

## Individual and Interactive Effects of Apigenin Analogs on G2/M Cell-Cycle Arrest in Human Colon Carcinoma Cell Lines

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**Abstract:** Apigenin has been previously shown to induce G2/M cell-cycle arrest in human colon cancer cell lines. The present study assessed the individual and interactive influence of seven apigenin analogs on cell cycle, cell number, and cell viability in human SW480 and Caco-2 colonic carcinoma cells. Cellular concentration of selected apigenin analogs was further assessed by high-performance liquid chromatography to assess cellular availability. The apigenin analogs studied were acacetin, chrysin, kampherol, luteolin, myricetin, naringenin, and quercetin. DNA flow cytometric analysis indicated that treatment with either chrysin or acacetin at 0 to 80  $\mu\text{M}$  for 48 h resulted in cell-cycle arrest at the G2/M phase in a dose-dependent manner in the SW480 cells but not in the Caco-2 cells. The percentage of SW480 cells at G2/M also increased when cells were treated with kampherol, luteolin, or quercetin between 5 and 30  $\mu\text{M}$ , but the percentage of cells in G2/M decreased at doses greater than 40  $\mu\text{M}$ . Cell number was significantly decreased in a time- and dose-dependent manner following the treatments with each analog except for naringenin and myricetin. The interactive effects of these analogs with apigenin were further assessed by combining each analog at doses from 0 to 80  $\mu\text{M}$  with apigenin at 20  $\mu\text{M}$ , a dose at which apigenin was found to double the proportion of SW480 cells in G2/M. When either acacetin, chrysin, luteolin, kampherol, or quercetin at doses between 5 and 30  $\mu\text{M}$  were combined with apigenin at 20  $\mu\text{M}$ , there was an increase of 22% in the proportion of G2/M cells over that observed with 20  $\mu\text{M}$  apigenin alone ( $P < 0.05$ ). At doses higher than 40  $\mu\text{M}$ , however, the interaction became antagonistic, and the proportion of cells in G2/M decreased below that observed with apigenin alone. Cell viability, as assessed by Trypan blue exclusion assay, significantly decreased by treatments with high doses of each agent or each agent combined with apigenin. Cellular concentration of apigenin, chrysin, or naringenin in SW480 cells significantly increased at doses of 40  $\mu\text{M}$  or greater, but it was not correlated with their impact on G2/M cell-cycle arrest. The induction of cell-cycle arrest by five of seven tested apigenin analogs and the additive induction by the combination of flavonoids at low doses sug-

gest that apigenin-related flavonoids may cooperatively protect against colorectal cancer through conjoint blocking of cell-cycle progression.

### Introduction

Consumption of fruits and vegetables that are abundant in flavonoids has been associated with reduced incidence of many forms of cancer including colorectal cancer (1,2). Flavonoids can be defined chemically as a substance composed of common phenylchromone structure (C6-C3-C6) with one or more hydroxyl substituents or other derivatives (3). These agents are universally present in fruits, vegetables, grains, nuts, tea, and wine. An average daily human intake of flavonoids has been estimated to be 1 g (4). Compelling data from epidemiological and animal studies have suggested that flavonoids could contribute to cancer prevention through many of their biological properties (1,5–7).

One of these promising properties is the perturbation of the cell-cycle progression. Research in our laboratory has been focused on apigenin (8). Apigenin is a flavone that is widely distributed in many fruits and vegetables, such as parsley, onions, orange, tea, chamomile, and wheat sprouts (9–12). Apigenin was shown to inhibit cell proliferation in many cancer cell lines (13–15). Apigenin was also anti-mutagenic against nitropyrene-induced genotoxicity in Chinese hamster ovary cells (16) and inhibited mitogen-activated protein kinase and the downstream oncogenes in v-H-ras-transformed NIH 3T3 cells (17). Dietary apigenin inhibited tumor necrosis factor-induced intercellular adhesion molecule-1 up-regulation (18). Previous studies conducted in our laboratory demonstrated that apigenin was a strong inhibitor of ornithine decarboxylase activity in the murine epidermis (19). Topical application of apigenin significantly reduced the number of benign papillomas and squamous cell carcinomas in the SENCAR skin carcinogenesis model initiated by 7,12-dimethylbenz[ $\alpha$ ]anthracene and promoted by 12-O-tetradecanoylphorbol-13-acetate (19). We also demonstrated that apigenin was equally effective at re-

ducing UVB radiation–induced squamous cell carcinomas in hairless SKH mice when applied prior to irradiation (20). The mechanisms by which apigenin protected against skin carcinogenesis in animal models seem to be related to the induction of cell-cycle arrest at the G2/M and/or G0/G1 phase (21,22). Recently we demonstrated that apigenin inhibited the growth of three human colon carcinoma cell lines in association with a reversible G2/M cell-cycle arrest (23). That arrest was associated with decreased activity of p34<sup>cdc2</sup> kinase and reduced accumulation of both p34<sup>cdc2</sup> and cyclin B1 proteins (23). Dietary apigenin has been further observed to protect against formation of aberrant crypt foci induced by azoxymethane in CF-1 mice and reduce cancer development in this model (Au et al., unpublished data). Taken together, these previous data suggest that apigenin may prevent colorectal cancer through blocking of cell-cycle progression.

