7

Phytoestrogens and inhibition of angiogenesis

THEODORE FOTSIS MD, PhD

Associate Professor
Laboratory of Biological Chemistry, Medical School, University of Ioannina, Greece

MICHAEL S. PEPPER MD, PhD

Assistant Professor

ROBERTO MONTESANO MD

Professor

Department of Morphology, University Medical Center, Geneva, Switzerland

ERKAN AKTAS MD

Postgraduate Student

STEPHEN BREIT PhD

Postdoctoral Researcher

LOTHAR SCHWEIGERER MD, PhD

Assistant Professor

Department of Haematology, Oncology and Endocrinology, Children's Hospital, University of Essen, Germany

SIRPA RASKU MSc

Research Associate

KRISTIINA WÄHÄLÄ PhD

Department of Chemistry, Organic Chemistry Laboratory, University of Helsinki, Finland

HERMAN ADLERCREUTZ MD, PhD

Director
Institute for Preventive Medicine, Nutrition and Cancer, Folkhälsan Research Centre, Department of Clinical Chemistry, P.O. Box 60, FI-00014, University of Helsinki, Helsinki, Finland

The consumption of a plant-based diet can prevent the development and progression of chronic diseases associated with extensive neovascularization, including the progression and growth of solid malignant tumours. We have previously shown that the plant-derived isoflavonoid genistein is a potent inhibitor of cell proliferation and in vitro angiogenesis. Moreover, the concentration of genistein in the urine of subjects consuming a plant-based diet is 30-fold higher than that in subjects consuming a traditional Western diet. We have also reported that certain structurally related flavonoids are more potent inhibitors than genistein. Indeed, 3-hydroxyflavone, 3′,4′-dihydroxyflavone, 2′,3′-dihydroxyflavone, fisetin, apigenin and luteolin inhibit the proliferation of normal and tumour cells as well as in vitro angiogenesis at half-maximal concentrations in the lower micromolar range. The wide distribution of isoflavonoids and flavonoids in the plant kingdom, together with their anti-angiogenic and anti-mitotic properties, suggest that these phytoestrogens may contribute to the preventive effect of a plant-based diet on chronic diseases, including solid tumours.

Key words: isoflavonoids; genistein; flavonoids; diet; inhibition; angiogenesis; pro-liferation.

ANGIOGENESIS

Angiogenesis, the generation of new capillaries from pre-existing vessels, is virtually absent in the healthy adult organism, in which it is restricted to a few conditions including wound healing and the formation of corpus luteum, endometrium and placenta. These conditions of physiological angiogenesis represent ordered, tightly regulated and self-limited processes (Klagsbrun and Folkman, 1990). However, in certain pathological conditions, angiogenesis is dramatically enhanced and loses its self-limiting capacity (Denekamp, 1990). Although pathological angiogenesis is seen during the development and progression of many diseases, such as rheumatoid arthritis, psoriasis and diabetic retinopathy, from a clinical perspective the most important manifestation of pathological angiogenesis is probably that induced by solid tumours (Folkman, 1985). Well-vascularized tumours expand both locally and by metastasis, while avascular tumours do not grow beyond a diameter of 1–2 mm (Folkman and Cotran, 1976; Klagsbrun and Folkman, 1990).

Although the mechanisms responsible for persistent angiogenesis are still unclear and certainly not the same in each disease, recent evidence indicates that they all lead to an imbalance between angiogenic factors and inhibitors (Liotta et al, 1991; Hanahan and Folkman, 1996). Recognition of the importance of angiogenesis has led to the search of angiogenesis inhibitors, which, by tilting the balance towards inhibition, can stop the process of persistent, unregulated pathological neovascular-ization (Crum et al, 1985; Ingber et al, 1990). Such compounds may find important applications in the treatment of angiogenic diseases, including cancer.

DIETARY INGESTED COMPOUNDS INHIBIT ENDOTHELIAL CELL PROLIFERATION

Dietary factors contribute to about one-third of potentially preventable cancers (Adlercreutz, 1990), and the long-known preventive effect of plant-based diets on tumourigenesis and other chronic diseases is well documented (Miller, 1990). Breast cancer, prostate cancer and endometrial cancer belong to the group of hormone-dependent cancers that, in addition to colon cancer and coronary heart disease, are among those chronic diseases which have a lower incidence in Asia than in Western countries (Rose et al, 1986). Migrants from Asia who maintain their traditional diet do not increase their risk of these diseases (Kolonel, 1988); however, an increased risk of these diseases accompanies a change towards a Westernized diet (Lee et al, 1991). These data indicate that certain plant-derived dietary groups might contain compounds exerting anti-mitotic and anti-tumourigenic effects, thereby offering anti-cancer protection to individuals consuming such diets. The identification and characterization of such compounds might provide us with additional chemotherapeutic agents for pharmacological intervention in cancer.

