasmid encoding ubiquitin were treated with vehicle or isorhamnetin (30 µM) for 1 h, and continuously exposed to CoCl₂ (100 µM) for 6 h. Immunoprecipitation assay was conducted using the cell lysates. HIF-1α immunoprecipitates were immunoblotted with anti-ubiquitin antibody (upper). Ten percent of precipitate with anti-HIF-1α antibody was immunoblotted by anti-HIF-1α antibody (middle). HIF-1α and β-actin were immunoblotted in cell lysates (lower). CoCl₂ with MG132 (10 µM) treated sample was used for positive control for detection of HIF-1α polyubiquitination by anti-ubiquitin antibody. The blots shown are representative of three different replicates. (B) Immunoprecipitation assay. HCT116 cells that had been transfected with the plasmid encoding ubiquitin were pre-treated with MG132 (10 µM) for 3 h and then treated with vehicle or isorhamnetin (30 µM) for 1 h, and continuously exposed to CoCl₂ (100 µM) for 6 h. Immunoprecipitation assay was conducted using the cell lysates. HIF-1α immunoprecipitates were immunoblotted with anti-ubiquitin antibody (upper). Ten percent of precipitate with anti-HIF-1α antibody was immunoblotted by anti-HIF-1α antibody (middle). HIF-1α and β-actin were immunoblotted in cell lysates (lower). The blots shown are representative of three different replicates. (C) Immunoblot analysis. HCT116 cells were treated with chloroquine (50 µM) or leupeptin (50 µg/mL) for 6 h, and continuously exposed to isorhamnetin (30 µM) for 6 h. The blots shown are representative of at least three different replicates; *p<0.05 when compared to the vehicle-treated cells; #p<0.05 when compared to the chloroquine or leupeptin-treated cells.
migration and invasion assays were conducted. Stimulation with 10% serum-condition significantly promoted the migration of HCT116 cells, but isorhamnetin markedly repressed the serum-induced in vitro cell migration (Fig. 6A). The inhibitory effect on the serum-induced cell migration was also observed with NAC treatment (Fig. 6A). Next, to demonstrate whether isorhamnetin affects cell invasion, in vitro cell invasion assay was performed using matrigel coated-invasion chamber in 10% serum-condition. Consistently, the invasive population of HCT116 cells was increased by serum treatment, but was notably suppressed in the presence of isorhamnetin or NAC (5 or 10mM) for 6h. Lysates from these cells were used to determine the protein level via immunoblotting. The blots shown are representative of at least three different replicates; **p<0.01 when compared to the vehicle-treated cells; ##p<0.01 when compared to the H2O2-treated cells.

**DISCUSSION**

The present study was designed to investigate the anti-cancer effects of isorhamnetin via HIF-1α inhibition on human colorectal cancer cells. When compared with quercetin, isorhamnetin effectively reduced the accumulation of HIF-1α protein under hypoxic condition. When administrated via oral routes, quercetin is metabolized to isorhamnetin by catechol-O-methyltransferase in the intestine and/or liver. Previous studies reported that isorhamnetin exhibits better intestinal absorption and hepatic metabolic stability, and a much longer T_max than quercetin. Therefore, as a therapeutic
agent, isorhamnetin is considered to have more favourable pharmacokinetic property than unmethylated quercetin.

Flavonoid-based compounds such as hispidulin, luteolin, and quercetin have been reported to inhibit HIF-1α in various cancer cells.9–11) Although studies on isorhamnetin have reported its anti-cancer efficacy, direct effects on HIF-1α regulation have not been identified. In this study, we observed that isorhamnetin inhibited HIF-1α following induction of HIF-1α accumulation in HCT116 and HT29 cells using hypoxia or CoCl2 (Fig. 2). In addition, isorhamnetin significantly reduced HIF-1α protein accumulated by overexpression in HEK293 cells (Fig. 5D). The inhibitory effect on HIF-1α mediated transcription by isorhamnetin was confirmed through mRNA levels of HIF-1α target genes including GLUT1, LDH A, CA-IX, and PDK1 (Fig. 3A). HIF-1α induces GLUT1, which increases glucose uptake, thereby enhancing the process of glucose utilization under hypoxia.5,7) In addition, HIF-1α upregulates the expression of LDH A, which catalyses the conversion of pyruvate to lactic acid.5,7) However, outflow of acid into the extracellular space causes acidosis. Therefore, HIF-1α induced CA-IX to regulate the intracellular pH in tumour growth and metastasis.5,7) In addition, HIF-1α induces the transcriptional expression of PDK1, inhibiting oxidative phosphorylation in mitochondria.26) Consequently, inhibition of HIF-1α by isorhamnetin contributes to suppress the expression of proteins involved in survival and metastasis of cancer cells.