To further develop potential strategies for colorectal cancer prevention by apigenin and apigenin-related flavonoids, we assessed the cell cycle in SW480 and Caco-2 human colon carcinoma cell lines that were treated with various apigenin analogs: acacetin, chrysin, luteolin, kampherol (or kaempferol), quercetin, myricetin, and naringenin (see chemical structures in Fig. 1). All the tested apigenin analogs ubiquitously occur in plant foods. For example, quercetin, luteolin, and kampherol are present in all leafy green vegetables (24). Naringenin usually occurs in the grapefruit (25). The combination of each analog with apigenin on cell-cycle arrest was addressed. In addition, cell number, cell viability, and cellular concentration of selected flavonoids were assessed.

## Materials and Methods

### Cell Culture and Treatment

Human colon carcinoma SW480 and Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in continuous culture in Dulbecco's Modified Eagle Media supplemented with 10% fetal bovine serum and 1% of a penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, MO) at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells (60–80% confluent) were treated with each analog at 0–80 μM alone or combined with apigenin at 20 μM for 24–48 h. Each apigenin analog was dissolved in dimethyl sulfoxide (DMSO) and mixed with fresh medium to achieve the desired concentration. The final DMSO concentration in all cultures was 0.2%. This concentration of DMSO did not alter cell growth or cell-cycle measurements when compared with the vehicle-free media.

### Cell-Cycle Analysis

Flow cytometric analysis for cellular DNA content was performed as previously described (23). Briefly, treated cells were harvested by trypsinization. After centrifugation, cells were fixed in 70% ethanol at 4°C, washed in 10 mM, pH 7.2 phosphate-buffered saline (PBS), and then resuspended in propidium iodide staining solution containing 20 mg/ml propidium iodide and 0.5 μg/ml RNase in 10 mM, pH 7.2 PBS at 37°C for 20 min before analysis by flow cytometry. Flow

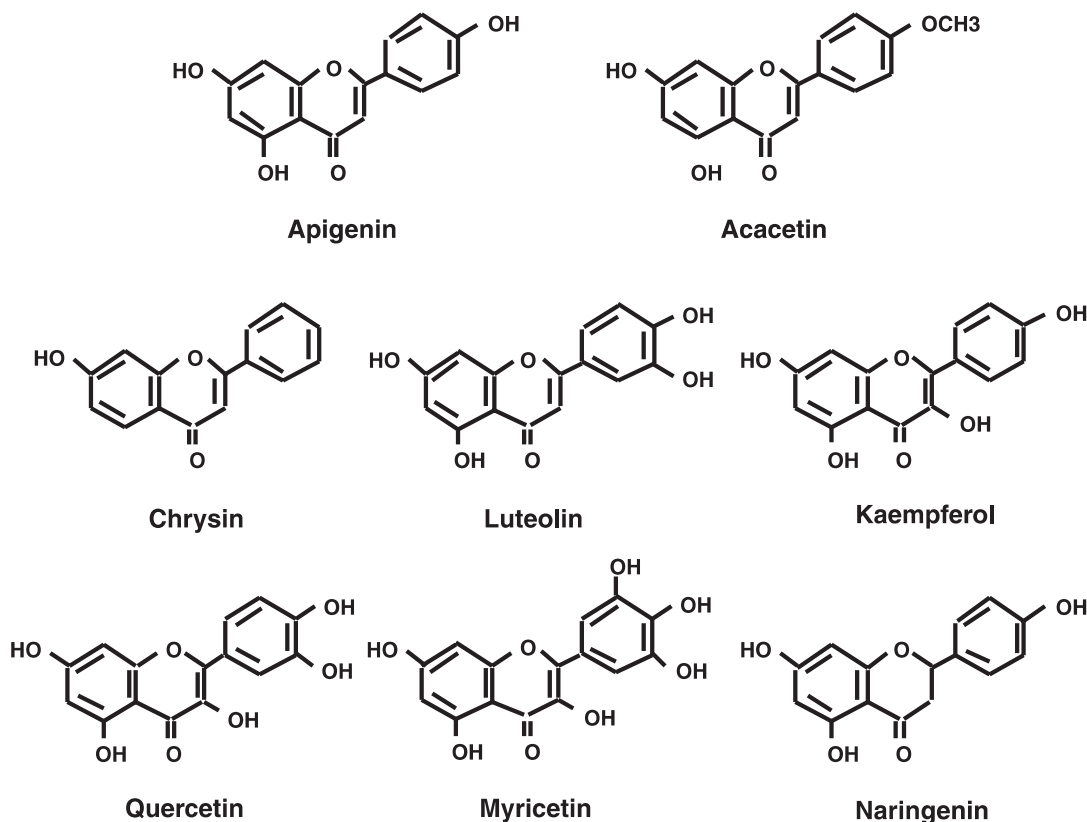


Figure 1. Chemical structures of apigenin and apigenin analogs tested in this study.

cytometric analysis was performed with a Beckman-Coulter flow cytometer (Miami, FL) with an excitation at 488 nm and an emission at 630 nm. Cell-cycle compartments were integrated using a Multicycle Program.

