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Differential inhibition of proliferation of human squamous cell carcinoma, gliosarcoma and embryonic fibroblast-like lung cells in culture by plant flavonoids

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We investigated the antiproliferative effect of two polyhydroxylated (quercetin and taxifolin) and two polymethoxylated (nobiletin and tangeretin) flavonoids against three cell lines in tissue culture. Tangeretin and nobiletin markedly inhibited the proliferation of a squamous cell carcinoma (HTB 43) and a gliosarcoma (9L) cell line at 2–8 μg/ml concentrations. Quercetin displayed no effect on 9L cell growth at these concentrations, while at 8 μg/ml it inhibited HTB 43 cell growth. Taxifolin slightly inhibited HTB 43 cell growth at 8 μg/ml, while moderately inhibiting HTB 43 cell growth at 2–8 μg/ml. The proliferation of a human lung fibroblast-like cell line (CCL 135) was relatively insensitive to low concentrations of the above flavonoids.

Key words: Antiproliferative effects, flavonoids, malignant cells, nobiletin, quercetin, tangeretin.

Introduction

The flavonoids, which are benzo-γ-pyrone (phenylchromone) derivatives, comprise a large class of naturally-occurring, low molecular weight polyphenolic compounds which display a wide range of pharmacodynamic properties.[1–4] They are present in all vascular plants and their consumption in the normal human diet amounts to 1 g or more per day.[5] Recently, there has been considerable interest in the antimutagenic and anticarcinogenic properties of plant flavonoids.[6,7] In addition, the possible anticancer activity of naturally occurring flavonoids has also received some attention. Edwards *et al.*[8] reported that quercetin (a pentahydroxy flavone containing vicinal hydroxy groups in the B ring) and another catechol-containing flavonoid (5,7,3′,4′-tetrahydroxy-3-glycosyl flavone) possessed antineoplastic activity toward Walker carcinoma 256. Molnar *et al.*[9] showed the antitumor activity of polyhydroxylated flavonoids towards NK/Ly ascites tumors in mice.

The hydroxylated flavonoids have been shown to inhibit the proliferation of several rapidly growing tumor cell lines *in vitro*. Suolinna *et al.*[10] demonstrated the inhibition by quercetin of aerobic glycolysis in Ehrlich ascites tumor cells. These authors found quercetin to inhibit the growth of L1210 and P388 leukemia and Ehrlich ascites tumor cells. Subsequent studies by several authors reported the growth-inhibitory effects of this flavonoid on NK/Ly ascites tumor cells,[9] HeLa cells,[11] gastric cancer cells (HGC-27, NUGC-2, MKN-7 and MKN-28),[12] MCF-7 human breast cancer cells,[13] human pharyngeal cancer cells,[14] colon cancer cells (COLON320 DM)[15] and ovarian cancer cells (OVCA 433).[16] Apigenin (4′,5,7-trihydroxyflavone) also appeared to diminish the growth of a human breast carcinoma cell line (ZR-75-1) in culture.[17] Interestingly, Mookerjee *et al.*[18] demonstrated that the methoxylated plant flavonoid tangeretin (which possesses no free hydroxyl groups), in addition to quercetin,

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inhibited human lymphocyte proliferation stimulated by the tumor promoter phorbol myristic acetate. Consistent with this observation we noticed that tangeretin and nobiletin (another polymethoxyflavonoid lacking free hydroxyl groups) significantly inhibited the proliferation of human squamous cell carcinoma (HTB 43) cells *in vitro* at concentrations ranging from 2 to 8 μg/ml, while quercetin displayed no inhibition at these concentrations.[19] In this context, we wished to compare the effects of the polymethoxylated flavonoids on the growth and survival of malignant human cells and normal cells in culture. We also thought it instructive to compare the tumor cell proliferation inhibitory action of polyhydroxylated and polymethoxylated flavonoids. In the following, we describe the effects of these plant flavonoids on the growth and proliferation of human squamous cell carcinoma (HTB 43), gliosarcoma (9L) and lung fibroblast-like cells (CCL 135) in culture.

Materials and methods

Monolayer cultures of human squamous cell carcinoma cells HTB 43, lung fibroblast-like cell CCL 135 and 9L goiosarcoma cells were grown in minimum essential medium containing 10% fetal calf serum and L-glutamine (398 μg/l). Cells growing in log phase were harvested by suspension in 0.25% trypsin–EDTA for 5 min, washed in medium and resuspended at a concentration of 2×10^3 cells/ml. Cells were plated at a concentration of 2×10^3 cells/well in a volume of 1 ml in 24-well plates. Cells were incubated at 37°C for 24 h to allow for attachment of cells to plates after which nobiletin, tangeretin, quercetin and taxifolin dissolved in dimethylsulfoxide (DMSO) were added in a final volume of 1 ml to provide concentrations of 2, 4 and 8 μg/ml. Control wells received 1 μl of 100% DMSO. Plates were then incubated at 37°C in a 5% CO_2 and 95% air atmosphere.

