

## Flavonoids Suppress Androgen-Independent Human Prostate Tumor Proliferation

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**Abstract:** *The present studies compared the effects of selected bioflavonoids on the proliferation of androgen-independent human prostatic tumor cells (PC-3). Complete growth retardation was observed in PC-3 cells treated with 100  $\mu$ M quercetin, kaempferol, and luteolin, while isomolar genistein, apigenin, and myricetin suppressed PC-3 proliferation by 73%, 70%, and 59%, respectively ( $p < 0.05$ ). Naringenin and rutin were not as effective and inhibited growth by <25%. Exposure to increasing concentrations of quercetin and kaempferol led to a dose-dependent decrease in proliferation. Refeeding kaempferol-treated cells (50  $\mu$ M) complete medium without the flavonoid resulted in a return toward control growth rates. Similar growth recovery was not observed in quercetin-treated cells. The antiproliferative response of PC-3 cells to quercetin and kaempferol was additive when supplemented to the medium at 25  $\mu$ M. A block in G<sub>2</sub>-to-M phase progression was observed after the addition of 25  $\mu$ M kaempferol. When quercetin reached 100  $\mu$ M, an increase in the proportion of cells in the S phase became apparent within 24 hours. Apoptosis was not evident, even when concentrations of quercetin or kaempferol were raised to 100  $\mu$ M. The present studies suggest that alterations in cell cycle progression contribute significantly to the antiproliferative effects of quercetin and kaempferol in PC-3 cells.*

### Introduction

Prostate cancer is the second leading cause of cancer mortality in men living in the United States (1). Race, age, and diet are identifiable risk factors associated with prostate cancer occurrence (1,2). While some dietary constituents are implicated in cancer promotion (1,2), increasing evidence suggests that certain essential and nonessential nutrients can suppress tumor development (1,3). Identifying these factors may be an effective noninvasive strategy for decreasing the incidence and severity of this disease.

Flavonoids, a group of naturally occurring polyphenolic substances found concentrated in the skins and outer layers of fruits and dark green leafy vegetables, are gaining widespread attention for their contribution to the anticarcinogenic properties associated with these foods (1,4,5). Over 4,000 flavonoids have been discovered and categorized according to structural class, hydroxylation pattern, other substitutions, degree of polymerization, and conjugation (5). Some of these flavonoids are known to possess anticarcinogenic properties (5–9). Specifically, laboratory data demonstrate that they promote metal chelation (6), scavenging of free radicals (7), alteration of phase I cytochrome *P*-450 (CYP) CYP 1A1 enzyme activation (8), and stimulation of phase II conjugation enzymes (9). Collectively, these mechanisms may lead to decreases in initiation and promotion of chemically induced carcinogenesis.

On a cellular level, flavonoids control a broad range of biochemical activities that culminate in their inhibition of tumor cell proliferation. These events include alterations in protein kinase C, phosphatidylyl-3-kinase, Na<sup>+</sup>-K<sup>+</sup>-ATPase, tyrosine kinase, and topoisomerase II enzyme activities (10–13). Downregulations in *ras*, *myc*, and *jun* oncogene expression and upregulation of p53 tumor suppressor function have also been documented (10,14,15). Although suppressions in neoplastic cell proliferation have been observed in several cell types (13,16–20), the signal transduction pathways affected appear to differ depending on the flavonoids used (8,16,17). Suppressions in cell division and/or the induction of apoptosis may account for these differences in response to selected flavonoids.

Cell division is controlled by specialized checkpoints that monitor the progression of cells through the various phases of the cell cycle (21,22). Stimulation of or interruption in checkpoint function results in cell cycle arrest and/or apoptosis (21). The ability to disrupt checkpoint function is characteristic of many antitumorigenic agents, including flavonoids (13,16,17,23–28). Although flavonoids have been shown to block G<sub>1</sub>-to-S and G<sub>2</sub>-to-M phase progression, the exact location of the block appears to differ de-

pending on the compound and the cell type (13,16,17,26, 27). Similarly, apoptosis is induced by some, but not all, flavonoids (13,16,26,27).