### Cell Number and Cell Viability Assays

Cell number was counted as previously described (23). Briefly, cells were detached with 0.025% trypsin and 0.03% ethylenediaminetetraacetic acid at 37°C for 10 min and then resuspended in PBS. The resuspended cells were diluted 1:1 in Trypan blue solution. The number of suspended cells was counted using a hemacytometer. All four 1-mm corner squares of the hemacytometer were counted and averaged. Cell viability was simultaneously evaluated by Trypan blue exclusion assay. Both Trypan blue-excluded and Trypan blue-containing cells were counted by a hemacytometer. Cell viability was expressed as percentage of the Trypan-blue excluded cells over the total cells. The concentration required to affect 50% of the viability ( $EC_{50}$ ) was estimated by probit data transformation and weighted linear regression analyses.

### Cellular Concentration Analysis

Cellular concentration of selected flavonoids was measured by high-performance liquid chromatography (HPLC) in SW480 cells after treatment with either apigenin, chrysin, or naringenin for 48 h. Briefly, cells were washed with cold PBS, detached by scraping, and collected in 2 ml PBS. The number of suspended cells was counted using a hemacytometer. An internal standard (100  $\mu$ l of 5  $\mu$ M flavone) was added to each sample, and the sample was homogenized for HPLC analysis (26,27). Samples were deproteinized by the addition of 8 ml of methanol (28) and then centrifuged at 370 g for 15 min. The supernatant was transferred to a glass tube, and the methanol was evaporated under  $N_2$  at 37°C. Sample was extracted three times by the addition of 3 ml of ethyl ether and vortexed, and the combined ethyl ether layers were evaporated to dryness under  $N_2$  in a water bath at 37°C. The residue was reconstituted with 50 ml HPLC-grade ethanol. The samples were analyzed by an HPLC system (Beckman model 126 Solvent Module, Fullerton, CA) with a C18 reversed-phase, 2.1  $\times$  250 mm microbore column (Alltech model Alltima C18, 5  $\mu$ m, Deerfield, IL) and a photodiode array detector (Beckman model 168). The mobile phase was initially 5% methanol (MeOH) in 0.1% trifluoroacetic acid (TFA; Sigma Chemical Company, St. Louis, MO). A gradient up to 60% MeOH in TFA was created over 5 min and then held for 34 min. Flow rate was 0.3 ml/min, and the agents tested were detected at OD 337 nm. Sensitivity was at a limitation of 0.05  $\mu$ M. We included an internal standard at the early step of extraction to minimize the variations between repeated measurements. The peak area indexed to internal standard was used to calculate the values of cellular levels. Cellular concentration was presented as nanograms per  $10^6$  cells.

### Statistics

The SAS statistical system 6.12 was used for statistical analysis. For G2/M arrest, cell number, or cell viability, the statistical significance of differences was determined by one-way ANOVA, and comparisons between groups were analyzed by Tukey's post hoc test. Data are presented as mean  $\pm$  SE. Differences were considered significant at  $P < 0.05$ . Cellular concentration data were statistically analyzed by General Linear model (GLM) and Tukey's multiple comparison tests and presented as mean  $\pm$  SE.

