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The Flavonol Isorhamnetin Exhibits Cytotoxic Effects on Human Colon Cancer Cells

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The aim of this study was to determine whether isorhamnetin, an immediate 3′-*O*-methylated metabolite of quercetin, affects proliferation, cell death, and the cell cycle of human colon carcinoma (HCT-116) cells. Isorhamnetin was found to be a potent antiproliferative agent in a dose- and time-dependent manner, with an IC_{50} of 72 μM after 48 h of incubation as estimated by MTT assay. Flow cytometry and fluorescence microscopy analysis showed that isorhamnetin exerted a stimulatory effect on apoptosis and necrosis. Isorhamnetin also increased the number of cells in G2/M phase. Serum deprivation appeared to potentiate the effects of isorhamnetin on cell death and facilitated cell cycle progression to G0/G1 phase. These results suggest that isorhamnetin might mediate inhibition of HCT-116 cell growth through the perturbation of cell cycle progression and are consistent with the notion that G2/M checkpoints could be a conserved target for flavonoids in human colon cancer cells, leading to apoptotic and necrotic death. These antiproliferative, apoptotic, necrotic, and cell cycle effects suggest that isorhamnetin may have clinically significant therapeutic and chemopreventive capabilities. To our knowledge, this is the first report of the effect of isorhamnetin on human colon cancer cells.

KEYWORDS: Isorhamnetin; cell growth; apoptosis; necrosis; cell cycle; colon cancer

INTRODUCTION

Colon cancer represents almost 10% of all tumors and is the third most common cancer in men in modern countries (after lung and prostate cancers) and the second in women (after breast cancer), with approximately 1 million new cases each year worldwide. Only 5−10% of the cases are due to genetic factors, while more than 70% are related to diet and lifestyle, suggesting that colon cancer rates could be substantially reduced by changes in dietary and lifestyle patterns (*1*). Epidemiological studies have associated a diet high in vegetables, fruits, cereals and seeds with a lower risk of colon cancer. In particular, food constituents such as polyphenols have been reported to reduce colon cancer risk experimentally (*2*).

One of the major polyphenols in the human diet is quercetin, a naturally occurring flavonoid that has antioxidative and antiproliferative effects in a wide variety of cancer cell lines (*3*). Once ingested, quercetin is extensively metabolized to form methylated, sulfated and glucuronidated phase II conjugates (*4*) (**Figure 1A**), which is a process common to many xenobiotics that restricts their potential toxic effects and facilitates biliary and urinary elimination by increasing their hydrophilicity. The 3′-hydroxyl group is an important target for phase II metabolism of quercetin (*5*), and conjugation of this hydroxyl group with the catechol moiety of quercetin is thought to attenuate its biological activity compared with aglycon. An intermediate 3′-*O*-methylated metabolite of quercetin is isorhamnetin (3′-*O*-methylquercetin) (**Figure 1B**), a flavonol which has recently been reported to highly accumulate in xenograft tumors (*6*) and to have antioxidant and antitumor activity on human hepatocellular (*7*) and human esophageal squamous (*8*) cancer cells. Isorhamnetin also prevents endothelial cell injuries caused by oxidized low-density lipoprotein (*9*) and angiotensin II (*10*), and inhibits adipogenesis through downregulation of PPARγ and C/EBPα in 3T3-L1 cells (*11*).

In addition to isorhamnetin metabolically derived from quercetin, different fruits and vegetables constitute a primary source of isorhamnetin (*12*). It is interesting to note that the bioavailability of isorhamnetin is thought to be higher than that of quercetin, because the absorption and metabolic stability of methylated flavonoids are dramatically increased when compared with unmethylated parent molecules (*13*, *14*), indicating that intrinsic biological activities of isorhamnetin should be tested thoroughly. In order to enhance understanding regarding the cytotoxicity of isorhamnetin against cancer cells in general and for treatment of colon cancer in particular, we investigated the *in vitro* chemotherapeutic potential of isorhamnetin on HCT-116 cells, an aggressive, microsatellite unstable, and growth hormone independent human colon cancer line.

MATERIALS AND METHODS

Materials. All cell culture reagents were purchased from Gibco (Madrid, Spain). Isorhamnetin was purchased from Extrasynthese

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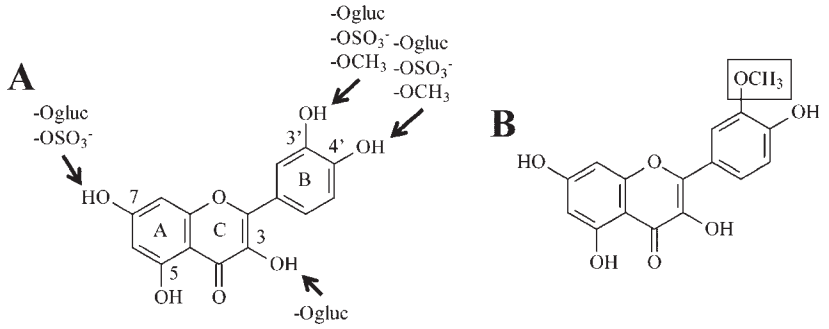

Figure 1. Structure of quercetin aglycon (**A**), numbering of relevant carbon atoms and the identified sites of conjugation, and isorhamnetin (**B**). Metabolites of quercetin include methylated ($-OCH_3$), sulfated ($-OSO_3^-$) and glucuronidated ($-$Ogluc) counterparts.

(Lyon, France). The purity of isorhamnetin (> 99%) was confirmed by HPLC-DAD−MS. A 100 mM stock solution of isorhamnetin was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C until cell culture studies. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide), DMSO, Triton X-100, EDTA, RNase-A, annexin V, and propidium iodide (PI) were purchased from Sigma-Aldrich (Madrid, Spain).

