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Review

The Antitumor Activities of Flavonoids

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Abstract. *The flavonoids are polyphenolic compounds found as integral components of the human diet. They are universally present as constituents of flowering plants, particularly of food plants. The flavonoids are phenyl substituted chromones (benzopyran derivatives) consisting of a 15-carbon basic skeleton (C_6-C_3-C_6), composed of a chroman (C_6-C_3) nucleus (the benzo ring A and the heterocyclic ring C), also shared by the tocopherols, with a phenyl (the aromatic ring B) substitution usually at the 2-position. Different substitutions can typically occur in the rings, A and B. Several plants and spices containing flavonoid derivatives have found application as disease preventive and therapeutic agents in traditional medicine in Asia for thousands of years. The selection of a particular food plant, plant tissue or herb for its potential health benefits appears to mirror its flavonoid composition. The much lower risk of colon, prostate and breast cancers in Asians, who consume more vegetables, fruits and tea than populations in the Western hemisphere do, raises the question of whether flavonoid components mediate the protective effects of diets rich in these foodstuffs by acting as natural chemopreventive and anticancer agents. An impressive body of information exists on the antitumor action of plant flavonoids. In vitro work has concentrated on the direct and indirect actions of flavonoids on tumor cells, and has found a variety of anticancer effects such as cell growth and kinase activity inhibition, apoptosis induction, suppression of the secretion of matrix metalloproteinases and of tumor invasive behavior. Furthermore, some studies have reported the impairment of in vivo angiogenesis by dietary flavonoids. Experimental animal studies indicate that certain dietary flavonoids possess antitumor activity. The hydroxylation pattern of the B ring of the flavones and flavonols, such as luteolin and quercetin, seems to critically influence their activities, especially the inhibition of protein kinase activity and antiproliferation. The different mechanisms underlying the potential anticancer action of plant flavonoids await further elucidation. Certain dietary flavonols and flavones targeting cell surface signal transduction enzymes, such as protein tyrosine and focal adhesion kinases, and the processes of angiogenesis appear to be promising candidates as anticancer agents. Further in vivo studies of these bioactive constituents is deemed necessary in order to develop flavonoid-based anticancer strategies. In view of the increasing interest in the association between dietary flavonoids and cancer initiation and progression, this important field is likely to witness expanded effort and to attract and stimulate further vigorous investigations.*

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The flavonoids, which are primarily benzo-γ-pyrone (phenylchromone) derivatives, comprise a massive group of polyphenolic compounds (1, 2). These natural antioxidants constitute more than 4,000 chemically unique and distinct moieties and enjoy almost ubiquitous distribution in the plant kingdom. The immensely diverse group broadly comprises distinct classes such as flavonols, flavans and proanthocyanidins, anthocyanidins, flavanones, flavones, isoflavones and neoflavonoids (Figure 1). They are universally present in

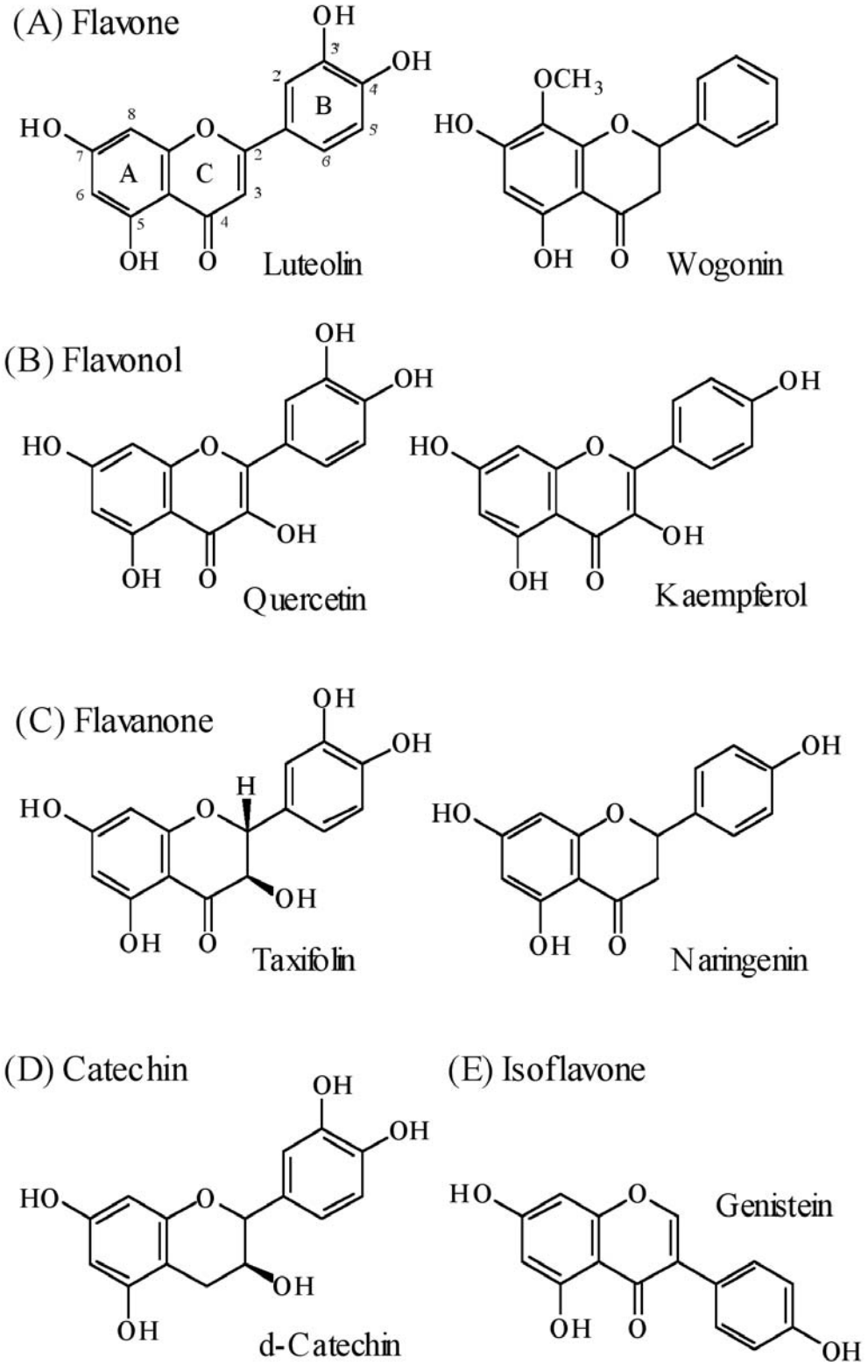

Figure 1. *The chemical structures of flavonoids. (A) Flavone: luteolin and wogonin; (B) Flavonol: quercetin and kaempferol; (C) Flavanones: taxifolin and naringenin; (D) Catechin: d-catechin; (E) Isoflavone: genistein.*