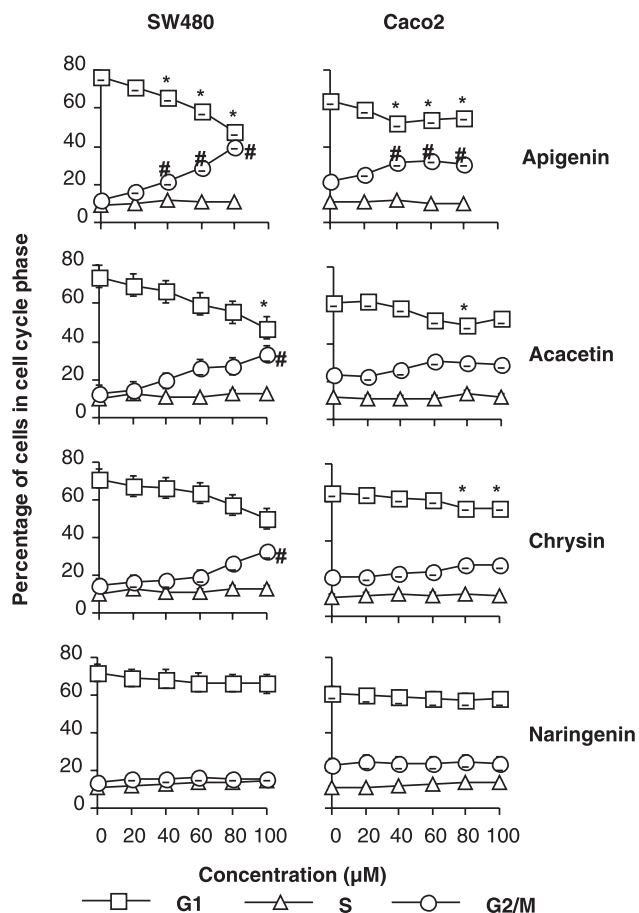
## Results

### Effects on Cell Cycle

DNA flow cytometric analyses of SW480 cells treated with apigenin at 0–80  $\mu$ M for 48 h showed an increase in the percentage of cells in the G2/M phase and a decrease in the percentage of cells in the G0/G1 phase; the proportion of cells in the S phase was not significantly altered (Fig. 2). None of the analogs significantly altered the distribution of cells in the phases of cell cycles at 24 h; therefore, only the 48-h data were presented in Fig. 2. In the SW480 cells there was a significant increase in the percentage of cells in G2/M at the following doses of these compounds in comparison with the vehicle control of that treatment (0  $\mu$ M): apigenin at 40–80  $\mu$ M, acacetin at 100  $\mu$ M, and chrysin at 100  $\mu$ M, whereas naringenin treatment had no effect. Although the Caco-2 cells exhibited a significant increase in the percentage of cells in the G2/M phase for apigenin at 40–80  $\mu$ M, they showed less sensitivity to arrest by any of other compounds when compared with the SW480 cells. Apigenin was more effective in arresting the SW480 cells than the Caco-2 cells, as was apparent from the higher dosage of apigenin required to attain a similar level of arrest in Caco-2 cells.

Figure 3 shows the changes of the proportion of SW480 cells in G2/M after treatment with either individual apigenin analog or the analog combined with apigenin at 20  $\mu$ M. The SW480 cells treated with chrysin or acacetin alone at 0–80  $\mu$ M for 48 h resulted in G2/M arrest in a dose-dependent manner when compared with the vehicle controls. The percentage of cells in the G2/M phase increased when cells were treated with kampherol, luteolin, or quercetin at  $<30$   $\mu$ M but decreased at doses of  $>40$   $\mu$ M. Naringenin and myricetin did not have a significant effect on G2/M cell-cycle arrest. It is notable that the increase in the proportion of cells in G2/M cells paralleled the decrease in the proportion of cells in G0/G1 cells. The proportion of cells in the S phase was not significantly affected, and there was no evidence of a pre-G0/G1 peak that might indicate the presence of appreciable apoptotic cells.

In comparison with individual influence on G2/M cell-cycle arrest, the interactive effects of each analog combined with 20  $\mu$ M apigenin are also presented in Fig. 3. Apigenin at 20  $\mu$ M for 48 h was generally shown to double the percentage



**Figure 2.** Comparison of cell-cycle arrest between SW480 and Caco-2 cells. Cells were cultured with apigenin or apigenin analogs for 48 h and then cell-cycle phases were monitored by DNA flow cytometry. Percentage of cells in the cell-cycle phase is presented as mean  $\pm$  SE from four independent experiments. Where error bars are not shown, they are smaller than the symbol. \* $P < 0.05$  vs. the vehicle controls for cells in the G0/G1 phase, # $P < 0.05$  vs. the vehicle controls for the cells in the G2/M phase.

of cells in G2/M when compared with the vehicle controls, and our previous studies demonstrated at least a doubling of the proportion of SW480 cells in G2/M with this dose of apigenin (23). After combination, acacetin, chrysin, luteolin, kampherol, and quercetin at doses of  $<30 \mu\text{M}$  significantly induced at least an additional 22% increase in the proportion of cells in the G2/M phase when compared with  $20 \mu\text{M}$  apigenin alone ( $P < 0.05$ ). At high doses, however, the interaction became antagonistic, and the proportion of cells in G2/M decreased to apigenin-alone (chrysin, acacetin, and kampherol) or to the vehicle-control levels (luteolin and quercetin). No significant effect on G2/M arrest, however, was found for the combination of apigenin with myricetin or naringenin.

### Effect on Cell Number

Cell number was counted after treatment with each apigenin analog at 0–80  $\mu\text{M}$  for 24–48 h. As shown in Fig. 4, the cell number, when treated with chrysin, acacetin,

kampherol, luteolin, or quercetin, resulted in a dose-response reduction. Statistically significant reduction was found at doses of  $\geq 20 \mu\text{M}$  after 48-h treatment for chrysin and acacetin but  $\geq 40 \mu\text{M}$  for kampherol, luteolin, and quercetin. The values of 50% inhibition concentration for chrysin, acacetin, kampherol, luteolin, and quercetin were about 30, 45, 60, 50, and 50  $\mu\text{M}$ , respectively. The cell number, however, was not significantly affected by the treatments with either naringenin or myricetin at any of the concentration regimens. In addition, similar influence was found on cell number when the analogs were combined with apigenin at  $20 \mu\text{M}$ . Values shown in Fig. 4 are pooled across the treatment alone or with apigenin because no significant difference was observed between these treatments.