The present studies were designed to investigate the ability of structurally related flavonoids (Figure 1) to inhibit the proliferation of an established human androgen-independent prostate (PC-3) cell line. Additional studies examined cell cycle arrest and apoptosis as possible mechanisms accounting for the ability of quercetin and kaempferol to modify PC-3 proliferation.

## Materials and Methods

### Chemicals

PC-3 cells were purchased from American Type Culture Collection (Rockville, MD). The apoptosis detection kit was purchased from Promega (Madison, WI). Flavonoids and other chemicals were purchased from Sigma Chemical (St. Louis, MO).

### Cell Culture

PC-3 cells were plated in 25-, 75- and 150-cm<sup>2</sup> tissue culture flasks and incubated in RPMI 1640 medium (pH 7.2; Sigma Chemical) supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin), and 1 µg/ml insulin (29). Cells were plated at 4 × 10<sup>3</sup>/cm<sup>2</sup> for 48 hours before treatment. Cells were grown under a humidified 5% CO<sub>2</sub> atmosphere at 37°C. Bioflavonoids were dissolved in dimethyl sulfoxide (DMSO) before addition to cultures. Control cultures were treated with DMSO. The maximum quantity of DMSO added to medium in these studies was 0.2%.

### Cell Proliferation

In all studies, cells were harvested by trypsinization (0.025% trypsin-EDTA), rinsed with RPMI 1640 medium containing fetal bovine serum to deactivate the trypsin, and centrifuged at 500 g. Cells were resuspended in RPMI 1640, and viable cells were counted on a hemocytometer using trypan blue exclusion. Growth inhibition was determined by the following equation, with the original count corresponding to the number of cells present after the 48-hour preincubation and before the addition of test compound

$$\text{growth inhibition (\%)} = \left[ 1 - \left( \frac{\text{final count}_{+\text{flavonoid}} - \text{original count}}{\text{final count}_{-\text{flavonoid}} - \text{original count}} \right) \right] \times 100$$

### Apoptosis and Cell Cycle Analysis

In these studies, PC-3 cells were harvested, rinsed in cold phosphate-buffered saline, and fixed in 1% methanol-free formaldehyde on ice for 20 minutes. Samples were centrifuged, resuspended in 70% ethanol, and stored at 2–8°C for subsequent analysis. Cell pellets were incubated in a terminal deoxynucleotidyl transferase buffer (Promega, Madison, WI) to determine DNA fragmentation. DNA fragments were stained by incorporating a fluorescein-conjugated dUTP onto the 3'-OH end of the DNA strand. Cellular DNA was stained by the addition of phosphate-buffered saline containing 200 U/ml ribonuclease and 18 µg/ml propidium iodide. Samples were incubated in the dark for ≥30 minutes before analysis. DNA fragmentation and the percentage of cells in the G<sub>1</sub>, S, and G<sub>2</sub>/M phases of the cell cycle were determined using a Coulter XL-MCL tabletop cytometer. The DNA of 10,000 cells was analyzed from each culture harvested.