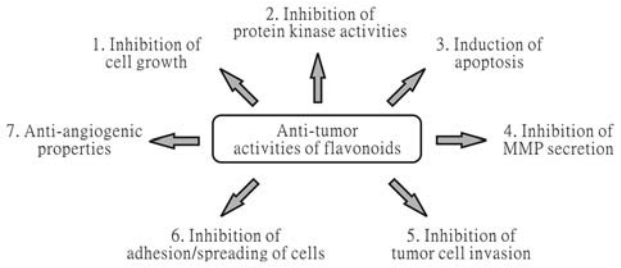

Figure 2. *Antitumor activities of flavonoids.*

fruits, cereals, legumes, vegetables, nuts, seeds, herbs, spices, stems and flowers, as well as in beverages such as tea, cocoa, beer and wine. As components of edible plants and plant foodstuffs, they constitute an integral part of the human diet (2-7). Early estimates for the average daily intake of mixed flavonoids in the U.S.A. were in the range of 0.5 to 1 g (4), expressed as glycosides, although later reports have indicated the figure to be frequently lower, at about 200 mg of mixed flavonoids (4). However, the consumption of such quantities may ensue pharmacologically significant levels in the plasma and body tissues.

The Dutch National Food Consumption Survey indicated an average intake of mixed flavonoids in the Netherlands of 23 mg/day, expressed as aglycones and essentially consisting of flavonols and flavones (free flavonoids and not glycosides), primarily originating from onions, apples and tea (8). The corresponding figure for free flavonoids in the U.S.A. could be much higher, even if one considers that the daily dietary consumption in this particular case is about 200 mg of mixed flavonoids (as glycosides). This becomes particularly significant since the absorption of flavonoid-glycosides by humans appears to be more efficient than that of the aglycones (9). Most of the flavonoids occur as conjugates and not simply as parent molecules. In addition, the total amount consumed, the kind and the source could show wide variation in other countries. For example, the dietary intake could be considerably higher in Asia, in vegetarian populations and in countries spanning the Mediterranean coast, where the diet is rich in olive oil, citrus fruits, grapes and greens. The dietary intake of flavonoids far exceeds that of vitamin E, a monophenolic antioxidant, and of β-carotene on a milligram/day basis (10).

The documented biological effects of dietary flavonoids include anti-inflammatory, anti-allergic, antimicrobial, hepatoprotective, antiviral, antithrombotic, cardioprotective, capillary strengthening, antidiabetic, anticarcinogenic and antineoplastic effects, among others (1, 2, 11-19). These dietary antioxidants exert significant immunomodulatory activities and show a propensity to critically influence a host of cellular inflammatory processes and immune functions, and cell surface signal transduction (11), which in disease states manifest as allergic reactions, inflammatory disorders, viral infection, tumor development and vascular dysfunction, among others. The much lower risk of colon, prostate and breast cancers in Asians, who generally consume more vegetables, fruits and tea than populations in the West do, raises the interesting, important and timely question of whether flavonoid components mediate the protective effects of diets rich in these foodstuffs by acting as natural chemopreventive and chemotherapeutic agents. Certain plant compounds, such as isoflavonoids, flavonoids and lignans, have received particular attention as putative cancer protective agents in populations with low incidences of breast and prostate cancer (20). Although the mechanisms underlying these potential chemopreventive and chemotherapeutic actions of dietary flavonoids await elucidation, some of the diverse pharmacological and cellular effects manifested by the flavonoids could have implications for these activities. Plant flavonoids possess the propensity to modify or modulate the activities of a host of enzyme systems critically involved in cell surface signal transduction, immune function, cellular transformation, tumor growth and metastasis, *etc.* (11, 18, 21-27). In particular, their ability to markedly modulate the activities of cellular protein tyrosine kinases might have significant implications for cancer protection. Since flavonoids display a vast array of cellular effects, several mechanisms might account for their potential anticancer actions.

Several food flavonoids not only inhibit the growth of tumor cells (11, 18, 28-30), but also induce cell differentiation (31). Earlier reports indicate that certain dietary flavonoids also exhibit potent antitumor activity *in vivo* (32, 33). The inhibitory effects of flavonoids on the growth of malignant cells could be a consequence of their interference with the protein kinase activities involved in the regulation of cellular proliferation and apoptosis (18, 34, 35). Additionally, the modulating effects of flavonoids on the cell cycle (36) and apoptosis (37, 38) are conceivably some of the factors that can mediate their antiproliferative activity. Furthermore, the possible anti-metastatic properties of flavonoids, such as the suppression of the secretion of MMPs (18, 34) and the modulation of epithelial cell migration (40), could also be relevant to their purported anticancer action. Limited epidemiological data exist on flavonoids and cancers, and we are yet to fully understand the association between dietary flavonoids and the incidence of cancers. Nevertheless, flavonoid-rich foods exhibit some protective characteristics. Finally, flavonoids are universally distributed in the plant kingdom (1-3), rendering them a very attractive target for further investigation.

The present review attempts to provide critical insights into the antitumor activities of dietary flavonoids, including the induction of apoptosis, suppression of protein tyrosine kinase activity, antiproliferation, antimetastatsis and anti-invasive effects and anti-angiogenesis (Figure 2). The current status of flavonoids that target cancers is explored.