Cell Culture. HCT-116, a colon adenocarcinoma cell line, was obtained from the American Type Culture Collection (Bethesda, MD; ATCC #CCL 247). Cells were grown at 37 °C with 5% CO_2 and 90% relative humidity in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Cell Viability Assay. Cell viability was assayed based on the ability of live cells to reduce MTT. HCT-116 human colon cancer cells were cultured in 96-well plates in triplicate at a density of 5×10^4 cells/well in 200 μL of medium. The cells were grown to 70% confluence, and the media supplemented with the indicated concentrations of isorhamnetin in DMSO. The final concentration of DMSO in culture medium was maintained at < 0.001%, and 5-fluorouracyl (5-FU, 5 μM) was used as a positive control. Cells were incubated for 24 and 48 h, and then 20 μL of the MTT solution (5 mg/mL in PBS) was added to each well. Cells were incubated for 3 h at 37 °C in a humidified chamber with 5% CO_2. The concentration of formazan was measured spectrophotometrically at 490 nm using a Multiskan Spectrum microplate reader (Thermo Labsystems, MA, USA). All MTT assays were carried out in three separate trials.

Cell Cycle Analysis by Flow Cytometry. Cells were grown in 24-well plates at 37 °C under 5% CO_2 until 90% confluence was reached. The medium was then changed, and isorhamnetin and 5-FU were added to the indicated concentrations. The cells were incubated at 37 °C for 24 and 48 h. After incubation, the cells were harvested, and washed three times with ice-cold PBS (pH 7.4). The supernatant was removed, and the cells were washed with 1.0 mL of PBS and centrifuged at 4 °C. Finally, the supernatant was carefully removed and the cell pellet was resuspended in 200 μL of 70% ice-cold ethanol and 200 μL of PBS and stored at −80 °C until further use. For use in flow cytometry experiments, the cell pellet was washed twice more with PBS. The cell pellet was suspended in 0.5 mL of staining reagent (50 μg/mL PI, 50 U/mL RNase, 0.1 mM EDTA, 0.1% Triton X-100, and PBS) and incubated for 30 min at 37 °C in the dark. DNA fluorescence was measured using a Becton Dickinson (BD) FACScanto II flow cytometer with an excitation wavelength of 488 nm and emission wavelength of 585 nm. Pulse width area signals were used to discriminate between G2 cells and cell doublets. The data were analyzed using FACSDiva Software (BD). The relative distribution of 10000 events for each sample was analyzed for background aggregates and debris, an indicator of apoptosis as well as G0/G1, S, and G2/M phases of the cell cycle.

Assessment of Apoptosis and Necrosis. Apoptosis and necrosis were assessed using a Vybrant Apoptosis Assay kit # 2 (Molecular Probes, Barcelona, Spain) according to the manufacturer's instructions. Apoptotic cells were labeled with annexin V conjugated to green-fluorescent Alexa Fluor 488 dye, and necrotic cells were labeled with red-fluorescent PI. These populations were analyzed using a BD FACScanto II flow cytometer with an excitation wavelength of 488 nm and a 530 nm filter for the detection of Alexa Fluor 488 and a 585 nm filter for PI detection. The data were analyzed using FACSDiva Software (BD). At least 10000 events for each sample were analyzed and gated according to light scattering properties.

For microscopy, cells were stained using a Vybrant Apoptosis Assay kit # 2 (Molecular Probes, Barcelona, Spain) according to the manufacturer's instructions and observed under an Olympus IX81 motorized inverted fluorescence microscope (Tokyo, Japan) equipped with FITC and Texas Red filtersets. Images were recorded using a MegaView II CCD camera (Soft Imaging System) and analyzed using a Cell R software (Olympus). Apoptotic cells were stained with annexin V conjugated to green-fluorescent Alexa Fluor 488 dye. Cells were dually stained with PI to mark necrosis.

Statistical Analysis. StatGraphics Plus software was used for statistical analyses. All quantitative data are represented as mean ± SD from triplicate experiments performed in parallel unless otherwise indicated. Mean values among treatment groups were compared by the ANOVA test followed by Duncan's multiple comparison test. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Isorhamnetin Induces Growth Inhibition of HCT-116 Human Colon Cancer Cells. Using the HCT-116 human colon cancer cell line, we first evaluated the effect of isorhamnetin on cell proliferation by MTT assay, which measures the appearance of a formazan product that is directly proportional to the number of living cells in the culture. HCT-116 cells were treated with different concentrations (0−100 μM) of isorhamnetin for 24 and 48 h. Cell viability in the presence of complete medium or the DMSO control alone was not affected (data not shown). However, HCT-116 cells were sensitive to isorhamnetin in a dose- and time-dependent manner (**Figure 2A**). The IC_{50} values of isorhamnetin for HCT-116 cells were 224 μM and 72 μM after 24 and 48 h of incubation, respectively. In addition, we examined whether serum deprivation could play a role in the survival of isorhamnetin-treated HCT-116 cells. Treatment of cells with 100 μM isorhamnetin for 48 h in serum-deprived medium led to a significant increase ($P < 0.05$) of cell death compared with cells grown in complete medium (**Figure 2B**).