### Effect on Cell Viability

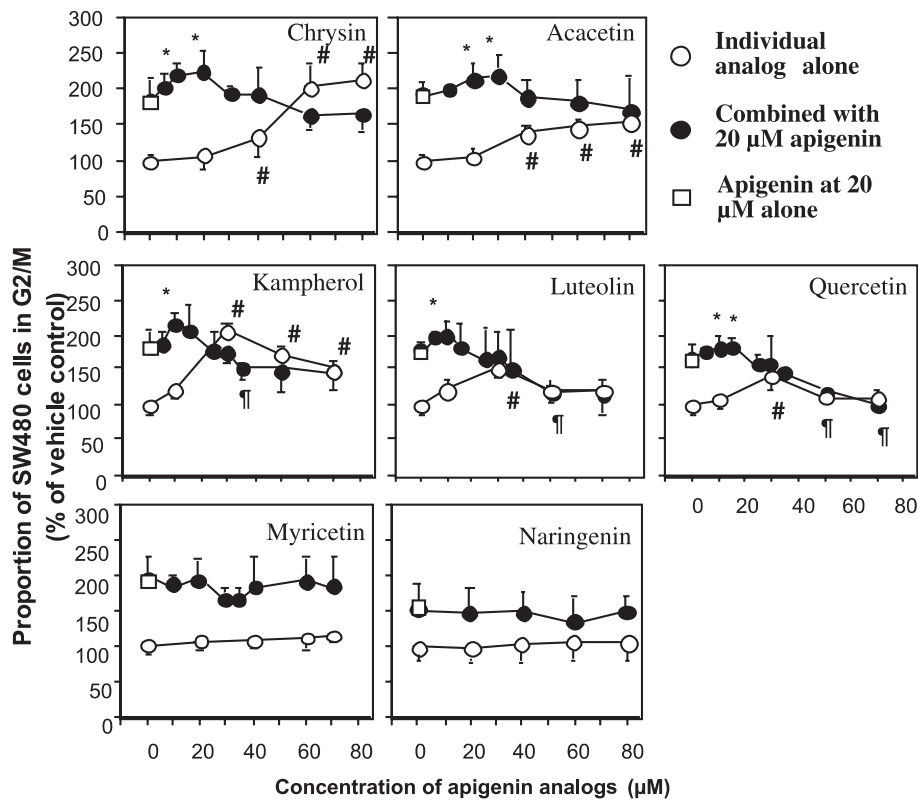
Cell viability was evaluated by measuring cellular exclusion of Trypan blue. As shown in Fig. 5, cells treated with chrysin, acacetin, kampherol, luteolin, or quercetin at doses of 60 or 80  $\mu\text{M}$  resulted in a significant reduction in cell viability. No significant toxic effect was observed for the treatment at lower concentrations. Based upon the trend of cell viability reduction, the  $\text{EC}_{50}$  values for the toxic potency of chrysin, acacetin, kampherol, luteolin, and quercetin were estimated at 165, 160, 100, 90, and 85  $\mu\text{M}$ , respectively. These values are rough estimates considering that the highest dose assessed was 100  $\mu\text{M}$ . Myricetin and naringenin did not significantly affect cell viability at any of the concentrations studied. In comparison with each analog alone, cell viability was not significantly changed by the combination with 20  $\mu\text{M}$  apigenin for all the tested agents except for chrysin. A stronger toxic effect was found for the combination of chrysin at 80  $\mu\text{M}$  with apigenin at 20  $\mu\text{M}$  when compared with chrysin alone.

### Effect on Cellular Concentration

SW480 cells were treated with either apigenin, chrysin, or naringenin for 48 h, and the cellular concentrations were then measured by HPLC. As shown in Fig. 6, the cellular concentrations of apigenin and chrysin increased in a dose-dependent manner. Naringenin also exhibited a significant dose-dependent increase in cellular concentration, but cellular concentration declined between 80 and 100  $\mu\text{M}$  of this flavonoid in the media. Significant increases of cellular concentrations were found for each agent at  $>40 \mu\text{M}$  when compared with the vehicle controls. Although no significant difference of cellular concentration was observed between apigenin and naringenin treatment, there was a significant difference of cellular concentrations between chrysin and apigenin or naringenin at 60  $\mu\text{M}$  or higher.

### Discussion

Studies on diet and cancer have revealed that a consistent pattern of consumption of diets rich in vegetables and fruits is