How flavonoids modulate protein kinase activities and cancer development is particularly focused on.

The influence of dietary flavonoids on mammalian biology: implications for cancer development

Inhibition of protein kinase activity. Flavonoids influence the activity of a host of mammalian enzyme systems, both *in vitro* and *in vivo*. Among the enzymes modulated by flavonoids, protein kinases have attracted intensive investigation and a wealth of data has accumulated (11). It is well known that the inhibitory effects of flavonoids on the growth of malignant cells could be a consequence of their interference with the protein kinase activities involved in the regulation of cell proliferation (18, 36, 40, 41). Over the last two decades, a series of investigations have assessed the inhibitory potencies of different flavonoids on various serine/threonine and tyrosine kinases. At the cellular level, flavonoids from different chemical classes exhibit inhibitory effects on protein kinase activity (18, 40).

Under ideal circumstances, the effectiveness of a kinase inhibitor would be related to the kinase which caused disease (21). In addition, a successful kinase inhibitor should target a kinase, which is a requirement for the proliferation or survival of the cancer cell. Therefore, in this report, the influence of flavonoids on the activities of three major kinases, PKC, EGFR and FAK, which can play important roles in cancers, is discussed. Certain flavonoids were shown to inhibit their activities. Protein kinase C (PKC), the ubiquitous, Ca^{2+} and phospholipid-dependent, multi-functional serine- and threonine-phosphorylating enzyme, plays a role in a gamut of cellular activities, including tumor promotion, mitogenesis, secretary processes, inflammatory cell function and T lymphocyte function (42-44). Certain dietary flavonoids turned out to be potent inhibitors of PKC *in vitro* (45-50). Out of different flavonoids examined, quercetin was the most efficient inhibitor of PKC (51-53). Earlier, Graziani *et al.* (54) documented that quercetin diminished the phosphorylating activity of the Rous sarcoma virus transforming gene product both *in vitro* and *in vivo*, and the inhibition was competitive towards the nucleotide substrates ATP and GTP. Ferriola *et al.* (49) demonstrated that fisetin, quercetin and luteolin were the most active flavonoid inhibitors of a partially purified rat brain PKC preparation. By employing histone and protamine as protein substrates, and diacylglycerol and tetradecanoylphorbol acetate (TPA) as activators, they found that fisetin and luteolin competitively blocked the ATP binding site on the catalytic unit of PKC. Flavonoids, impairing the activities of other ATP-utilizing enzymes, cause inhibition by competitively binding to the ATP binding site. Both luteolin and quercetin possess a double bond between C2 and C3 in ring C (unsaturated C-ring), and -OH groups on C3' and C4' in its B-ring. However, luteolin does not have an -OH substitution on C3. Based on our data and that of others (18, 34, 40, 51), we consider that quercetin and luteolin possess the same ability to bind to ATP binding sites in PTK. However, we require a more systematic study, employing X-ray crystallography of luteolin and quercetin and known kinase data, in order to elucidate the detailed structure-function relationship involved in the interaction of flavonoids with the ATP binding site.

Protein phosphorylation/dephosphorylation is an important regulatory mechanism in the action of many hormones and growth factors, which transmit their message *via* activation of cellular PTKs (55-60). One can trace the history of protein tyrosine kinases back to 1911, when Peyton Rous first observed that chicken tumor could be transplanted using a cell-free filtrate (22). However, it was not until more recent advances in molecular biology that we recognized that the transformation activity was due to the presence of the Rous sarcoma virus (23). The developments in this case led to the finding that the *src* oncogene product ($pp60^{src}$) possessed intrinsic protein kinase specific to tyrosine residues. Since the discovery of the *src* oncogene as a protein tyrosine kinase, numerous tyrosine kinases have been found, and now over 100 are identified (23). The development of cancer comprises a multi-stage process involving progressive genetic changes in the positive or negative regulation of cell proliferation and survival. The activation of oncogenes or the inactivation of tumor suppressor genes, as many as four or five different genes, may be required for the development of a tumor. Tyrosine kinases could involve themselves in any one or more of the sequential steps in tumor development, such as progression, metastasis and angiogenesis (24). There is a general tendency to implicate the loss of PTK regulatory mechanisms in the growth of neoplastic lesions (25). The down-regulation of specific PTKs, in turn, could impair the growth of a specific tumor (25).

There is an elevation in the expression of PTKs, including several membrane-associated oncogene products, in tumor cells (22, 55-60). Moreover, there is an association of this increase with the development of various cancers (61, 62). Among the PTKs investigated, the role of epidermal growth factor receptor (EGFR) tyrosine kinase and focal adhesion kinase (FAK) in the regulation of the growth of cancer cells has commanded extensive investigations (26, 27), with the resultant accumulation of well-documented results (26-28). EGFR is a cell surface glycoprotein composed of a single polypeptide chain of 170 kDa, which binds to EGF (63, 64). The activated EGFR tyrosine kinases can phosphorylate a number of substrates, such as serine/threonine kinases, MAP kinases and raf (64). Since EGF can stimulate the growth of tumor cells, one would anticipate an inhibition in tumor cell growth as a result of a reduction in EGFR tyrosine kinase

activity (18, 34). At this juncture, one should note that, if agents could exert a decisively suppressive effect upon EGFR tyrosine kinase activity, their impact at the level of autophosphorylation would be the preferred site of action, because it would culminate in the blockade of the signaling pathway (34, 40, 65). Thus, it is imperative to assess whether flavonoids that possess antiproliferative activity exert any direct action upon the autophosphorylation of EGFR and of its intrinsic kinase activity.