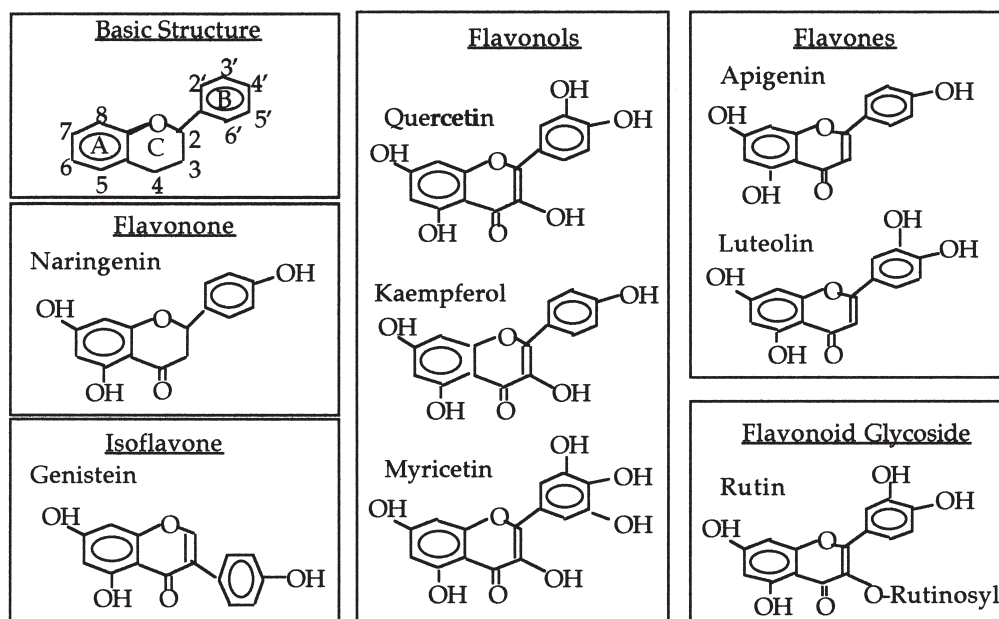
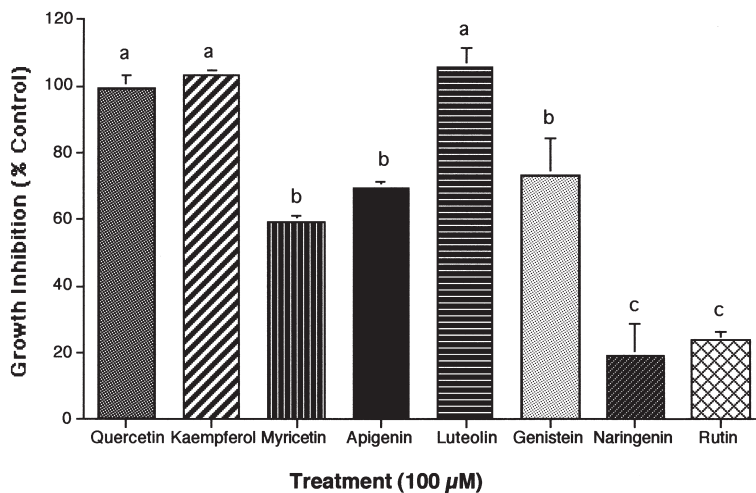


Figure 1. Flavonoid chemical structures.



**Figure 2.** Effect of various bioflavonoids on PC-3 tumor cell growth. PC-3 cells were initially plated at  $4 \times 10^3/\text{cm}^2$  in flasks 48 h before a 48-h treatment with 100  $\mu\text{M}$  selected flavonoids. Values are means  $\pm$  SEM of 3 observations/treatment. Values not sharing a common superscript (a–c) differ ( $p < 0.05$ ). Maximum SEM for all measurements was  $\pm 11.5\%$ .