Isorhamnetin Induces Apoptosis and Necrosis of HCT-116 Human Colon Cancer Cells. As resistance to apoptosis is critical for cell survival and also contributes to drug resistance of cancer cells, we studied whether isorhamnetin induced apoptosis in HCT-116 cells. There was a dose- and time-dependent increase in apoptosis (**Figure 3A**) and necrosis (**Figure 3B**), as visualized by the Annexin V/PI staining assay, with maximum apoptosis and necrosis observed at a dose of 75 μM isorhamnetin for 24 and 48 h. 5-Fluorouracyl was used as a positive control for apoptosis and necrosis. These findings were further confirmed by fluorescence microscopy experiments in which treatment of cells with isorhamnetin enhanced green and red fluorescence when compared with control cells, indicating that the flavonol increased apoptosis

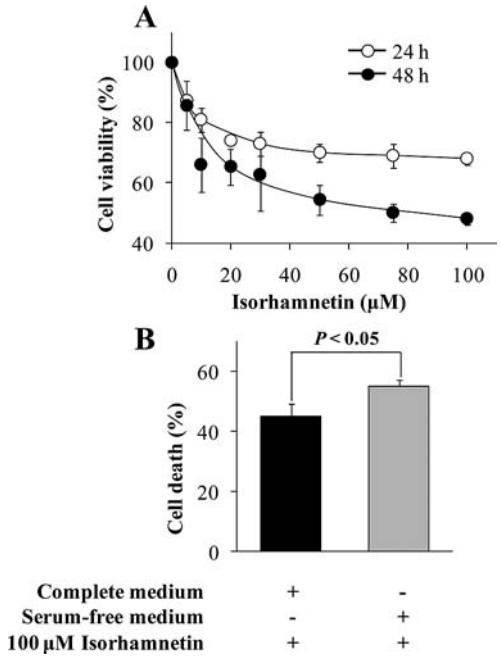

Figure 2. Effect of isorhamnetin on viability of HCT-116 human colon cancer cells assessed by MTT assay. (**A**) HCT-116 cells were treated with isorhamnetin (0−100 μM) in complete medium for 24 and 48 h. (**B**) HCT-116 cells were treated with 100 μM isorhamnetin in the presence (complete medium) or absence (serum-free medium) of 10% FBS for 48 h. Cell viability was assessed by MTT assay as described in Materials and Methods. In panel **A**, HCT-116 cells treated with complete medium were taken as 100% of cell viability. The data represent the average median of at least three independent experiments (by triplicate) ± SD. $P < 0.05$ was determined by one-way analysis of variance followed by Duncan's test.

and necrosis (**Figure 4A**). Proportions of apoptotic and necrotic cells following treatment of cells with 75 μM isorhamnetin for 24 h are shown in representative plots of FACS analysis (**Figure 4B**).

Isorhamnetin Induces G2/M Growth Arrest of HCT-116 Human Colon Cancer Cells. Apoptosis and cell cycle deregulation are closely related events, and disruption of cell cycle progression may ultimately lead to apoptotic/necrotic death. Isorhamnetin, at doses up to 75 μM, doubled the fraction of HCT-116 cells in G2/M phase after 24 h (**Figure 5A** and **5B**) and 48 h (data not shown) of treatment compared with controls. The G0/G1 phase was correspondingly decreased after isorhamnetin treatment, suggesting that isorhamnetin may induce G2/M growth arrest in HCT-116 cells. These results also indicate that isorhamnetin-induced apoptosis and necrosis in HCT-116 cells is likely to involve modulation of cell cycle progression. Isorhamnetin-treated HCT-116 cells also remained at G0/G1 phase in parallel cultures in serum-deprived medium (**Figure 5C**).

DISCUSSION

Flavonoids are a group of naturally occurring polyphenol compounds that are ubiquitous in the vegetable kingdom and are widely present in the human diet. In addition to their antioxidant function, their antiproliferative effects are considered to be among the most therapeutically utilizable of these flavonoid activities, which are related to their structure (*3*). These compounds commonly have the basic skeleton of phenylbenzopyrone ($C_6-C_3-C_6$) consisting of 2 aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated central pyran ring, or C ring (**Figure 1A**). Quercetin is one of the major dietary flavonoids with antiproliferative efficacy on a range of cancer cells including