In 1987, Akiyama *et al.* (35) proposed that the isoflavone, genistein, was a specific inhibitor of PTK. Genistein inhibited growth and induced differentiation in human HL-60 and K-562 leukemia cells (66). In addition, this isoflavone inhibited EGFR tyrosine kinase activity in human epidermoid carcinoma A431 cells that constitutively express EGFR (67). Furthermore, investigators hypothesized that genistein was responsible for controlling cell cycle progression in human breast (68, 69) and prostate (38) cancer cell lines. In addition to genistein, the flavone, luteolin (18, 34) and the flavonol, quercetin (18, 34, 51), effectively suppressed EGFR tyrosine kinase. We found that these two flavonoids were more potent than genistein in these cancer cells. Butein inhibited the EGF-induced autophosphorylation levels of EGFR in human Hep G2 cells, but had no significant impact on the activities of serine- and threonine-specific kinases, such as PKC and PKA (70). In our laboratory, we observed that quercetin and luteolin caused the transinactivation of EGFR tyrosine kinase and marked reduction in the phosphotyrosyl level of 125-, 65-, 60- and 42 kDa cellular proteins. We further identified the 125 kDa protein as focal adhesion kinase (34).

Focal adhesion kinase (FAK) is a non-receptor and non-membrane-associated PTK, originally isolated from v-Src-transformed chick-embryo fibroblasts (71). The human FAK, which does not contain Src homology 2 (SH2) or SH3 protein interaction domains, is an important regulator of cellular signaling pathways essential to cell survival and the cell cycle, as well as to cell motility (71). Overexpression of FAK occurs in a great number of human tumors, however, and it is reportedly an important determinant of tumorigenesis, cellular invasiveness and metastasis (71). The characteristics of FAK reveal an association between elevated FAK expression in human tumor cells and increased cell invasiveness (71-74); on the other hand, the inactivation of FAK accompanies suppressed cell proliferation and migration *in vitro*. Increased tumor invasiveness shows an association with augmented cell migration (73). The inhibition of FAK expression decreases cell motility. In growing, integrin-stimulated, or migrating cells, there is a high degree of phosphorylation of FAK in a number of tyrosine residues, *in vivo* (74, 75). Mechanistically, the stimulation of phosphorylation of FAK at Tyr-397 creates an SH2-binding motif that is required for FAK function in promoting cell motility (71, 73). The expression of PTEN, a tumor suppressor, leads to FAK dephosphorylation and inhibition of cell motility (74, 75). Furthermore, there is often an amplification of FAK overexpression and epidermal growth factor receptor (EGFR) tyrosine-kinase activities in tumor cells (75). Studies have demonstrated that the diminution of the activity or disabling the function of FAK impairs both growth and metastasis in a variety of tumors (40, 72-76).

In a previous study in our laboratory, we elaborated the importance of FAK function in the promotion of EGF-stimulated cell motility by resorting to flavonoid treatment in order to accomplish a reduction in FAK expression and phosphorylation (40). Tumor cells treated with luteolin or quercetin impeded the phosphorylation of FAK. In addition, our data clearly demonstrated that tumor cells responded to quercetin and luteolin by parallel reductions in the levels of phosphorylated FAK and the secreted matrix metalloproteinases (MMPs), that may lead to the suppression of invasive potential and cell migration. This is the first demonstration, to our knowledge, of this kind for these dietary flavonoids. While the molecular mechanisms operative in the regulation of tumor cell MMP secretion by FAK remain unclear, our results strongly suggest that blockade of the EGFR-signaling pathway may contribute to the net effect. Knowledge is lacking on how the flavonoids enter the cell and exert their action in the compartment where the kinases are localized. One possibility is that the flavonoids have no effect on kinases in quiescent cells and only interfere with the ATP-binding site when the enzyme translocates upon activation (11).

Antiproliferative activity. Over the last three decades, there has been considerable interest in assessing the tumor cell growth inhibitory effect of flavonoids. Quercetin, a flavonol, is the most prominent flavonoid known, being particularly abundant in fruits and vegetable, with an estimated daily intake of 25-30 mg in Europe (8). Suolinna *et al.* (28) demonstrated, in 1975, that quercetin exerted growth inhibitory effects *in vitro* on malignant tumor cell lines, such as Ehrlich ascites cells, L1210 and P-388 leukemia cells. These studies prompted the evaluation of the *in vivo* antitumor activity of dietary flavonoids, in particular that of quercetin. In later studies, Edwards *et al.* (32) explored the cytotoxic and *in vivo* antineoplastic activity of flavonols, flavones and isoflavones, and documented that quercetin and another catechol-containing flavonoid (5,7,3',4'-tetrahydroxy-3-glycosyloxyflavone) possessed antineoplastic activity towards Walker carcinoma 256. Subsequently, Molnar *et al.* (33) reported the antitumor activity of polyhydroxylated flavonoids towards NK/LY ascites tumors in mice. In 1989, we evaluated the antineoplastic activity of polyhydroxylated flavonols and reported the *in vivo* inhibition of the growth of

head and neck carcinoma cells implanted in an animal model (17). Quercetin, in addition to apigenin, also inhibited tumor development in other animal models (77).

The polyhydroxylated flavonoid, quercetin exerted potent growth inhibitory effects on several malignant tumor cell lines *in vitro*, such as NK/LY ascites tumor cells, HeLa cells (78), gastric cancer cells (HGC-27, NUGC-2, MKN-7 and MKN-28) (79), colon cancer cells (COLO 320 DM) (79, 80), human breast cancer cells (18, 80), human squamous and gliosarcoma cells (17, 30) ovarian cancer cells (29), human epidermoidal cancer (A431), human liver cancer cells (Hep G2) and human pancreatic cancer cells (18, 40). Quercetin was synergistic with cisplatin in its antiproliferative effect *in vitro*, possibly due to inhibition of PKC (82); such an interaction was also evident *in vivo*. This flavonol was a hypothermic sensitizer of HeLa cells (83). Quercetin and other flavonoids also inhibited the induction of heat shock proteins in HeLa cell and colon cancer cell cultures at the level of mRNA accumulation (81). Kioka *et al.* (85) reported that quercetin impaired the expression of the multidrug resistance gene (*MDR1*) in the human hepatocarcinoma cell line Hep G2. The flavonol also inhibited the increase of P-glycoprotein synthesis and *MDR1* mRNA accumulation in these cells caused by exposure to arsenite (85). This appears to be the first report to describe the inhibition of *MDR1* expression by any chemical.