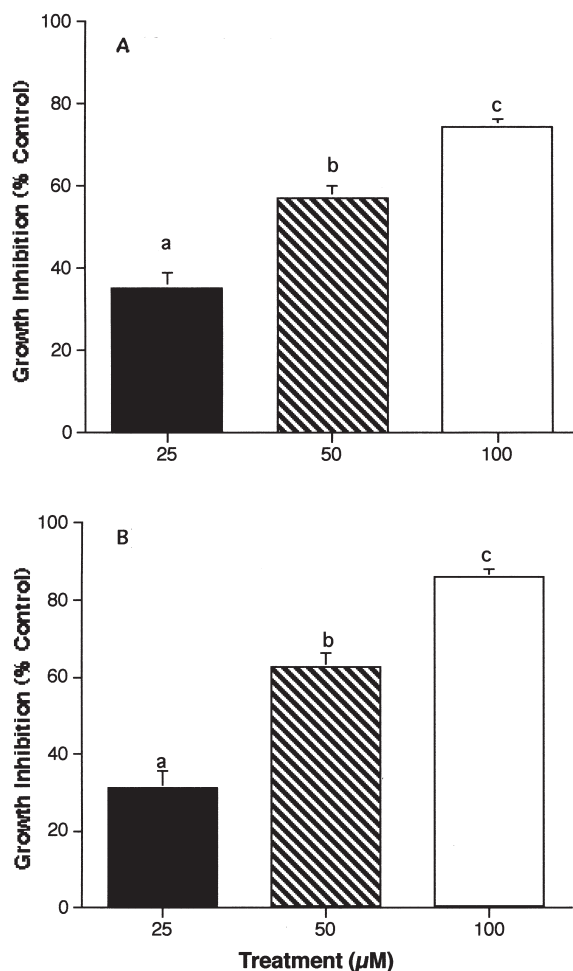
### Statistical Analysis

Analysis of variance statistics were applied to the data using the Statistical Analysis System (SAS) for Windows (version 6.12, 1996). Comparisons among treatments were calculated using Tukey's honestly significant difference test. Treatment mean differences with  $p < 0.05$  were considered statistically different.

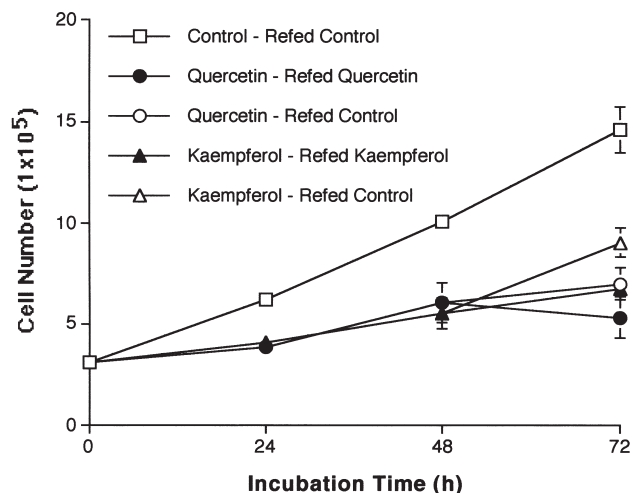
### Results

The structurally related bioflavonoids examined in the present studies for their effects on PC-3 cell proliferation are presented in Figure 2. These flavonoids were found to differ widely in their ability to inhibit PC-3 cell growth. Adding 100  $\mu\text{M}$  genistein, apigenin, and myricetin inhibited cell growth by 73%, 70%, and 59%, respectively, while isomolar naringenin and rutin inhibited proliferation by  $<25\%$ . Treatment of cells with 100  $\mu\text{M}$  quercetin, kaempferol, and luteolin caused complete growth inhibition. Because of their higher antitumorigenic potential, the remaining results focus on the antiproliferative effects of quercetin and kaempferol.

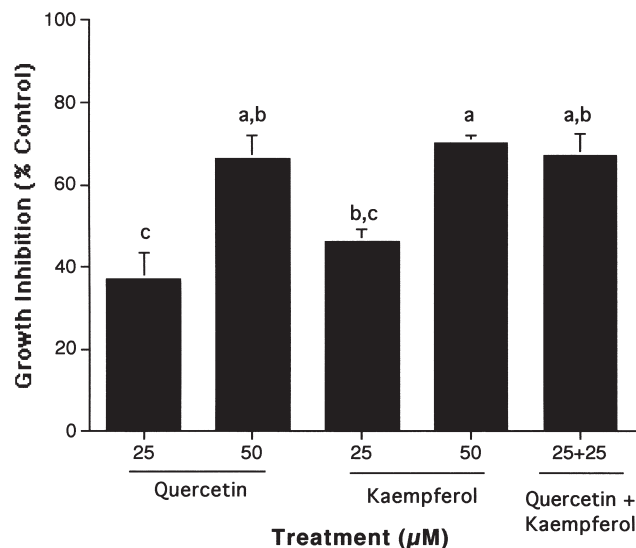
Dose-response experiments revealed that exposure to increasing concentrations of quercetin or kaempferol progressively decreased PC-3 cell proliferation (Figure 3). The equations and significance of correlation describing the relationship between the concentration of quercetin or kaempferol added to the incubation medium and the degree of growth inhibition are as follows:  $y = 0.502x + 26.385$  ( $r = 0.968$ ), and  $y = 0.696x + 18.920$  ( $r = 0.963$ ), respectively. Figure 4 illustrates that the antiproliferative effects of kaempferol are reversible. Refeeding kaempferol-treated cells with complete medium without the flavonoid resulted in a return toward control growth rates within 24 hours. Similar growth recovery was not observed in quercetin-treated cells. Growth continued to be depressed in cells refeed medium containing quercetin or kaempferol. The simultaneous addi-