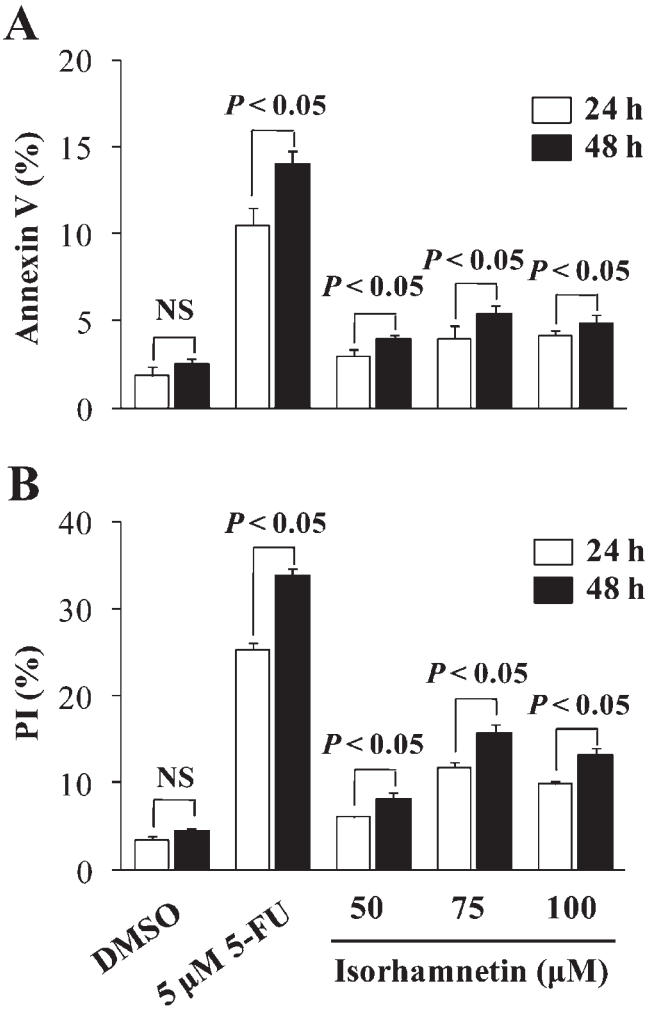

Figure 3. Effect of isorhamnetin on apoptosis and necrosis of HCT-116 human colon cancer cells assessed by FACS analysis. HCT-116 cells were treated with dimethylsulfoxide (DMSO), 5 μM 5-fluorouracyl (5-FU), or isorhamnetin (50, 75, and 100 μM) in complete medium for 24 and 48 h. Cells were stained with annexin V (**A**) or propidium iodide (PI) (**B**), and subjected to FACS analysis as described in Materials and Methods. The data represent the average median of at least three independent experiments (by triplicate) ± SD. $P < 0.05$ was determined by one-way analysis of variance followed by Duncan's test.

colon cancer cells *in vitro* (*15*, *16*) and *in vivo* (*17*, *18*). The effects of quercetin may in large part be mediated by its metabolites, in particular isorhamnetin (3′-*O*-methylquercetin) (*19*). According to pharmacokinetic studies in animal models, isorhamnetin remains elevated in plasma longer than quercetin and is not transformed back into its unmethylated parent molecule (*20*). The oral bioavailability, hepatic metabolic stability, and tissue uptake of quercetin has also shown to be dramatically enhanced by methylation (*13*). More importantly, it has been suggested that methyl capping of flavonoid free hydroxyl groups could greatly improve their intrinsic cancer chemotherapeutic activity (*14*). However, the antiproliferative effects of isorhamnetin in colon cancer cells are still not understood. Our study aimed to examine the effects of isorhamnetin in HCT-116 cells.

Our results show that isorhamnetin can induce both dose- and time-dependent growth inhibitory effects in HCT-116 cells. Isorhamnetin was previously shown to inhibit proliferation of human hepatocellular carcinoma BEL-7402 cells (*7*), human esophageal tumor cell line Eca-109 (*8*), and murine Lewis lung cancer (LLC) cells (*21*). We found that isorhamnetin exhibited