In one of our earlier reports, we observed the potent suppressive effect of the flavonol, fisetin, on the growth of HTB 43 squamous cell carcinoma cells (86). Quercetin also showed an antiproliferative effect against this cell line. Interestingly, the supplementation of the cell culture medium with low concentrations of ascorbic acid enhanced the growth suppressive activity of both fisetin and quercetin in these cancer cells. The protection of the polyhydroxylated flavonoids, fisetin and quercetin, from oxidative degradation by ascorbic acid may explain the observed protective action.

We documented an interesting observation on the anticancer activity of citrus flavones in different cells. The polymethoxylated flavones, tangeretin and nobiletin, exhibited such effects in human squamous cell and glio-sarcoma cells in culture at low concentrations (5-20 μM) (30, 87). Interestingly, these flavones had no notable effect on the growth of normal human diploid, fibroblast-like lung cells (CC1 135) in culture for a corresponding period and at similar concentrations. Since nobiletin and tangeretin displayed little effect on actively dividing cells, it is possible that these flavonones have preferential growth-inhibitory effects on tumor cells. The growth-suppressive activity of the polymethoxylated flavonoids may, in part, be ascribed to their chemical stability. Quercetin may undergo autoxidation and can also be oxidatively degraded, while methylation of the phenolic groups, as in the case of tangeretin and nobiletin, would be expected to confer greater stability to the flavonoids. Mori *et al.* (78) observed that the presence of a 3'- or 4'-methoxyl group enhanced the cytotoxicity of flavanes against HeLa cells.

One may surmise that plant flavonoids are potential inhibitors of tumor cell proliferation since they are known to inhibit several biochemical events associated with cellular growth. For instance, quercetin impeded aerobic glycolysis in tumor cells (28). The flavonol inhibited DNA, RNA and protein synthesis in Ehrlich ascites tumor cells (45) and NY68-infected chick embryo fibroblasts by quercetin (88) and increased cyclic AMP levels (45). Quercetin abolished the loss of density-dependent inhibition of growth in NY68-infected chick embryo fibroblasts (88).

In the case of human gastric (79) and colon cancer cells (80), growth inhibition by quercetin appears to involve interference with cell cycle events. In addition, tumor cell growth inhibition due to the interaction of quercetin with nuclear type II estrogen-binding sites is also a distinct possibility, as proposed by Markaverich *et al.* (89). The presence of these binding sites in several primary tumors (90-92) suggests that quercetin can also exert antitumor effects *in vivo*. Quercetin effectively competed for [^{3}H]17β-estradiol binding (10^{-8}-10^{-5} M) in acute lymphoid (91) and myeloid leukemias cells (93), which harbor type II EBS. There was a good correlation between the relative binding affinity of quercetin for type II EBS and cell growth inhibition. Quercetin (10 μM) was effective in inhibiting the *in vitro* incorporation of bromodeoxyuridine in transitional cell carcinoma cells of the bladder exhibiting type II EBS (91), similar to type II EBS from other organs.

The ability of quercetin to inhibit various tyrosine kinases might be a distinct mechanism underlying its action on tumor cell proliferation, as emphasized in the previous section. Glossmann *et al.* reported that quercetin inhibited the activity of a tyrosine-specific protein kinase, considered to be responsible for the transformation of non-malignant fibroblasts to sarcoma cells (94). The preliminary studies of Cunningham *et al.* (95) indicated that quercetin diminished the growth of Abelson-transformed NIH 3T3 cells, which express the Abelson tyrosine protein kinase. Ferry and coworkers undertook a phase I clinical trial to evaluate the antitumor effect of quercetin (96). The plasma concentrations of quercetin resulting from *i.v.* bolus administration inhibited lymphocyte protein tyrosine phosphorylation, and there was indication of anticancer activity (96). Singhal *et al.* (97) reported enhanced signal transduction in human breast cancer cells. Quercetin caused a striking decrement in this activity, thus suggesting a novel target for chemotherapy.

Our laboratory accrued evidence to show that the blockade of the EGFR-signaling pathway by the PTK inhibitors, luteolin and quercetin, results in significant growth inhibition of A431 and MiapaCa-2 cells *via* the induction of apoptosis (18, 34, 40). We documented that quercetin and luteolin

transinactivated EGFR tyrosine kinase activity, with marked reduction in the phosphotyrosyl level of 170, 125, 65, 60 and 42 kDa cellular proteins, and induced apoptosis. Our findings clearly indicated that A431 and MiaPaCa-2 cancer cells respond to quercetin and luteolin by a parallel reduction in cellular protein phosphorylation and cellular proliferation. To our knowledge, this is the first report of its kind to uncover the effects of a dietary flavonoid such as luteolin on EGF-mediated phosphorylation events, proliferation and apoptosis in tumor cells. Based on the evidence accrued, one could implicate the above proteins in growth signal transduction and utilize the subtle changes in their phosphorylation, as effected by flavonoids, as a reliable guide to predict growth response. The modulation of the EGF-mediated signaling pathway and associated EGFR kinases appears to be a critically important, intrinsic component of quercetin- and luteolin-induced growth suppression, even though other mechanisms could also have contributed to the net effect.

Avila *et al.* (98) reported that quercetin strongly inhibited, in a time- and dose-dependent fashion, the expression of the mutated p53 (tumor suppressor gene) protein, which is the only form present at high levels in the human breast cancer cell line MDA-MB468. Quercetin prevented the accumulation of newly synthesized p53 protein without affecting the steady-state mRNA levels of p53.