**Figure 3.** Effect of increasing concentrations of quercetin (A) and kaempferol (B) on PC-3 cell growth. Each symbol represents mean growth inhibition 48 h after treatment. Values are means  $\pm$  SEM of 3 observations/treatment. Values not sharing a common superscript (a–c) differ ( $p < 0.05$ ). Maximum SEM for all measurements in both experiments was  $\pm 5\%$ .



**Figure 4.** Recoverability of quercetin- or kaempferol-treated cells. PC-3 cells were initially plated at  $4 \times 10^3/\text{cm}^2$  in flasks 48 h before a 48-h treatment with 0 or 50  $\mu\text{M}$  quercetin or kaempferol, then cells were refed control medium with or without flavonoid for an additional 24 h. Values are means  $\pm$  SEM of 3 observations/treatment. Maximum SEM for all measurements was  $\pm 1.14 \times 10^5$  cells.



**Figure 5.** Effect of combined concentrations of quercetin and kaempferol on PC-3 cell growth. PC-3 cells were initially plated at  $4 \times 10^3/\text{cm}^2$  in flasks 48 h before a 48-h treatment with 25 or 50  $\mu\text{M}$  quercetin or kaempferol or 25  $\mu\text{M}$  quercetin + 25  $\mu\text{M}$  kaempferol. Values are means  $\pm$  SEM of 3 observations/treatment. Maximum SEM for all measurements was  $\pm 6.3\%$ . Values not sharing a common subscript (a-c) differ ( $p < 0.05$ ).

tion of 25  $\mu\text{M}$  quercetin and kaempferol to the culture medium resulted in growth inhibition comparable to that when either was provided at 50  $\mu\text{M}$  (Figure 5).

Examination of cell cycle distribution found that whereas no change in cycle progression occurred in quercetin-treated cells, kaempferol (25  $\mu\text{M}$ ) exposure resulted in an approximately twofold increase in the proportion blocked within the  $G_2/M$  phase compared with untreated controls (Table 1). A slightly lower  $G_2/M$  arrest was observed in cells cultured in the presence of quercetin and kaempferol (25  $\mu\text{M}$ ). To maxi-

**Table 1.** Effect of Quercetin and Kaempferol on the Percentage of Cells in the Various Phases of the Cell Cycle<sup>a-c</sup>

Treatment	Cycle Phase		
	$G_1$	S	$G_2/M$
Control	$63.8 \pm 2.7^*$	$28.7 \pm 4.0^*$	$7.6 \pm 1.6^*$
Quercetin (25 $\mu\text{M}$ )	$54.2 \pm 1.1^\dagger$	$33.6 \pm 1.8^*$	$12.2 \pm 1.1^\ddagger$
Quercetin (50 $\mu\text{M}$ )	$57.0 \pm 0.3^{*\dagger}$	$33.5 \pm 1.5^*$	$9.5 \pm 1.2^{*\ddagger}$
Kaempferol (25 $\mu\text{M}$ )	$47.5 \pm 0.4^\dagger$	$31.7 \pm 0.8^*$	$20.8 \pm 1.1^{\ddagger\ddagger}$
Kaempferol (50 $\mu\text{M}$ )	$47.5 \pm 1.0^\dagger$	$31.0 \pm 1.7^*$	$21.5 \pm 1.8^{\ddagger\ddagger}$
Quercetin (25 $\mu\text{M}$ ) + kaempferol (25 $\mu\text{M}$ )	$50.9 \pm 2.9^\dagger$	$34.3 \pm 4.0^*$	$14.8 \pm 1.5^\ddagger$

a: Values are means  $\pm$  SEM of 3 replicates per treatment, except 50  $\mu\text{M}$  quercetin, which had 2 replicates.