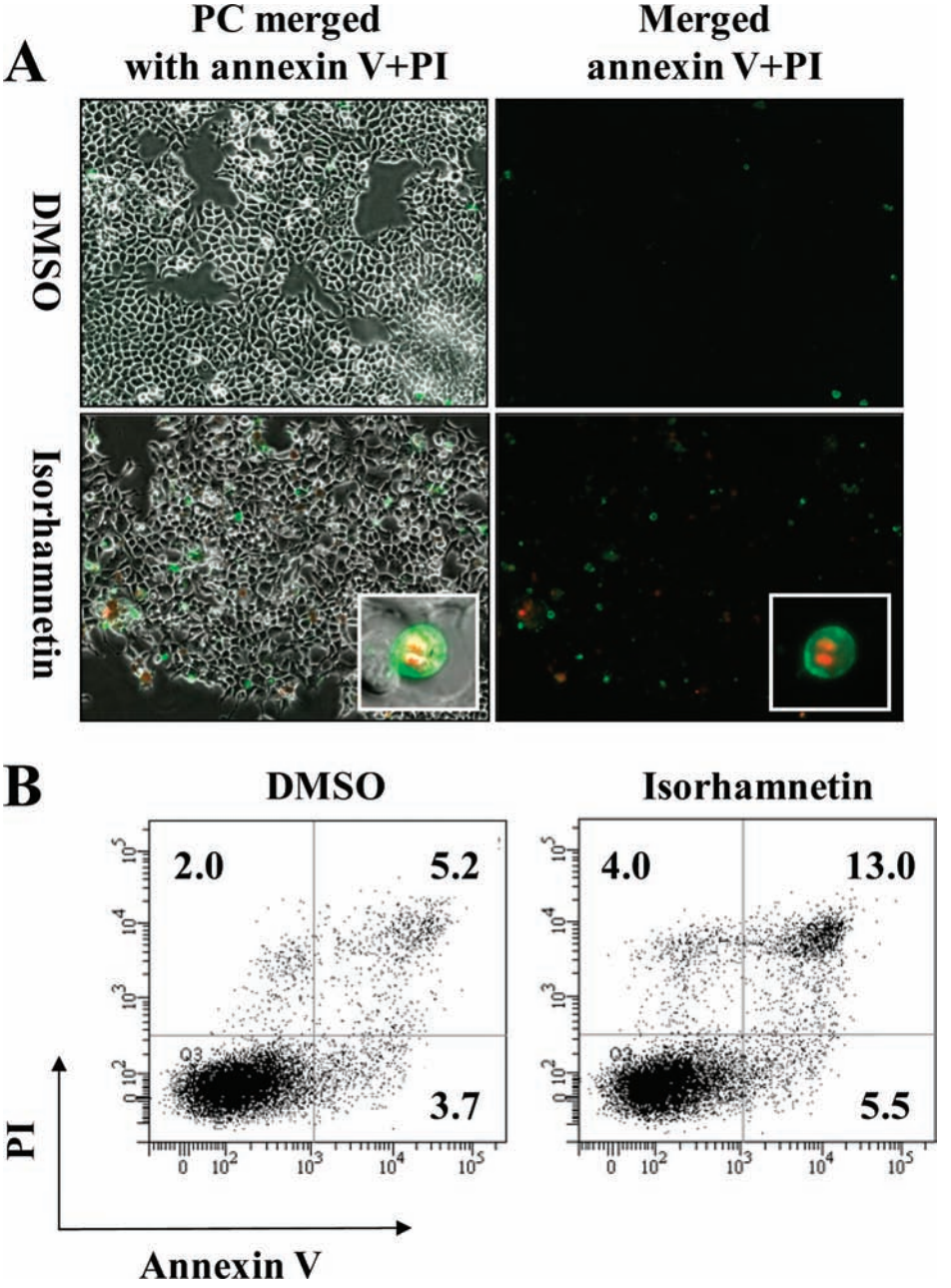

Figure 4. Representative pictures and FACS analysis of HCT-116 human colon cancer cells treated with isorhamnetin and stained with annexin V and propidium iodide (PI). HCT-116 cells were treated with dimethylsulfoxide (DMSO) or 75 μM isorhamnetin in complete medium for 24 h. Cells were stained with annexin V and PI, and then subjected to phase contrast (PC) and fluorescence microscopy (**A**) or FACS analysis (**B**) as described in Materials and Methods. The data are the relative number of annexin V^{high}, PI^{high}, and annexin V/PI^{high} cells.

potent cytotoxicity against HCT-116 cells, with an IC_{50} (72 μM) after a 48 h incubation 2- to 3-fold lower than those measured in BEL-7402 cells (IC_{50} 235 μM, 72 h) (*7*) and Eca-109 cells (IC_{50} 126 μM, 48 h) (*8*). In a recent study, IC_{50} for quercetin in HCT-116 cells under similar experimental conditions was >100 μM (*22*). Our study provides support for the superior potency of isorhamnetin compared with quercetin in inducing cell death in HCT-116 cells. Inhibition of cell growth by flavonoids has been suggested to be mediated by different mechanisms of action such as regulation of oncogene and tumor suppressor gene expression, inhibition of signal transduction pathways involving Nrf, NF-κB, AP-1, Wnt/β-catenin, MAPK and growth factors, and induction of cell-cycle arrest and apoptosis involving the p53, Bcl-2 and caspase families (*3*). Therefore, we investigated whether isorhamnetin could induce apoptosis or affect cell cycle progression in HCT-116 cells.

Our results show that isorhamnetin can induce both dose- and time-dependent apoptosis and necrosis in HCT-116 cells. These effects were assessed using two methods, flow cytometry and fluorescence microscopy. Isorhamnetin was previously shown to induce apoptosis in LLC cells through an activation of the mitochondria pathway, involving the loss of transmembrane potential, upregulation of Bax, and cleavage of procaspase-9 and PARP, and resulting in a significant decrease in the size and weight of tumors excised from LLC bearing C57BL/6 mice (*21*). Isorhamnetin has also been shown to induce apoptosis in Eca-109 cells through a decrease in the ratio of Bcl-2 to Bax (*8*). In addition, it was shown that chromosomal condensation precedes the formation of isorhamnetin-induced apoptotic bodies in BEL-7402 cells (*7*). In HCT-116 cells, quercetin was found to trigger apoptosis via NAG-1 under the control of the transcription factor Sp1 and p53 (*23*). In our study, necrotic HCT-116 cells