Cell growth determination was made by counting cells excluding trypan blue using a hemocytometer. Cell viability was also determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as the assessable end-point, employing a colorimetric assay.[20] On days 3, 5 and 7 cells were harvested with 0.25% trypsin–EDTA in duplicate from wells representing each treatment condition; the cells were counted in a hemocytometer. The MTT reduction assay was performed in duplicate wells by removing culture medium from each well and adding 1.0 ml of 0.5 mg/ml of MTT in Tris–dextrose buffer. The culture plates were incubated at 37°C for 3 h to allow formation of the indicator formazan crystals. After the incubation period, the overlying tetrazolium solution was removed and 1 ml of acidified isopropanol was added to each well to dissolve the cell-associated formazan. The absorbance of the formazan solution was then measured at 570 nm. Linear regression analysis was performed to determine the correlation between cell viability obtained by trypan blue exclusion and tetrazolium reduction on days 3, 5 and 7.

Results

We studied the effects of four flavonoids (both polymethoxylated and polyhydroxylated), i.e. tangeretin, nobiletin, quercetin and taxifolen (Figure 1), on the growth and survival of squamous cell carcinoma HTB 43 cells in culture. As shown in Figure 2, control cells increased from the initial

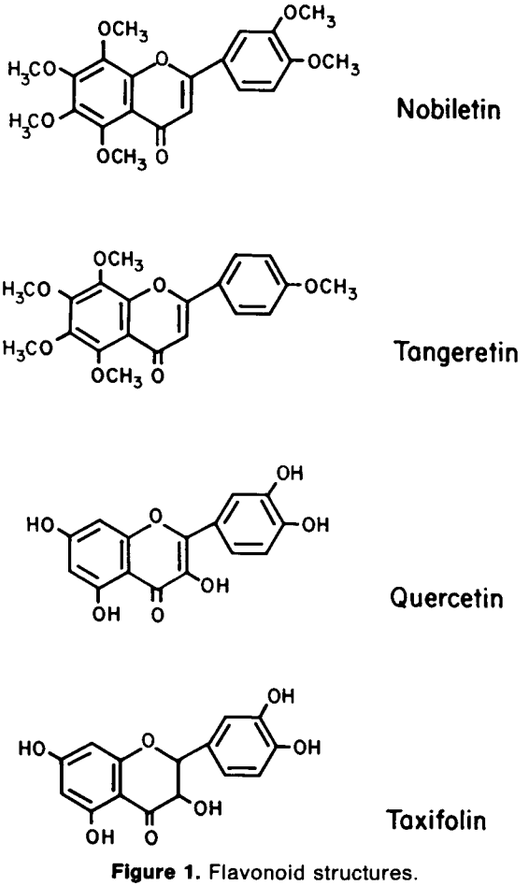

Figure 1. Flavonoid structures.

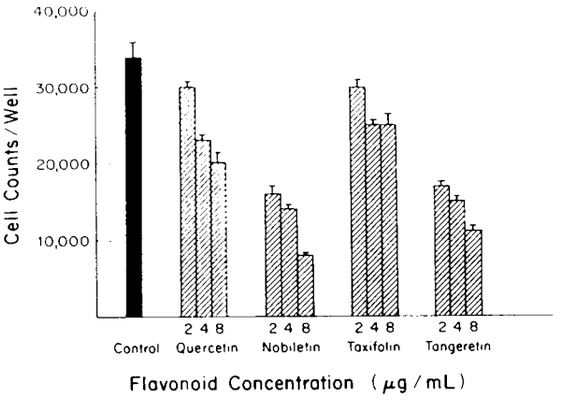

Figure 2. The inhibitory effects of flavonoids on the *in vitro* proliferation of human squamous cell carcinoma (HTB 43). Data (±SE of mean, $N = 3$) are from one of three experiments, all of which gave similar results. Numbers 2, 4 and 8 signify concentrations (in μg/ml).