b: Unsynchronized cells were initially plated at  $4 \times 10^3/\text{cm}^2$  48 h before a 24-h treatment with 0, 25, and 50  $\mu\text{M}$  quercetin or kaempferol and the combination of these flavonoids at 25  $\mu\text{M}$ .

c: Values within a phase of the cycle that do not share a common superscript (\*, †, ‡) differ significantly ( $p < 0.05$ ).

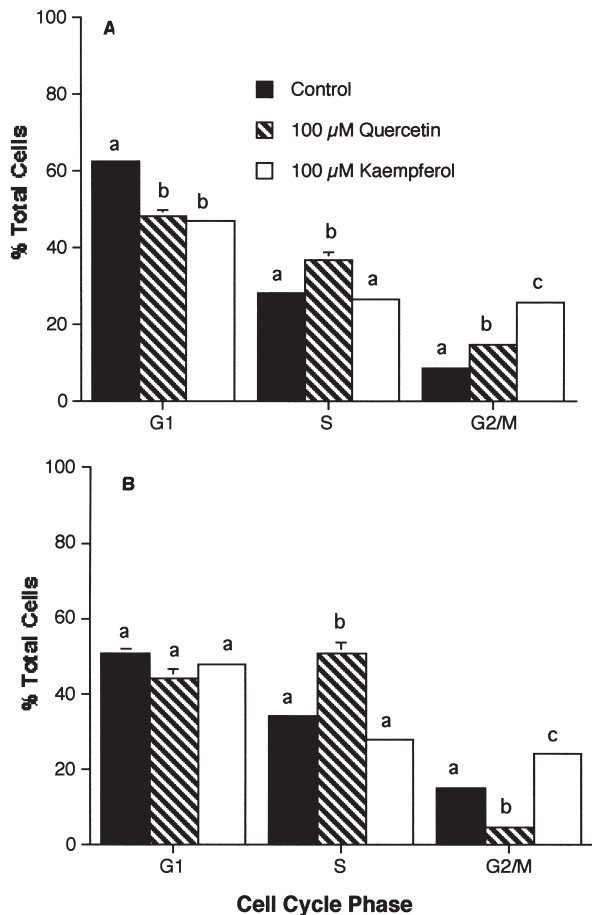
mize detection of these cell cycle alterations, quercetin and kaempferol were added to the medium at 100  $\mu\text{M}$ . Figure 6 reveals that exposure to 100  $\mu\text{M}$  quercetin triggered an increase (29% and 48% at 24 and 48 h, respectively) in the proportion of cells located in the S phase compared with controls. In contrast, kaempferol (100  $\mu\text{M}$ ) treatment induced a comparable increase (24% or 26%) in the proportion blocked in the  $G_2/M$  phase by 24 and 48 hours, respectively (Figure 6). In response to fluctuations in control profiles, the magnitude of this  $G_2/M$  increase differed and was 1.6 and 2.8 times that occurring in untreated cells (9% or 15%) for 24 and 48 hours, respectively.

DNA fragmentation was not induced after exposure to 100  $\mu\text{M}$  quercetin or kaempferol (Figure 7). Prolonging the exposure to quercetin or kaempferol did not influence the degree of DNA fragmentation.

## Discussion

Prostate cancers typically belong to a class of tumors that are androgen hormone dependent (30,31). Clinically, estrogen replacement therapy and androgen withdrawal have been used as effective strategies for decreasing the growth of these tumors (31). Androgen ablation can lead to the development of androgen-insensitive tumors that are less responsive to conventional chemopreventive strategies (30,31). The present studies reveal that flavonoid treatment may offer an alternative strategy for suppressing androgen-insensitive tumor growth. While the mechanism by which flavonoids modify the growth of these cells remains to be determined, increasing evidence suggests that they change the estrogen receptor and hormone-responsive genes (1).