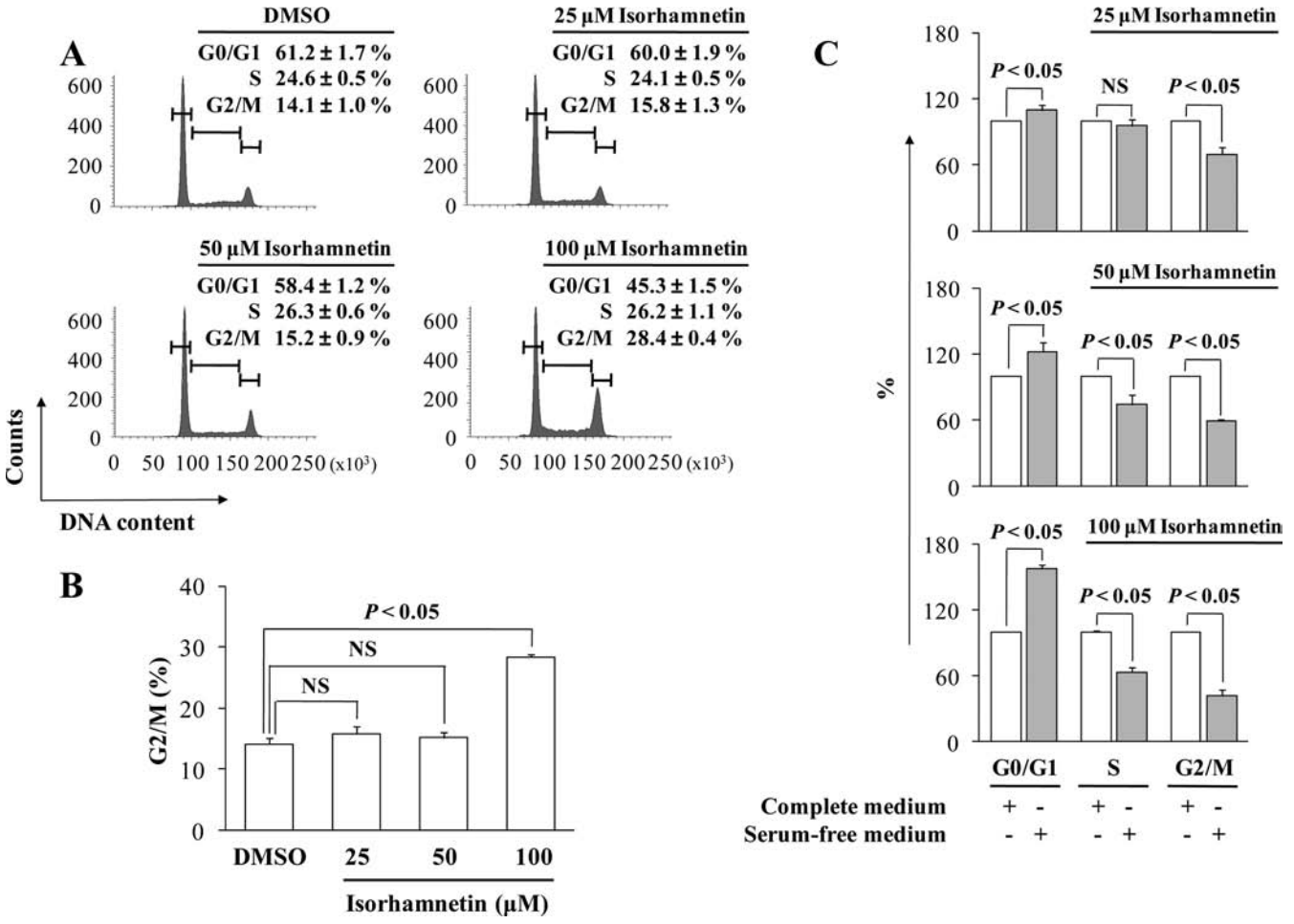

Figure 5. Effect of isorhamnetin on cell cycle distribution of HCT-116 human colon cancer cells assessed by FACS analysis. HCT-116 cells were treated with dimethylsulfoxide (DMSO) or isorhamnetin (25, 50, and 100 μM) in the presence (complete medium) (**A**, **B**) or absence (serum-free medium) (**C**) of 10% FBS for 24 h, and then subjected to FACS cell cycle analysis as described in Materials and Methods. In panel **C**, HCT-116 cultures treated with isorhamnetin in complete medium were taken as 100% of cells in the G0/G1, S, or G2/M phase of the cell cycle. The data represent the average median of at least three independent experiments (by triplicate) ± SD. $P < 0.05$ was determined by one-way analysis of variance followed by Duncan's test.

constituted the main fraction of the dead and dying HCT-116 cells after isorhamnetin treatment. Similar findings have been recently described for quercetin in rat neurons and in a human neuroblastoma cell line SK-N-AS, in which the number of necrotic cells exceeded the number of apoptotic ones (*24*). Morphological changes that occur with necrotic cell death include cell swelling, formation of cytoplasmic vacuoles and blebs, disrupted organelle membranes and lysosomes, and the eventual disruption of cell membrane and release of cytoplasmic contents into the cell surroundings. Whereas apoptosis and necrosis have been considered respectively as controlled or accidental modes of cell death, there is now mounting evidence that necrotic cell death is also regulated by a set of signaling pathways (*25*). Our results suggest that isorhamnetin can induce the activation of multiple cell death mechanisms, including both apoptosis and necrosis in HCT-116 cells. Additional studies will be required to ascertain the detailed mechanisms by which isorhamnetin initiates cell death in human colon cancer cells.

We found that isorhamnetin altered cell cycle progression in HCT-116 cells, with entry into G0/G1 being most strongly inhibited. Therefore, isorhamnetin-treated HCT-116 cells were growth arrested at G2/M. Isorhamnetin was previously reported to prevent Eca-109 cells from entering G2/M (*8*). Conflicting observations are common with regard to the stage-specific cell cycle arrest caused by flavonoids (*3*). For example, quercetin was recently shown to induce cell cycle arrest at G0/G1 in human breast carcinoma SK-Br3 cells through hypo-phosphorylation of pRb, which was accompanied by the induction of CDK inhibitor p21 (*26*). In contrast, quercetin blocked human SW480 colon cancer cells at G2/M through suppression of cyclin D_1 and survivin at both the transcriptional and protein expression levels (*15*). Cell cycle arrest of SW480 cells at G2/M was also reported when cells were treated with quercetin and with structurally related analogues of quercetin (*27*). Furthermore, an increase in the number of cells in G2/M was observed in CO115 human colon cancer cells following quercetin treatment (*28*). The negative regulation of the complex cyclin B/cdk1 and other positive regulators of cell cycle progression could impair CDK activities and contribute to the isorhamnetin-induced suppression of G2/M transition in HCT-116 cells (*29*). Our findings suggest that isorhamnetin might mediate growth inhibition of HCT-116 cells through the perturbation of the cell cycle progression and are consistent with the notion that G2/M checkpoints could be a conserved target for flavonoids in human colon cancer cells, leading to cell growth inhibition and possible apoptotic and necrotic death (*30*).

Serum deprivation potentiated the effects of isorhamnetin on cell death and facilitated cell cycle progression to G0/G1. It is possible that the enhanced effects observed in the absence of serum may be due to binding of isorhamnetin with serum proteins

thus lowering the active concentration of isorhamnetin (*31*). These findings are also in agreement with cytotoxic effects involving both nuclear-dependent and nuclear-independent processes (*32*) and the effect of serum deprivation stress in synchronizing cell cultures to arrest in G0/G1 (*33*). The question of whether both observations are concurrent or whether the sensitivity of starved HCT-116 cells to isorhamnetin is time-dependent requires further investigation.

All of these biological activities were exerted *in vitro* by isorhamnetin at micromolar concentrations above the concentration range expected in human plasma after the intake of dietary quercetin or isorhamnetin (*31*). However, these values were within that observed in mouse plasma after the intake of quercetin supplements (*6*). There is no agreement between effective concentrations *in vitro* and those *in vivo*. For example, long-term exposure to dietary polyphenols is thought to likely produce significant concentrations in plasma and tissues and a biological impact, even if the intake levels are low (*31*). Moreover, isorhamnetin could exert their effects both systemically and locally within the lumen of the intestinal tract. Data from ileostomy studies support this notion, showing that polyphenols may reach the colon in substantially large amounts (*34*). Therefore, it will be important to design studies, including appropriate biomarkers that examine the efficacy of isorhamnetin against colon cancer *in vivo* by reaching bioactive steady-state plasma and intraluminal levels for targeting colonic epithelial cells.