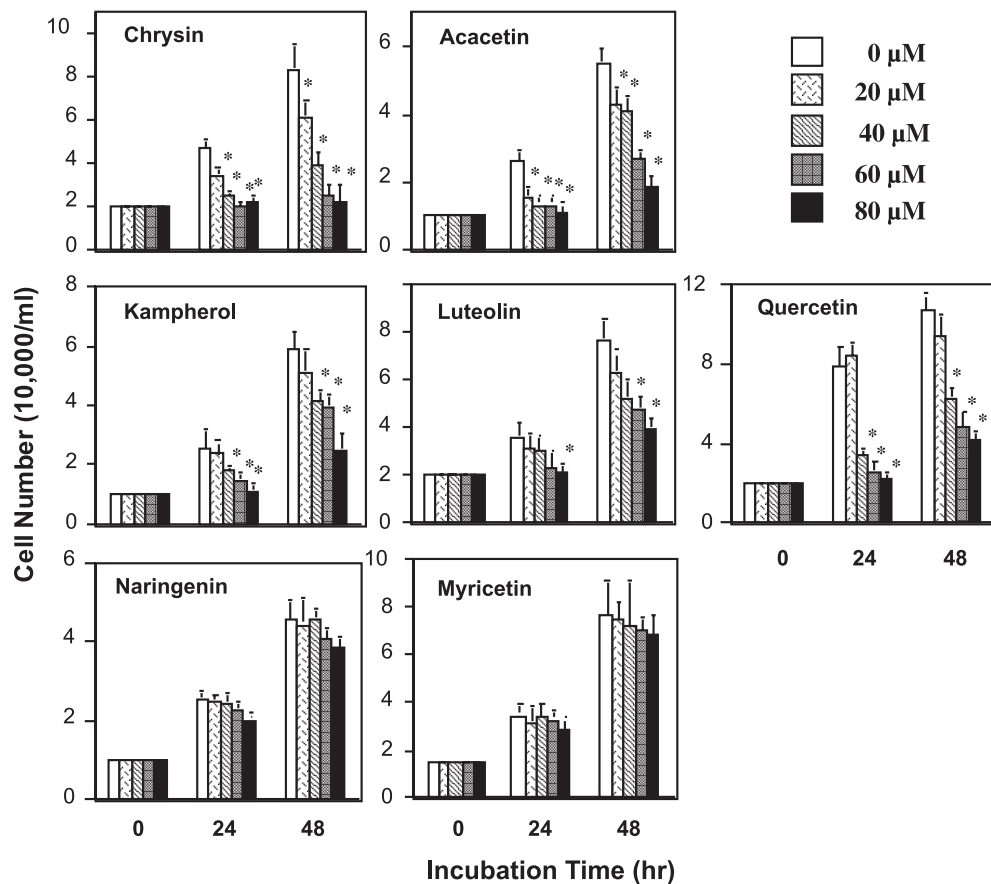


**Figure 3.** Individual and combined effect of apigenin analogues on G2/M cell cycle arrest in SW480 cells. SW480 cells were cultured with indicated agent individually or combined with 20  $\mu\text{M}$  apigenin for 48 hr, then the cell cycle was monitored by a DNA flow cytometric analysis. The percentage of the G2/M proportion of SW480 cells over the vehicle controls is presented as means  $\pm$  SE from four to five independent experiments. Where error bars are not shown, they are smaller than the symbol. The proportion of SW480 cells at G2/M phase in 48-hr vehicle controls was  $16.5 \pm 3.3\%$  (100% on the y-axis). #  $P < 0.05$  vs. the vehicle controls, \* significantly greater than 20  $\mu\text{M}$  apigenin alone ( $P < 0.05$ ), significantly less than 20  $\mu\text{M}$  apigenin alone ( $P < 0.05$ ).

associated with a reduced cancer risk (1,2). Flavonoids are a major class of nonnutritive components of plant-derived diets, and they are widely distributed in plant-based foods and have been suggested to play a role in cancer prevention (1). Current studies in our laboratory focusing on apigenin as a potential anticancer agent have demonstrated that 1) apigenin inhibited colon cancer cell growth by inducing G2/M cell-cycle arrest (23) and 2) apigenin prevented carcinogen-induced aberrant crypt foci formation, a preneoplastic lesion for colorectal cancer, in a murine model (unpublished data). Apigenin modestly inhibited tumors in azoxymethane-induced CF-1 mice. However, it was not effective against genetically induced tumors in *Apc<sup>Min</sup>* mice (Au et al., unpublished data). In studies by Tatsuta et al. (29), injections of apigenin did not alter AOM-induced colon carcinogenesis but inhibited cancer metastasis induced by this protocol. To further develop a strategy for colon cancer prevention by apigenin, we assessed the influence of seven apigenin analogs both individually or combined with apigenin on cell-cycle perturbation, cell number, cell viability, and cellular concentration in human colon carcinoma SW480 or Caco-2 cells. SW480 and Caco-2 were selected to represent different genetic abnormalities of human colon cancer. As mentioned in our previous publication (23), both cell lines have truncated or mutant adenomatous polyposis coli (*APC*) genes. In addition, SW480 cells have mutated *p53* (Arg to His-273 and

Pro to Ser-309) and mutated *ras* (Val to Glu-12) genes. Caco-2 cells express wild-type *ras* but mutated *p53*. The differences in induction of G2/M arrest by apigenin between these two colon carcinoma cell lines have been previously demonstrated, suggesting that flavonoids might be differentially effective against tumors with specific mutational spectra (23).

In addition to antioxidation and apoptosis induction (30,31), many flavonoids have been demonstrated to perturb the cell cycle in cultured human cancer cell lines. We have previously demonstrated that apigenin induced cell-cycle arrest at the G2/M phase in three human colonic carcinoma cell lines, that is, SW480, HT-29, and Caco-2 (23). Of the three cell lines, SW480 had the greatest sensitivity to apigenin. Similar results were found in this study. Because the three cell lines have different genetic abnormalities, such as truncated or mutant *APC* or *p53* or *ras* genes, the different responses suggest that apigenin may be more effective in controlling growth of tumors with certain mutational spectra and less effective in wild-type normal cells. This suggestion has been further supported by our recent studies that apigenin demonstrated a limited cytostatic effect in transfected HT-29 cells with activated wild-type *APC* gene (Chung et al., unpublished data). All of these studies were conducted in transfected colon cancer cells, which may not equate with the impact in normal colonic epithelial cells.