HIF-1α is unstable at normal oxygen conditions, and protein degradation is recognized as a key step in controlling the amount of HIF-1α protein.5) Under normal oxygen conditions, the two proline residues (402, 506) of HIF-1α are hydroxylated by PHD.5) The hydroxylated HIF-1α is recognized by the von Hippel–Lindau tumour suppressor protein, leading to ubiquitination and proteasomal degradation.6) Based on these previous reports, we examined whether the inhibitory effect on HIF-1α by isorhamnetin results from accelerated protein degradation. However, isorhamnetin did not affect ubiquitination of HIF-1α (Fig. 4B). Isorhamnetin lowered the level of HIF-1α even if proteasomal degradation was reduced by MG132 (Fig. 4B). Moreover, isorhamnetin also decreased HIF-1α accumulation by lysosome inhibition (Fig. 4C).

According to the study by Li et al., dihydrotanshinone I (DHTS) inhibits HIF-1α protein synthesis rather than promot-
ing its degradation process.\textsuperscript{27} DHTS suppresses the signal cascade of mammalian target of rapamycin (mTOR)/p70 S6 kinase/ELF4E binding protein-1 and extracellular signal-regulated kinase (ERK), which inhibits protein synthesis of HIF-1\textalpha.\textsuperscript{27} Another independent study indicated that phosphatidylinositol 3-kinase (PI3K) and Akt are involved in the HIF-1\textalpha protein synthesis; hence, HIF-1\textalpha can be suppressed when PI3K-Akt is inhibited.\textsuperscript{28} Based on these previous findings, isorhamnetin might have the potential to inhibit HIF-1\textalpha through a protein synthesis inhibition. Therefore, it is necessary to validate the signalling pathways that regulate protein synthesis such as mTOR, ERK, or PI3K. In addition, we observed that isorhamnetin reduced mRNA level of HIF-1\textalpha (Supplementary Fig. 2). Several studies have been reported that a naturally occurring compound with antioxidant activity including curcumin or aingerprint represses induction of mRNA synthesis of HIF-1\textalpha.\textsuperscript{29,30} Further detailed study is needed to understand the inhibition mechanisms of HIF-1\textalpha mRNA or protein synthesis by isorhamnetin.

Isorhamnetin activates Nrf2 to increase the binding of the promoter region of the gene antioxidant response element.\textsuperscript{31} This induces a variety of antioxidant proteins such as glutamate cysteine ligase and Sestrin2, and shows a cytoprotective effect.\textsuperscript{32} In addition, isorhamnetin induces heme oxygenase-1 to reduce the free radicals, which result in the inhibition of cyclooxygenase-2 and inflammatory response.\textsuperscript{33} We also observed the result that isorhamnetin decreased ROS in colorectal cancer cells and suppressed the accumulation of HIF-1\textalpha caused by free radicals (Figs. 5A–C). Based on the experiment with the antioxidant NAC, the possibility that isorhamnetin inhibited cancer cell migration and invasion due to its antioxidant effect was confirmed (Fig. 6). These results indicate that isorhamnetin suppresses ROS, which can be increased easily in a low-oxygen environment. Finally, this could contribute to the suppression of HIF-1\textalpha in cancer cells.

In summary, the present study shows that the antioxidant effect of isorhamnetin inhibits HIF-1\textalpha and contributes to the inhibition of in vitro cancer cell migration and invasion. This suggests the possibility that isorhamnetin could be used as an anti-cancer agent. Moreover, the presented mechanisms can explain the anti-cancer efficacy of various natural compounds having an antioxidant effect.

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Conflict of Interest The authors declare no conflict of interest.

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