In conclusion, we demonstrated that isorhamnetin inhibited cell growth, arrested the cell cycle at G2/M, and induced both apoptosis and necrosis in HCT-116 human colon cancer cells. These antiproliferative and cell cycle effects suggest that isorhamnetin may have clinical significance with therapeutic and chemopreventive capabilities. Studies to further elucidate synergistic or additive effects of this flavonol in conjunction with conventional chemotherapeutic regimens are ongoing in our laboratory. Further investigations are needed to establish detailed molecular mechanisms of isorhamnetin-induced cytotoxicity in HCT-116 cells. To our knowledge, this is the first report of the effects of isorhamnetin on human colon cancer cells.

LITERATURE CITED

(1) Duijnhoven, F. J. B.; De Mesquita, H. B.; Ferrari, P.; Jenab, M.; Boshuizen, H. C.; Ros, M. M.; Casagrande, C.; Tjønneland, A.; Olsen, A.; Overvad, K.; Thorlacius-Ussing, O.; Clavel-Chapelon, F.; Boutron-Ruault, M. C.; Morois, S.; Kaaks, R.; Linseisen, J.; Boeing, H.; Nöthlings, U.; Trichopoulou, A.; Trichopoulos, D.; Misirli, G.; Palli, D.; Sieri, S.; Panico, S.; Tumino, R.; Vineis, P.; Peeters, P. H.; van Gils, C. H.; Ocké, M. C.; Lund, E.; Engeset, D.; Skeie, G.; Suárez, L. R.; González, C. A.; Sánchez, M. J.; Dorronsoro, M.; Navarro, C.; Barricarte, A.; Berglund, G.; Manjer, J.; Hallmans, G.; Palmqvist, R.; Bingham, S. A.; Khaw, K. T.; Key, T. J.; Allen, N. E.; Boffetta, P.; Slimani, N.; Rinaldi, S.; Gallo, V.; Norat, T.; Riboli, E. Fruit, vegetables, and colorectal cancer risk: the European Prospective Investigation into Cancer and Nutrition. *Am. J. Clin. Nutr.* **2009**, *89*, 1441−1452.

(2) Davis, C. D.; Milner, J. A. Gastrointestinal microflora, food components and colon cancer prevention. *J. Nutr. Biochem.* **2009**, *20*, 743−752.

(3) Huang, W. Y.; Cai, Y. Z.; Zhang, Y. Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention. *Nutr. Cancer* **2010**, *62*, 1−20.

(4) Cao, J.; Zhang, Y.; Chen, W.; Zhao, X. The relationship between fasting plasma concentrations of selected flavonoids and their ordinary dietary intake. *Br. J. Nutr.* **2010**, *103*, 249−255.

(5) Van der Woude, H.; Boersma, M. G.; Vervoort, J.; Rietjens, I. M. C. M. Identification of 14 quercetin phase II mono- and mixed conjugates and their formation by rat and human phase II in vitro model systems. *Chem. Res. Toxicol.* **2004**, *17*, 1520−1530.

(6) Zhang, L.; Angst, E.; Park, J. L.; Moro, A.; Dawson, D. W.; Reber, H. A.; Eibl, G.; Hines, O. J.; Go, V. L.; Lu, Q. Y. Quercetin aglycone is bioavailable in murine pancreas and pancreatic xenografts. *J. Agric. Food Chem.* **2010**, *58*, 7252−7257.

(7) Teng, B. S.; Lu, Y. H.; Wang, Z. T.; Tao, X. Y.; Wei, D. Z. In vitro anti-tumor activity of isorhamnetin isolated from Hippophae rhamnoides L. against BEL-7402 cells. *Pharmacol. Res.* **2006**, *54*, 186−194.

(8) Ma, G.; Yang, C.; Qu, Y.; Wei, H.; Zhang, T.; Zhang, N. The flavonoid component isorhamnetin in vitro inhibits proliferation and induces apoptosis in Eca-109 cells. *Chem. Biol. Interact.* **2007**, *167*, 153−160.

(9) Bao, M.; Lou, Y. Isorhamnetin prevents endothelial cell injuries from oxidized LDL via activation of p38MAPK. *Eur. J. Pharmacol.* **2006**, *547*, 22−30.

(10) Sanchez, M.; Lodi, F.; Vera, R.; Villar, I. C.; Cogolludo, A.; Jimenez, R.; Moreno, L.; Romero, M.; Tamargo, J.; Perez-Vizcaino, F.; Duarte, J. Quercetin and isorhamnetin prevent endothelial dysfunction, superoxide production, and overexpression of p47phox induced by angiotensin II in rat aorta. *J. Nutr.* **2007**, *137*, 910−915.

(11) Lee, J.; Jung, E.; Lee, J.; Kim, S.; Huh, S.; Kim, Y.; Kim, Y.; Byun, S. Y.; Kim, Y. S.; Park, D. Isorhamnetin represses adipogenesis in 3T3-L1 cells. *Obesity* **2009**, *17*, 226−232.

(12) Vasanthi, H. R.; Mukherjee, S.; Das, D. K. Potential health benefits of broccoli- a chemico-biological overview. *Mini-Rev. Med. Chem.* **2009**, *9*, 749−759.