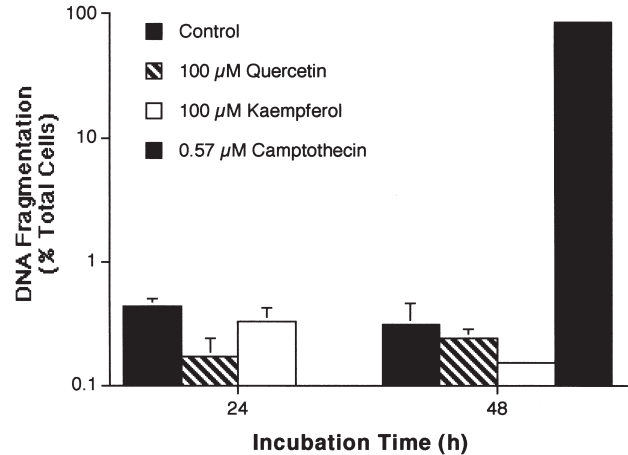
These studies reveal that flavonoids are inhibitors of androgen-independent human prostate tumor proliferation. Although not all the flavonoids examined were equally effective in depressing growth, substantial suppressions



**Figure 6.** Effect of 100  $\mu\text{M}$  quercetin or kaempferol on 24- (A) and 48-h (B) PC-3 cell cycle distribution. Quercetin significantly increased percentage of cells in S phase, while kaempferol induced a G<sub>2</sub>/M phase arrest. Values are means  $\pm$  SEM of 3 observations/treatment, except kaempferol at 48 h, which had 2 replicates. Values within a phase of cell cycle at each time that do not share a common superscript (a–c) differ significantly ( $p < 0.05$ ). Maximum SEM for all measurements was  $\pm 2.6\%$ .

were achieved after exposure to quercetin, kaempferol, luteolin, myricetin, apigenin, and genistein. One factor reported to influence these differences in efficacy is the arrangement of chemical groups around the flavonoid nucleus (32,33). In these studies, maximum growth inhibition was achieved by flavonoids possessing the C ring 2,3-double bond and 4-carbonyl group. Absence of the 2,3-double bond in naringenin, which is otherwise structurally similar to apigenin, drastically reduced its ability to suppress proliferation. Whether removal of the 4-carbonyl group would also lead to decreases in efficacy remains to be determined.

Comparisons between the growth inhibition caused by quercetin and its glycoside rutin reveal that addition of a sugar moiety to the C ring 3-position is another factor decreasing efficacy. Quercetin was approximately fourfold more effective in reducing PC-3 prostate tumor cell proliferation than rutin. Loss of the 3-hydroxyl group from the C ring is thought to explain this decrease in biological activity (34,35). However, examination of quercetin and luteolin



**Figure 7.** Impact of 100  $\mu\text{M}$  quercetin or kaempferol on inducing apoptosis in PC-3 cells after 24 or 48 h of exposure. Treatment of PC-3 cells with camptothecin, a compound known to stimulate apoptosis, served as a positive control for detection of DNA fragments. Values are means  $\pm$  SEM of 3 observations/treatment, except kaempferol at 48 h, which had 2 replicates. Maximum SEM for all measurements was  $\pm 6.0\%$ . Quercetin and kaempferol failed to induce apoptosis.

reveals that removal of this residue was not a factor determining efficacy in these studies. Recently, Plumb and co-workers (35) reported that the sugar residue addition interferes with the ability of the B ring's 3',4'-dihydroxy catechol configuration to act as a hydrogen donor. Impaired antioxidant activity may account for the reduced antiproliferative effects of rutin compared with other flavonoids examined in the present studies.