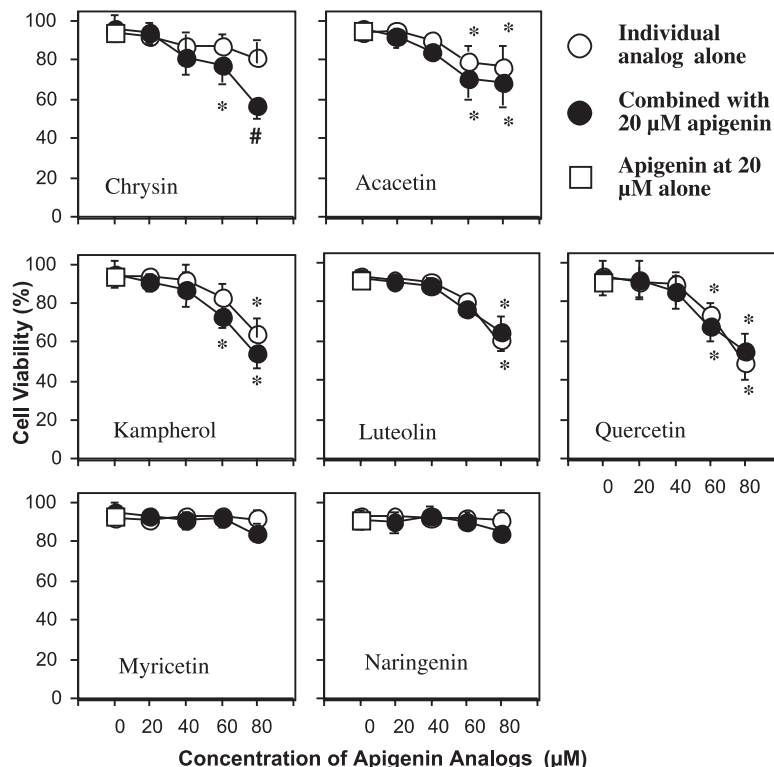
**Figure 4.** Effect of each apigenin analog alone or combined with 20  $\mu$ M apigenin on cell number. After 48-h treatment with the combination, SW480 cells were detached by trypsinization, and the cell number was counted by a hemacytometer. Values are pooled across the inclusion of apigenin because 20  $\mu$ M apigenin alone did not significantly alter the cell numbers and interactions with the analogs were not observed. Mean  $\pm$  SE from four to seven independent experiments is shown. \* $P < 0.05$  vs. the vehicle controls.

In addition to apigenin, both acacetin and chrysin showed stronger cell-cycle arrest ability in SW480 cells than that in Caco2 cells. The concentration of apigenin required to double the proportion of SW480 cells in the G2/M phase for 48 h was  $\sim 20$   $\mu$ M. Apigenin has uniformly inhibited colon cancer cell growth and induced cell-cycle arrest, but the concentration that was effective in doubling G2/M cells differed between studies. We demonstrated a significant increase in G2/M cells in Fig. 3 and in our previous publication (23). Although a repeated study shown in Fig. 2 was not significant for apigenin at 20  $\mu$ M, a 50% increased accumulation in G2/M cells was consistently observed.

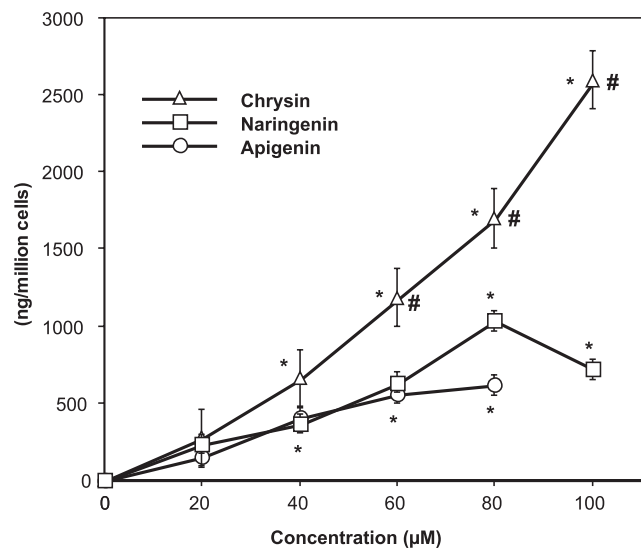
This study demonstrated that all of the apigenin analogs tested except myricetin and naringenin were able to arrest cell cycle at the G2/M phase. Both chrysin and acacetin were found to block SW480 cells at G2/M in a dose-dependent manner. The percentage of cells in G2/M was also increased by treatments with kampherol, luteolin, or quercetin at doses of  $<30$   $\mu$ M but decreased to the vehicle control levels at the higher doses. The observation of apigenin's ability to perturb SW480 cells is consistent with an earlier report from our laboratory (23). To achieve an arrest of G2/M relative to apigenin, higher doses of acacetin and chrysin were required. A 20- $\mu$ M dose of apigenin treatment was required to obtain a

50% increased accumulation in G2/M in SW480 cells after 48 h compared with 40  $\mu$ M for acacetin and 70  $\mu$ M for chrysin. A 25% accumulation in G2/M in Caco-2 cells was obtained with 21  $\mu$ M apigenin compared with 57  $\mu$ M for acacetin. A 100- $\mu$ M dose of chrysin only elicited 19% accumulation in G2/M. Although the efficacy of G2/M cell-cycle arrest by each analog tested was less than that of apigenin, the interactive effects of each analog at 0–80  $\mu$ M combined with apigenin at 20  $\mu$ M showed that acacetin, chrysin, luteolin, kampherol, and quercetin at low doses significantly increased the proportion of cells in the G2/M phase in comparison with apigenin alone. At higher doses, however, the relationship became antagonistic and the proportion of cells in G2/M decreased.