(13) Wen, X.; Walle, T. Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metab. Dispos.* **2006**, *34*, 1786−1792.

(14) Walle, T. Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption. *Mol. Pharmaceutics* **2007**, *4*, 826−832.

(15) Shan, B. E.; Wang, M. X.; Li, R. Q. Quercetin inhibits human SW480 colon cancer growth in association with inhibition of cyclin D1 and survivin expression through Wnt/beta-catenin signaling pathway. *Cancer Invest.* **2009**, *27*, 604−612.

(16) Lea, M. A.; Ibeh, C.; Han, L.; Desbordes, C. Inhibition of growth and induction of differentiation markers by polyphenolic molecules and histone deacetylase inhibitors in colon cancer cells. *Anticancer Res.* **2010**, *30*, 311−318.

(17) Miyamoto, S.; Yasui, Y.; Ohigashi, H.; Tanaka, T; Murakami, A. Dietary flavonoids suppress azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-db/db mice. *Chem. Biol. Interact.* **2010**, *183*, 276−283.

(18) Kyle, J. A.; Sharp, L.; Little, J.; Duthie, G. G.; McNeill, G. Dietary flavonoid intake and colorectal cancer: a case-control study. *Br. J. Nutr.* **2010**, *103*, 429−436.

(19) Spencer, J. P.; Kuhnle, G. G.; Williams, R. J.; Rice-Evans, C. Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. *Biochem. J.* **2003**, *372*, 173−181.

(20) Park, K. H.; Choo, J. J.; Choe, S. N. Tissue concentrations of quercetin and its metabolite isorhamnetin following oral administration of quercetin in mice. *Korean J. Food Sci. Technol.* **2005**, *37*, 90−94.

(21) Lee, H. J.; Lee, H. J.; Lee, E. O.; Ko, S. G.; Bae, H. S.; Kim, C. H. Ahn, K. S.; Lu, J.; Kim, S. H. Mitochondria-cytochrome C-caspase-9 cascade mediates isorhamnetin-induced apoptosis. *Cancer Lett.* **2008**, *270*, 342−353.

(22) Li, L.; Henry, G. E.; Seeram, N. P. Identification and bioactivities of resveratrol oligomers and flavonoids from Carex folliculata seeds. *J. Agric. Food Chem.* **2009**, *57*, 7282−7287.

(23) Lim, J. H.; Park, J. W.; Min, D. S.; Chang, J. S.; Lee, Y. H.; Park, Y. B.; Choi, K. S.; Kwon, T. K. NAG-1 up-regulation mediated by EGR-1 and p53 is critical for quercetin-induced apoptosis in HCT116 colon carcinoma cells. *Apoptosis* **2007**, *12*, 411−421.

(24) Jakubowicz-Gil, J.; Rzeski, W.; Zdzisińska, B.; Piersiak, T.; Weiksza, K.; Glowniak, K.; Gawron, A. Different sensitivity of neurons and neuroblastoma cells to quercetin treatment. *Acta Neurobiol. Exp.* **2008**, *68*, 463−476.

(25) Hitomi, J.; Christofferson, D. E.; Ng, A.; Yao, J.; Degterev, A.; Xavier, R. J.; Yuan, J. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell* **2008**, *135*, 1311−1323.

(26) Jeong, J. H.; An, J. Y.; Kwon, Y. T.; Rhee, J. G.; Lee, Y. J. Effects of low dose quercetin: cancer cell-specific inhibition of cell cycle progression. *J. Cell. Biochem.* **2009**, *106*, 73−82.
(27) Wang, W.; VanAlstyne, P. C.; Irons, K. A.; Chen, S.; Stewart, J. W.; Birt, D. F. Individual and interactive effects of apigenin analogs on G2/M cell-cycle arrest in human colon carcinoma cell lines. *Nutr. Cancer* **2004**, *48*, 106−114.
(28) Murtaza, I.; Marra, G.; Schlapbach, R.; Patrignani, A.; Künzli, M.; Wagner, U.; Sabates, J.; Dutt, A. A preliminary investigation demonstrating the effect of quercetin on the expression of genes related to cell-cycle arrest, apoptosis and xenobiotic metabolism in human CO115 colon-adenocarcinoma cells using DNA microarray. *Biotechnol. Appl. Biochem.* **2006**, *45*, 29−36.
(29) Lindqvist, A.; Rodríguez-Bravo, V.; Medema, R. H. The decision to enter mitosis: feedback and redundancy in the mitotic entry network. *J. Cell Biol.* **2009**, *185*, 193−202.
(30) Auyeung, K. K.; Ko, J. K. Novel herbal flavonoids promote apoptosis but differentially induce cell cycle arrest in human colon cancer cell. *Invest. New Drugs* **2010**, *28*, 1−13.
(31) D'Archivio, M.; Filesi, C.; Vari, R.; Scazzocchio, B.; Masella, R. Bioavailability of the polyphenols: status and controversies. *Int. J. Mol. Sci.* **2010**, *11*, 1321−1342.
(32) Steiger-Barraissoul, S.; Rami, A. Serum deprivation induced autophagy and predominantly an AIF-dependent apoptosis in hippocampal HT22 neurons. *Apoptosis* **2009**, *14*, 1274−1288.
(33) Chou, R. C.; Langan, T. J. In vitro synchronization of mammalian astrocytic cultures by serum deprivation. *Brain Res. Protoc.* **2003**, *11*, 162−167.
(34) Gonzalez-Barrio, R.; Borges, G.; Mullen, W.; Crozier, A. Bioavailability of anthocyanins and ellagitannins following consumption of raspberries by healthy humans and subjects with an ileostomy. *J. Agric. Food Chem.* **2010**, *58*, 3933−3939.

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