Quercetin and kaempferol are two of the predominant flavonoids present in fruits and vegetables (36,37). Although exact intakes of these flavonols remain unknown, total flavonoid consumption in Western societies is estimated at 23 mg/day (37). In the present studies, quercetin and kaempferol were effective in suppressing proliferation. Concentration and duration of exposure significantly affected the ability of PC-3 cells to survive. Quercetin's antiproliferative effects were found to continue even after the compound was removed from the culture medium. In contrast, cells were found to acclimate to kaempferol (50  $\mu\text{M}$ ) exposure, as demonstrated by their return toward normal proliferation rates when refed complete medium without the flavonoid. Whereas the antiproliferative effects of kaempferol were not permanent, suppressions in PC-3 proliferation continued after repeat flavonoid exposure.

Suppression of cell proliferation through alterations in division and apoptosis is well documented (21,22). Clearly, apoptosis was not a mechanism mediating antiproliferative effects of quercetin or kaempferol in the present studies. Suppressions in cell division did, however, correspond to the observed depressions in growth. The ability of quercetin to arrest cells in the S phase was consistent with earlier reports demonstrating blocks in G<sub>1</sub>-to-S and G<sub>2</sub>-to-M phase progression (16,26,27). These studies provide evidence that kaempferol alters the progression of PC-3 cells from the G<sub>2</sub> to the

M phase of the cell cycle. Maximum elevations in the G<sub>2</sub>/M population were achieved using 25 μM kaempferol. Exposure to 100 μM kaempferol induced similar increases in the G<sub>2</sub>/M population at 24 and 48 hours. Since the antiproliferative effects of kaempferol are not permanent, the duration of this G<sub>2</sub>/M arrest likely relates to the rate of its metabolism by the cell.

Cyclin-cyclin-dependent protein kinase complexes regulate the progression of cells through the various phases of the cell cycle (21,22). Specifically, p34<sup>cdc2</sup> kinase activation is known to modulate G<sub>2</sub>-to-M phase progression (38). A number of antitumorigenic compounds block cells in the G<sub>2</sub>/M phase by suppressing p34<sup>cdc2</sup> kinase activity (23–25). Evidence that flavonoids modify p34<sup>cdc2</sup> activity comes from Lopley and others (28), who revealed that apigenin depressed kinase activation by suppressing cyclin B<sub>1</sub> protein expression and increasing the amount of the inactive hyperphosphorylated p34<sup>cdc2</sup>. Similar disruptions in p34<sup>cdc2</sup> kinase activity have been observed after genistein treatment (16). It remains to be determined whether the ability of kaempferol to arrest cells in the G<sub>2</sub>/M phase relates to suppressions in p34<sup>cdc2</sup> kinase activity.

Synergistic suppressions in tumor proliferation have been documented in cells exposed simultaneously to flavonoid and nonflavonoid antitumorigenic agents (19,20). To our knowledge, the present studies were the first to examine the impact of flavonoid combinations on prostate tumor proliferation. The simultaneous addition of quercetin and kaempferol did not enhance their antiproliferative effects but, instead, resulted in their additive inhibition of PC-3 growth. While alterations in different enzyme systems and cell cycle transitions are thought to account for the increased potency of flavonoid/nonflavonoid anticancer combinations (19,20), the ability of quercetin and kaempferol to modify different phases of the cell cycle did not lead to enhanced antiproliferative effects in these studies. Recent data from Ciolino and associates (8) revealed that although quercetin and kaempferol are ligands of the aryl hydrocarbon receptor, only quercetin induced CYP 1A1 transcription. Competition for a common cellular receptor and/or differences in their ability to modify the receptor's function may account for additive antiproliferative effects observed after simultaneous exposure to quercetin and kaempferol.

In summary, the present studies demonstrate that flavonoids are effective inhibitors of androgen-independent prostate tumor proliferation. While the presence of the 2,3-double bond on the flavonoid C ring was clearly a factor determining efficacy, the exact role of the 4-carbonyl in mediating tumor inhibition remains unknown. In these studies, the ability of quercetin to suppress proliferation related to the induction of an S phase arrest, whereas kaempferol blocked G<sub>2</sub>-to-M phase progression. Simultaneous exposures to quercetin and kaempferol did not lead to an enhancement in these antitumorigenic effects. Additional studies are needed to clarify the mechanism(s) by which quercetin and kaempferol block cell cycle progression.

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