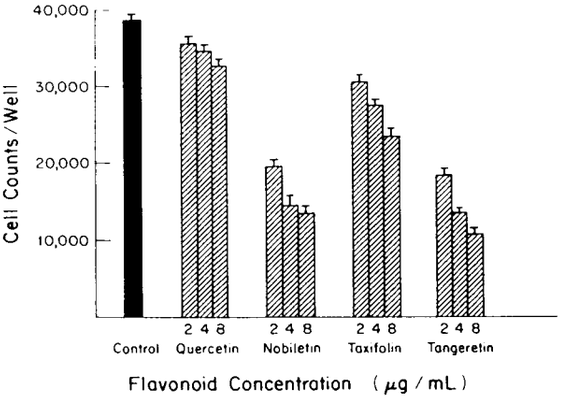

Figure 3. The inhibitory effects of flavonoids on the *in vitro* proliferation of 9L gliosarcoma cells. Data (±SE of mean, $N = 3$) are from one of three experiments, all of which gave similar results. Numbers 2, 4 and 8 signify concentrations (in μg/ml).

inoculation of 2500 to approximately 33 750 cells/well at 72 h. The methoxy-substituted flavonoids, nobiletin and tangerretin, caused a concentration-dependent decrease in cell growth over a 72 h period. At a concentration of 2 μg/ml, the percentage of growth inhibition by tangeretin and nobiletin was 50 and 53%, respectively, at 72 h. At a concentration of 4 μg/ml the extent of growth inhibition by these two flavonoids was 55 and 58%, respectively. At 8 μg/ml the corresponding values for the two flavonoids were 67 and 75%, respectively. Quercetin at 2 μg/ml suppressed cell growth by 12% at 72 h, while at 4 and 8 μg/ml, the percentages of inhibition were 32 and 41%, respectively. Taxifolin (dihydroquercetin) was not effective in inhibiting the growth of HTB 43 cells. At 2 μg/ml, taxifolin inhibited growth by 12%, while at 4 and 8 μg/ml, the extent of inhibition was 26 and 24%, respectively. Thus, the polymethoxylated flavonoids exerted significant growth inhibition of human squamous cell carcinoma cells at all the concentrations tested and were also more potent than the polyhydroxylated flavonoids in their antiproliferative effect under the conditions employed for cell growth.

Tangeretin and nobiletin also exerted concentration-dependent growth inhibitory effects on cultured gliosarcoma cells (Figure 3). The percentages of growth inhibition by these two flavonoids at 2 μg/ml were 51 and 48%, respectively, at 72 h. The inhibition at 4 μg/ml was 60 and 62%, respectively, while the values at 8 μg/ml were 64 and 73%, respectively. Quercetin at 8 μg/ml inhibited cell growth by 16% at 72 h of growth, while the values at 2 and 4 μg/ml were 9 and 10%, respectively. At 2 μg/ml taxifolin showed a 22% growth inhibition at 72 h, while at 4 and 8 μg/ml, the inhibition was 33 and 49%, respectively. Nobiletin and tangeretin dramatically inhibited cell growth at 120 and 168 h (not shown). It is noteworthy that the flavonoids studied herein exhibit similar effects on the growth and survival of human malignant cells of diverse origin, suggesting that a common mechanism of action may be operative in different malignant cells.

That the flavonoids studied impaired the growth and survival of HTB 43 cells was ascertained by the MTT reduction assay. This assay showed a concentration-dependent inhibition of cellular proliferation. There was an excellent correlation between cell counts obtained by trypan blue exclusion and cell viability as determined by MTT reduction (Figure 4). The correlation coefficient (r value) obtained by linear regression analysis was 0.93 for 72 h of cell growth. The values for 120 and 168 h were 0.95 and 0.96, respectively.

Both the polymethoxylated and polyhydroxylated flavonoids exerted a diminished inhibitory effect on the growth and survival of CCL 135 cells (a normal human embryonic, fibroblast-like cell line) as compared with that of the malignant human cells studied, as shown above. The CCL 135 grew rapidly in culture, increasing from an initial inoculation of 2500 to about 130 000 cells/well in 72 h (Figure 5). At a concentration of 2 μg/ml, tangeretin and nobiletin exerted 7 and 11% inhibition at 72 h. At 4 μg/ml, nobiletin showed only 9% inhibition. At this concentration, however,

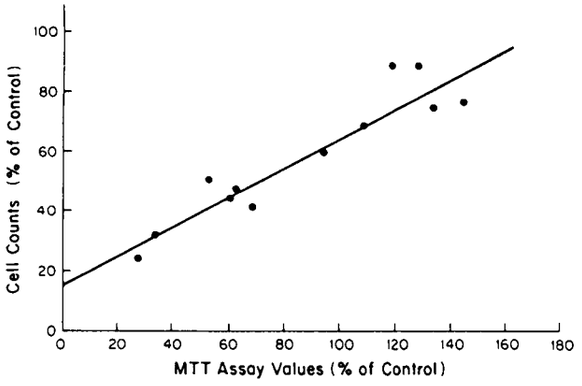

Figure 4. Linear relationship curve showing the correlation between MTT reduction values (viability) and cell counts. The *r* values were obtained by linear regression analysis.