Recently, the topic of "why drinking green tea could prevent cancer" (99) has drawn tremendous attention, because it contains polyphenol derivatives with reported pro-apoptotic activity (100). Epidemiological studies suggest that the tea polyphenols or its key components, the catechins, which are flavanols, possess suppressive effects on human cancers (101). The major catechins in green tea are EC, ECG, EGC and EGCG (102), while the principal modified (oxidized) catechins in black tea are thearubigins, followed by theaflavins. The low toxicity, moderate cost and natural abundance make it an attractive substance for investigations. Oral administration of epigallocatechin, (EGCG), one of the principal food flavonoid constituents, and the most abundant catechin in green tea, inhibited the metastasis of B16 melanoma cell lines, such as B16-F10 and B16, in both experimental and spontaneous systems (103). Chen *et al.* (104) reported that EGCG showed a pronounced growth inhibitory effect on cancer cells, but not on their normal counterparts. There is evidence (105) linking the anticancer activities of tea polyphenols with the direct inhibition of the anti-apototic Bcl-2-family of proteins. We are yet to understand the various mechanisms of action of other tea polyphenols (106-111).

Genistein caused marked inhibition of the growth of estrogen receptor-negative human breast carcinoma cell lines MDA-468 (67) and MCF-7, and the estrogen receptor-positive cell line MCF-7-D40 (IC_{50} values of 6.5 to 12 μg/mL) (67), showing thereby that the action of the isoflavone was independent of the estrogen receptor presence. The effects of biochanin A and daidzein were less pronounced, while genistein and daidzein glycosides exhibited no noticeable activity. Interestingly, there was no effect on the growth-suppressing activity of genistein and biochanin A on MCF-7-D40 cells, which overexpresses gp 170, the gene product responsible for multidrug resistance.

There may be a link between the low incidence of breast cancer in Asian women with the high isoflavone-containing soy intake from their diet (112). Genistein treatment significantly reduced prostatic tumor development in 74% of the studies using animal models, as noted in a recent review on the impact of isoflavones on *in vitro* and *in vivo* models of cancer (109). Subcutaneous administration of the food phytoestrogen isoflavonoid, formononetin, resulted in the stimulation of mammary gland proliferation in BALB/c female mice with associated changes in vaginal cytology (113). Estrogen receptor expression showed a 2-fold increase in the treated mice, while the plasma prolactin increase was 1.7-fold. The reported effects may be related to the route of administration and it is doubtful if high enough plasma concentrations of the compound could be achieved by oral dosing. These results raise the question of whether the estrogenic activity of this isoflavonoid surpasses its growth suppresssive effects.

Polyphenol components of red wine such as catechin, epicatechin, quercetin and resveratrol, depressed the proliferation of human breast cancer cells at picomolar concentrations (108). These compounds also potently inhibited the growth of human prostate cancer cells (109) and altered surrogate markers of proliferation such as serum PSA (114).

Induction of apoptosis. Cell death in multicellular organisms occurs by two distinct mechanisms, apoptosis and necrosis (115-118). Apoptosis, also called programmed cell death, plays a cardinal role in embryonic development, metamophorphosis, hormone-dependent atrophy, as well as in the maintenance of tissue homeostasis. This process plays an important role in physiological processes such as differentiation (119) and immune system regulation (120). Apoptosis is the result of complex signal transduction pathways, bringing about gene-mediated cell death. Being a process regulated by specific gene activity, apoptosis is sensitive to mutations. Therefore, defects in apoptosis can be responsible for human diseases including cancer (121). Certain morphological features, such as loss of membrane asymmetry and detachment, condensation of the cytoplasma and nucleus, DNA fragmentation and poly(ADP-ribose) polymerase degradation, characterize the apoptotic program (118).

Apoptosis is one of the important pathways through which anticancer agents inhibit the growth of tumor cells. Resistance

of tumor cells to cytostatic agents is a major problem in the treatment of advanced cancers. Understanding the signaling pathways that control cytostatic agent-induced apoptosis in tumor cells is critical to ultimately improving anticancer therapy. At present, only a few potential anticancer agents such as the flavonoids seem to cause apoptosis. However, we have yet to fully understand the different mechanisms responsible for the antitumor effect of flavonoids. Therefore, it is necessary to glean insights into the specific mode of action contributing to flavonoid-induced apoptosis.

Genistein caused apoptosis in human myelogenous leukemia HL-60 cell cultures, within 8 h (122). This isoflavone completely blocked the ability of EGF, TGF-α, and basic fibroblast growth factor (bFGF) to suppress apoptosis in cultured rat ovarian granulosa cells (123). Bergamaschi *et al.* (124) documented that both genistein and the PTK inhibitor, tyrphostin, induced apoptosis in human leukemic cell lines M07e and HL-60. Additional observations with the tyrosine phosphatase inhibitor sodium orthovanadate, prompted the authors to conclude that the balance between tyrosine kinases and phosphatases determines the fate of the cell.

Wei *et al.* (37) reported that quercetin induced apoptosis, characterized by typical morphological changes, in certain tumor cell lines. Quercetin also inhibited the synthesis of heat shock protein (HSP) 70 in these cell lines. There was an association between this effect and the induction of quercetin-induced apoptosis. The citrus flavone, tangeretin (5,6,7,8,4'-pentamethoxyflavone), induced apoptosis in HL-60 cells, at concentrations greater than 2.7 μM; the flavone had little effect on the mitogen-stimulated blastogenic response of human peripheral blood mononuclear cells (125). The presence of Zn^{2+}, a known inhibitor of the apoptosis-requiring enzyme, endonuclease, abrogated apoptosis in these cells. These studies also indicated a requirement for protein synthesis for the induction of apoptosis.

In our previous studies, we found that quercetin and luteolin induced apoptosis in a wide range of tumor cells such as A431, MiaPaCa-2, Hep G2 and MCF 7 (18). To our knowledge, this is the first demonstration of its kind for the flavone, luteolin. We demonstrated that, in A431 cells and in MiaPaCa-2 cancer cells, luteolin at 15-25 μM led to growth retardation and induced various features of apoptosis such as oligonucleosomal DNA cleavage, cytoplasmic blebbing and PARP degradation. The addition of EGF to the luteolin-treated A431 cells abrogated the induction of apoptosis (18); we obtained similar results for MiaPaCa-2 tumor cells. Our unpublished observations, however, indicated that the addition of EGF did not prevent apoptosis in tumor cells that did not express EGFR, such as HT-29 and HTB 43. Our results indicated that the blockade of the EGFR-signaling pathway by the PTK inhibitors quercetin and luteolin significantly inhibited the growth of MiaPaCa-2 cells and induced apoptosis. Our findings, as those of previous reports, support the hypothesis that tyrosine phosphorylation is a "balancing act" between the activation and inhibition of apoptosis in tumor cells (18, 126). Even though the molecular basis for this observation remains to be explored, the evidence we had accrued supported the contention that changes in the tyrosine phosphorylation levels of certain proteins may well be intimately linked to this process.