The cell number data revealed a significant decrease when cells were treated with chrysin, acacetin, kampherol, luteolin, or quercetin but not with myricetin or naringenin, suggesting that cell-cycle arrest might cause the reduction in cell number. However, the proportion of cells in G2/M decreased in the high doses of each agent individually or combined with apigenin at 20  $\mu$ M. Because apigenin at 20  $\mu$ M did not reveal a significant reduction in cell number as we discussed previously, this interference may imply a cytotoxic effect by each analog except for naringenin and myricetin. To reveal the in-



**Figure 5.** Individual and combined effects of apigenin analogs on cell viability. Cell viability was assessed by Trypan blue exclusion assay after 48-h treatment with either the indicated agent alone or combined with 20  $\mu\text{M}$  apigenin. The percentage of viable cells over total cells is presented as mean  $\pm$  SE from three or four independent experiments. Where error bars are not shown, they are smaller than the symbol. \* $P < 0.05$  vs. the vehicle controls and # $P < 0.05$  vs. individual agent at the same concentration.



**Figure 6.** Cellular concentrations of apigenin or analogs in SW480 cells for 48-h treatment. Mean  $\pm$  SE from four independent experiments. \* $P < 0.01$  for all three treatments vs. the vehicle controls. # $P < 0.05$  for chrysin vs. either apigenin or naringenin treatments.

fluence of possible cytotoxicity on cell number, cell viability was assessed by counting the cellular exclusion of Trypan blue. Cell viability was not significantly affected by the treatments at low doses of each analog when compared with vehicle controls. It was significantly decreased in treatments with

chrysin, acacetin, kampherol, luteolin, or quercetin only at high doses (60–80  $\mu\text{M}$ ). This suggests that treatments at low doses reduced cell number mainly through cytostatic effects, but high doses introduced cytotoxicity.

Because a significant difference on cell-cycle arrest was found between apigenin and apigenin analogs, we measured the cellular concentrations of apigenin, chrysin, and naringenin, respectively, to further determine whether cellular availability of each compound would correlate with the inhibition of cell cycle or the reduction in cell number. We used an HPLC procedure to quantitate the flavonoids. Although cellular concentration of apigenin, chrysin, and naringenin significantly increased with their concentrations in media, chrysin showed higher concentration than apigenin and naringenin. There was no difference between apigenin and naringenin cellular concentrations, although they expressed completely different impacts on cell-cycle arrest. No correlation was observed between cellular concentration and cell-cycle arrest with these three compounds. Therefore, cell-cycle arrest seems to be independent of cellular concentration. It is possible that the tested concentrations may not have reached the required thresholds for each analog. We did not observe any metabolite peaks from this HPLC procedure. A recent study reported that apigenin was rapidly absorbed, conjugated, and excreted in Caco-2 cells (32).

Data using apigenin and apigenin analogs to assess cell cycle and cell number illustrated that structural features affected the efficacy in inhibiting growth of human colon cancer cell

lines. This is brought out most dramatically by the differences seen between apigenin and naringenin. The absence of G2/M arrest by naringenin, considering it was the only analog with the saturated C2-C3 bond, suggested the essentiality of the bond for cell-cycle arrest to be induced with the compounds tested. Naringenin, at a high dose of 710  $\mu$ M, has been required to inhibit proliferation in the HT-29 colon cancer cell line (33). Also, the reduced effectiveness of chrysin compared with apigenin suggested the efficacy of a functional group at the 4' position on the B-ring. Comparing apigenin and acacetin, it was observed that the type of functional group attached to the 4' position affected the analog's activity in inducing cell-cycle arrest but did not have an effect on reduction in cell number. However, myricetin gave an unexpected result. Myricetin did not influence cell-cycle arrest or cell number. These observations may suggest a link to solubility because myricetin possesses six hydrogen groups and would be expected to be more water soluble than the other analogs studied.

Although little quantitative information is available on the bioavailability of flavonoids in human subjects, an average daily human intake of flavonoids has been estimated to be 1 g (4). By using a column-switching HPLC method, Nielsen and Dragsted reported that levels of apigenin in urine samples were up to 21  $\mu$ mol/day for human subjects who received an intervention with parsley containing 150–180  $\mu$ mol apigenin per day (34,35). Nothing is known about the tissue or cellular levels of apigenin in vivo. Considering that a mixture of flavonoids is present in foods, this study, by using a combination of apigenin analogs, seems to be relevant to normal subjects consuming vegetables and fruits.

In summary, the individual induction of cell-cycle arrest in G2/M by five of seven apigenin analogs tested and the synergistic arrest of cell cycle by a combination at low concentrations suggest that dietary apigenin-related flavonoids may cooperatively protect against colorectal cancer through jointly blocking of cell-cycle progression.

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