tangeretin exhibited 30% inhibition of cell growth. At 8 μg/ml, both nobiletin and tangeretin exerted significant growth inhibition (57 and 71%, respectively). At 2 and 4 μg/ml, quercetin inhibited cell growth by 11 and 15%, respectively; at 8 μg/ml, however, quercetin diminished cell growth by 70%. In contrast, taxifolin depressed cell growth by only 23% at this concentration. The extents of inhibition by taxifolin at 2 and 4 μg/ml were 22 and 26%, respectively.

In spite of their marked effect on the proliferation of squamous cell carcinoma and gliosarcoma cells at lower concentrations, the polymethoxylated flavonoids do not seem to impair the growth of normal human embryonic cells at similar concentrations. Whether these flavonoids exert a preferential proliferation-inhibiting action on malignant tumor cells remains to be establishesd.

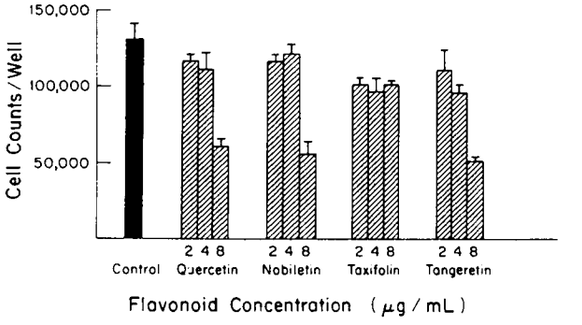

Figure 5. The inhibitory effects of flavonoids on the *in vitro* proliferation of human embryonic fibroblast-like lung cells (CCL 135). Data (±SE of mean, $N = 3$) are from one of three experiments, all of which gave similar results. Numbers 2, 4 and 8 signify concentrations (in μg/ml).

Discussion

Our data indicate that both tangeretin and nobiletin inhibit the growth of human gliosarcoma and squamous cell carcinoma cells. Interestingly, Mori *et al.* indicated that the presence of a single methoxyl group at certain positions enhanced the growth-inhibitory effect of flavones against HeLa cells. In the case of tangeretin and nobiletin, however, all the hydroxyl groups in the flavone structure are methoxylated. Bracke *et al.*[21] reported the inhibition by tangeretin of the invasion of MO_4 cells (Kristen murine sarcoma virus transformed fetal mouse cells) into embryonic chick heart fragments *in vitro*. The flavonoid appeared to be chemically stable in the cell culture medium and inhibited the growth of MO_4 aggregates in suspension culture at a concentration of 100 μM. In the present investigation, however, growth inhibition of HTB 43 and 9L cells by tangeretin and nobiletin was found at far lower concentrations (5–20 μM).

Because of its free phenolic groups, quercetin is more polar than tangeretin and nobiletin and, therefore, its uptake by cells could be less than that of the polymethoxylated flavonoids. This could account for the relatively lesser cell growth-inhibitory effect of quercetin at lower concentrations. The introduction of several methoxyl groups would result in a decrease in hydrophilicity of the flavonoid and would facilitate enhanced uptake by cell membranes, thus accounting for the higher proliferation-inhibiting activity of tangeretin and nobiletin.

The higher growth-suppressive activity of the polymethoxylated flavonoids observed here with respect to malignant cells may partly be ascribed to their chemical stability in the cellular environment. Methylation of the phenolic groups, as in the case of tangeretin and nobiletin, can confer greater stability to these compounds. The lack of hydroxyl groups would render them resistant to chelation by metal ions with subsequent oxidative degradation, thus making them freely available at cellular sites of interaction.

Flavonoids possessing vicinal dihydroxy (catechol) and trihydroxy (pyrogallol) configurations in their B-ring are known to undergo autoxidation in aqueous solution.[22,23] Autoxidation of such flavonoids to their corresponding quinones, followed by intracellular reduction in the presence of molecular oxygen (redox cycling), may generate oxygen radicals which are cytotoxic. Such reactive oxygen species can directly damage DNA by strand scission.[24] Quercetin which contains a catechol

configuration in the B-ring can autoxidize in an aqueous medium, although it is slow at pH 7.5.[22] Such autoxidation and the resultant toxic oxygen radical production may be partially implicated in its cytotoxic activity against HTB 43 cells. In the case of tangeretin and nobiletin, however, other mechanisms of cell growth inhibition have to be invoked.