Several reports, including ours, indicate that quercetin exerts its anticancer activity by inducing apoptosis as a consequence of cell cycle block, suggesting that the failure of malignant cells to bypass quercetin-dependent cell cycle arrest triggers apoptosis (11, 18, 40, 127-129). Shukla and Gupta obtained similar results with the flavone, apigenin (129). They observed that the modulation of cell cycle machinery, disruption of mitochondrial function and NF-kappa B inhibition were the molecular events contributing to apigenin-mediated growth inhibition and induction of apoptosis in DU145 prostate cancer cells.

Mukhtar and Ahmad observed that EGCG induced apoptosis and cell cycle arrest in human A431 cells (101). Importantly, they found that the apoptotic response of EGCG was specific to cancer cells (101). Evidence presented indicated that baicalin impaired the proliferation of LNCap, PC3 prostate cancer cells *via* apoptosis and cell cycle arrest (130). It is worth noting that EGCG was able to significantly impair DNA synthesis in A431 cells (131). Although not well reviewed, data exist on the anti-apoptotic effects of quercetin (132).

Inhibition of metastasis/migration/angiogensis. The spread of cancer through metastasis represents one of the gravest dangers of the disease (133-135). In human cancers of the breast, liver, colon, lung and ovary, the production of certain matrix metalloproteinases (MMPs) correlates with cancer invasion/metastasis (135-140). At least 20 genes encoded different MMPs (141), which one can categorize into four subclasses based on structural organization and substrate specificity: collagenases, gelatinases, stromelysins and membrane-type MMPs (135, 142). MMPs belong to a rapid growing family of zinc-dependent endopeptidases that are capable of degrading a variety of ECMs (143, 144). The signal or pre-peptide domain is usually rich in hydrophobic amino acids and targets the enzyme to the endoplasmic reticulum for possible excretion from the cell. The propeptide contains a highly conserved PRCGVPD sequence that contains cysteine residues, which interact with the zinc atom in the catalytic site to maintain the enzyme in an inactive state. Activation of the MMP occurs with proteolytic removal of the propetide domain (145, 147). Collectively, MMPs degrade most components of the extracellular matrix. Tumor cells probably need more than

one MMP, as well as more general degradative enzymes to cross the tissue barriers they encounter. There are multiple levels in the regulation of the activities of MMPs , including the expression and secretion of MMPs, and the activation processes of MMPs (135, 142-147). Endogenous inhibitors such as α2-macroglobulin, and the tissue inhibitors of metalloproteinases (TIMP) (147) cause inhibition of MMPs *in vivo*, once activated. Four structure-related tissue inhibitors, TIMP-1 to TIMP-4, regulate MMP activity (147-149). The secretion of MMPs is necessary for tumor invasion, as indicated by the observations that treatment with antibodies or inhibitors against MMPs abolished the invasive behavior of certain tumor cells (143-145). Therefore, one would expect to limit the metastatic potential of cancer cells by the suppression of the secretion and of the action of the activated MMPs in cancers.

The last decade has witnessed a major effort to generate clinically useful proteinase inhibitors for cancer treatment. Investigators are currently pursuing TIMPs, endogenous inhibitors of MMPs, which provide *in vivo* control of MMP activity, as gene therapy molecules in the treatment of cancers. Also, there is remarkable activity in the development of synthetic inhibitors of MMPs, including pseudopeptides that mimic MMP substrates and non-peptide molecules that bind the catalytic zinc (149). Examples of synthetic MMP peptide inhibitors are BB94 and marimastat (146, 149, 150). Most peptide inhibitors of MMP are non-selective because many MMPs recognize the collagen-mimicking amino acid sequences Lle-Ala-Gly, Leu-Leu-Phe, or Leu-Phe (150).

Despite the potential for MMP inhibition as a treatment for cancer metastasis, the results of investigations on MMP inhibitors obtained with animal models seem to be disappointing. Certain studies report that flavonoids influence the level of MMPs in different ways. In several cell types, flavonoids fit the description as agents down-regulating the biosynthesis of MMPs (151-153). Quercetin, for instance, diminishes the invasion of murine melanoma cells by decreasing pro-MMP-9 *via* the PKC pathway (154). Recently, we illustrated that both luteolin and quercetin were able to decrease the secretion of MMP-2 and MMP-9 in several cancer cell lines such as A431 (18), MiaPaCa-2 (40) and A549 (Lee *et al.*, unpublished data). In addition, quercetin showed a dose-dependent decrement in the gelatinolytic activity of pro-MMP-9 (154). Genistein inhibited the invasion of highly metastatic MDA-AM 231 breast cancer cell *in vitro*, an observation showing an association with the down-regulation of MMP-9 activity (155-157). Ende and Gebhardt observed that certain flavonoids, such as luteolin, quercetin and apigenin, could inhibit MMP-2 and -9 activities (158). Interestingly, flavonoids with an increasing number of hydroxyl groups or substitutions (glucoside) exerted a stronger inhibitory effect on MMP-2 and -9 (158). MMPs were subject to inhibition by fruit extracts from raspberries, blackberries and grapes, since they all contain high levels of flavonoids and polyphenols (159). EGCG, a prominent flavanol in green tea, was also a potent inhibitor of MMP-2 and -9 (160-162). It is worth emphasizing that detailed studies are lacking on the possibility of direct inhibition of MMPs by flavonoids. However, there is evidence indicating that the inhibition is of a non-competitive type (158).

Accumulated evidence support the hypothesis that tyrosine phosphorylation is a balancing act between the activation and inhibition of MMP activities, although the mechanism underlying the regulation of the secretion of MMPs lacks full understanding at this juncture (10, 28). One of our previous reports documented that EGF stimulated the secretion of MMP-2 and -9 from A431 cells, while luteolin and quercetin suppressed this action (10, 28). Consequently, agents that inhibit EGFR tyrosine kinase activity may have potential in the prevention of tumor metastasis. The above data (18, 40) suggested that targeting specific genes that regulate the expression of MMP presented a rational approach to the treatment of cancer metastasis. Scholar and Toews reported that the tyrosine kinase inhibitor genistein could inhibit tumor invasion, possibly *via* repression of tyrosine phosphorylation (163, 164); the flavone might be suppressing tyrosine kinase-mediated secretion of MMPs involved in the tumor invasion (18).

Bracke *et al.* (165) reported the binding of the flavanol, (+)-catechin, laminin, one of the ECM molecules involved in the invasion and metastasis of malignant tumor cells. Contact of cells with this component appreciably impacted the adhesion of a variety of invasive and non-invasive cell types to cell surfaces. Prior treatment of the laminin-coated surfaces with a high dose of (+)-catechin (0.5 mM) impaired the effect of laminin (166) on the morphology and adhesion of two different cell types, MO4 (Kristen murine sarcoma virus-transformed fetal mouse cells) and M5076 (a mouse reticulum cell sarcoma). These authors also observed that tangeretin inhibited the invasion of MO4 cells into embryonic chick heart fragments *in vitro* (166). The omission of the flavone from the culture medium reversed the observed anti-invasive effect, and the compound appeared to be chemically stable in the cell culture medium. Other reports indicated that low concentrations of genistein had the potential to inhibit a very invasive BALB/c mammary carcinoma from invading a basement membrane-like material (Matrigel) with no effect on growth (163). In another case, 3,7-dimethoxyflavone reversibly inhibited the invasion of MCF-7/6 human mammary carcinoma cells into embryonic chick heart fragments in organ culture in a nontoxic fashion (167).

Neovascularization, which involves angiogenesis, is obligatory for the progression of metastasis. During efforts to find and characterize possible dietary inhibitors of

angiogenesis, Fotsis *et al.* (168) identified different isoflavones, including genistein, by the fractionation of urine from healthy human volunteers consuming a soy-rich vegetarian diet. Out of the different isoflavones examined for their inhibitory action on endothelial cell proliferation and *in vitro* angiogenesis, genistein turned out to be the most potent with IC_{50} values of 5 and 150 μM, respectively. The detection of a high excretion of genistein in the urine of vegetarian subjects led to the suggestion that genistein is contributory to the preventive action of a plant-based diet on chronic diseases, including solid tumors and inflammatory conditions (168, 169) by inhibiting neovascularization. Genistein may thus represent a new class of diet-derived anti-angiogenic compounds. Fotis' group also observed that flavones and flavonols, such as 3-hydroxyflavone, 3',4'-dihydroxyflavone, 2',3'-dihydroxyflavone, fisetin, apigenin and luteolin, also inhibited *in vitro* angiogenesis, in addition to tumor cell growth (170).

The secretion of neovascularization/angiogenesis agents by mast cells (171) and the stimulation of mast cell migration by tumor-derived peptides (172) indicate that mast cells may be involved in tumor growth and metastasis (173, 174). The potent inhibition of mast cell activation and proliferation by several flavonoids (174) may also contribute to their antitumor effects. Mast cells release TNF, which induces endothelial adhesion molecule expression (174, 175). Initial studies from our laboratories indicated that quercetin impaired the TNF-stimulated induction of endothelial cell adhesion molecules (176). Reports on the effects of other flavonoids, such as apigenin (177) and genistein (166-167), appeared to be consistent with our findings. Genistein markedly diminished both the basal levels and bFGF-induced levels of plasminogen activator (PA) and its physiological inhibitor, PAI-1 (178). The angiogenic factor, bFGF, stimulates the production of urokinase-type, PA and PAI-1 in vascular endothelial cells. Plasmin, formed from plasminogen (*via* PA), causes stepwise proteolytic degradation of matrix proteins, an obligatory step in neovascularization (178).

Conclusion

Dietary flavonoids critically influence several cellular and immune processes associated with the development and progression of cancer. It is clear that these food components possess the propensity to modulate a variety of biological events associated with cancer progression and development, such as cell proliferation, apoptosis, cell differentiation and neovascularization. *In vitro* studies have established an association between flavonoid-induced modulation of protein kinase and MMP activities with apoptosis, cellular proliferation and tumor cell invasive behavior. Certain dietary flavonoids display *in vivo* antitumor activity and depress *in vivo* angiogenesis.

It is important to understand how the flavonoids enter the cells and accumulate in certain cellular organelles and tissues. Further studies should help elucidate the various mechanisms by which flavonoids can markedly and decisively impact the activities of important mammalian enzymes such as protein kinase C, tyrosine and focal adhesion kinases, and MMPs, relevant to cancer cell proliferation and metastasis. It is necessary to perform both *in vitro* and *in vivo* assessments employing concentrations of flavonoids approximating to those in the diets and also at subpharmacological concentrations. Knowledge garnered from these studies may help in designing a potent, non-toxic, chemotherapeutic strategy against cancer. Most of the plant flavonoids occur as conjugates in the diet, with the possible exception of flavans, flavanols and proanthocyanidins. However, typical flavonoids generally employed in cancer-related studies are the unconjugated (parent) moieties. It is imperative to investigate the conjugated derivatives in order to assess the anticancer activity of dietary flavonoids. The introduction of certain functional groups such as glucose or sulfate to the unsubstituted flavonoids would increase their solubility during *in vitro* evaluation. Appreciation of the role of these substituents may greatly aid in the selection of potent flavonoid moieties in chemotherapeutic evaluation, and in anticancer drug design and development.

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