Hydroxylated flavonoids may inhibit tumor cell growth by interacting with nuclear type II sites. Markaverich *et al.*[13] reported that quercetin and another hydroxylated flavonoid, luteolin, specifically competed for [^{3}H]estradiol binding to nuclear type II sites in rat uterine preparations. They also found that these two flavonoids inhibited the growth of human breast cancer (MCF-7) cells in culture. Since estradiol-17β stimulated nuclear type II sites and the proliferation of MCF-7 cells, these authors indicated that flavonoid inhibition of MCF-7 cell growth might be mediated through an interaction with nuclear type II sites. Since tangeretin and nobiletin do not possess any free hydroxyl groups, these polymethoxylated flavones may inhibit tumor cell growth by other mechanisms. In exploring the potential mechanisms of antitumor effects of flavonoids, an important and interesting question arises as to the potential effects of inhibitory flavonoids on cell cycle-associated events. Cytofluorimetric studies indicated that quercetin induced a G_1 block in human gastric cancer cells (HGC 27) in culture,[12] while it induced a block at the G_1/S boundary in human colon cancer cells (COLO320 DM).[15] Our preliminary studies employing flow cytometry of propidium bromide-stained HTB 43 cells have not yet indicated any consistent effect of the methoxylated flavonoids tested. We are continuing these studies.

The potential inhibitory effect of plant flavonoids on protein tyrosine kinases may also account for the antitumor action of these compounds. Quercetin was found to inhibit the activity of a tyrosine-specific protein kinase thought to be responsible for the transformation of non-malignant fibroblasts to sarcoma cells.[25] The preliminary studies of Cunningham *et al.*[26] indicated that quercetin inhibited the growth of Abelson-transformed NIH-3T3 cells which express the Abelson tyrosine protein kinase. In preliminary studies, we have observed that quercetin and certain other hydroxylated flavonoids impair tyrosine protein kinase activity of isolated HTB 43 cell membranes. We are currently examining whether these flavonoids can inhibit this activity in intact HTB 43 cells in culture. Preliminary studies showed that the polymethoxylated flavonoids did not inhibit this activity in isolated HTB 43 membranes. We are currently examining this problem in detail. At this juncture, it is of interest to recall our earlier observations on the inability of polymethoxylated flavonoids to inhibit protein kinase C activity *in vitro*.[27]

The efficacy of the hydroxylated flavonoids to impair cell growth may be related to their relative stability under the conditions of cell growth employed, their ability to enter cells, and susceptibility to oxidative degradation. It is evident that quercetin is ionized at slightly above neutral pH since ionization of the hydroxyl groups results in a bathochromic shift in the flavonoid spectrum.[28] It is reasonable to expect ionization of one or more of the hydroxyl groups of quercetin at near-neutral pH. Since an ionized molecule may not cross the cell membrane as readily as a non-ionized one, ionization of quercetin may also contribute to its decreased cellular uptake, thus accounting for its diminished inhibitory effect on cell growth.

Hydroxylated flavonoids, like quercetin, possess the propensity to chelate metal ions.[29,30] Metal ions like Cu(II) can oxidize quercetin in aqueous media.[31] Chelation of the vicinal hydroxyl groups of quercetin by Cu(II) would result in its conversion to a quinone.[31] Such a chelation may have an impact on the cytotoxic activity of quercetin and related flavonoids.

The question of the stability of quercetin arises when evaluating the cell growth-inhibiting potential. Based on their observations, Ueno *et al.*[32] suggested that quercetin was degraded autocatalytically by self-generated superoxide anions at above neutral pH. In this context, reducing agents and antioxidants may enhance the stability of quercetin and hence its antiproliferative activity. We are currently looking into these possibilities.

Conclusion

The polymethoxylated flavonoids, tangeretin and nobiletin, inhibited the growth of human malignant (squamous cell carcinoma and gliosarcoma) cells in tissue culture. In general, they were more potent than the polyhydroxylated flavonoid, quercetin, in inhibiting tumor cell proliferation. This may be attributed to their greater stability and increased cellular uptake. The flavonoids studied exerted a preferential antiproliferative effect on these malignant cells as compared with a normal, fibroblast-like human lung cell. Since the *in vitro* antitumor activity

of several flavonoids has now been demonstrated, it may be well worthwhile to screen these naturally-occurring compounds for their anticancer action *in vivo*.

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