That dietary ingested compounds could be modulating the proliferation of tumour cells and pathological angiogenesis appeared to us an important possibility meriting further investigation. If dietary compounds were to inhibit angiogenesis, this could explain, at least in part, the long-known preventive effect of plant-based diets on tumourigenesis and other chronic diseases, such as inflammation (Adlercreutz, 1990). We have, therefore, examined this possibility by screening the urine of human subjects consuming a diet rich in plant products for the presence of anti-mitotic and anti-angiogenic compounds (Fotsis et al, 1993, 1995). Towards this purpose, we have used a purification procedure (Figure 1A) previously developed by us to separate lipophilic compounds of steroid- or steroid-like structure (Fotsis, 1987; Fotsis and Adlercreutz, 1987; Adlercreutz et al, 1991a). Figure 1B outlines the various fractions (1–6) obtained from the purification of urine and their effect on basic fibroblast growth factor (bFGF)-stimulated proliferation of bovine brain-derived capillary endothelial cells (BBCE). Fractions 1, 2 and 4 were able to inhibit the proliferation of endothelial and neuroblastoma cells. Fraction 4 was not further examined as it contains neutral steroids (Fotsis and Adlercreutz, 1987), which are known angiogenesis inhibitors (Crum et al, 1985).

THE ISOFLAVONOID GENISTEIN INHIBITS THE IN VITRO PROLIFERATION OF ENDOTHELIAL CELLS

Fraction 1, which exhibited strong inhibition of the in vitro proliferation of endothelial cells, contained diphenolic compounds (Figure 1). Using gas chromatography-mass spectroscopy (GC-MS), initial identification was carried out by comparing the mass spectra of the different peaks with those of reference mass spectra collections. Synthetic standards of potential
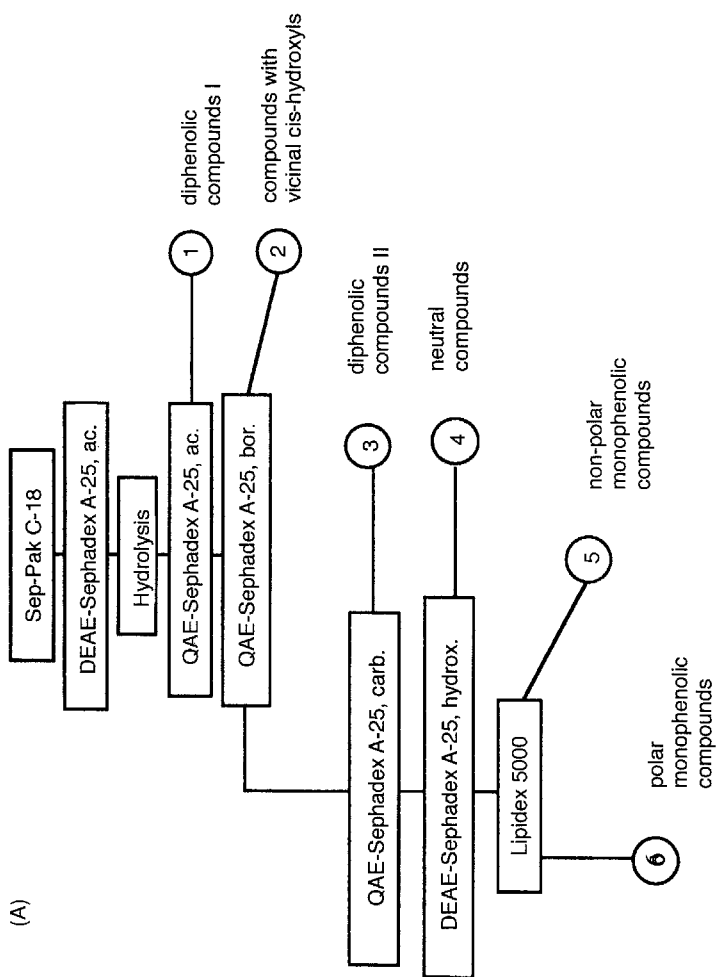
(A)

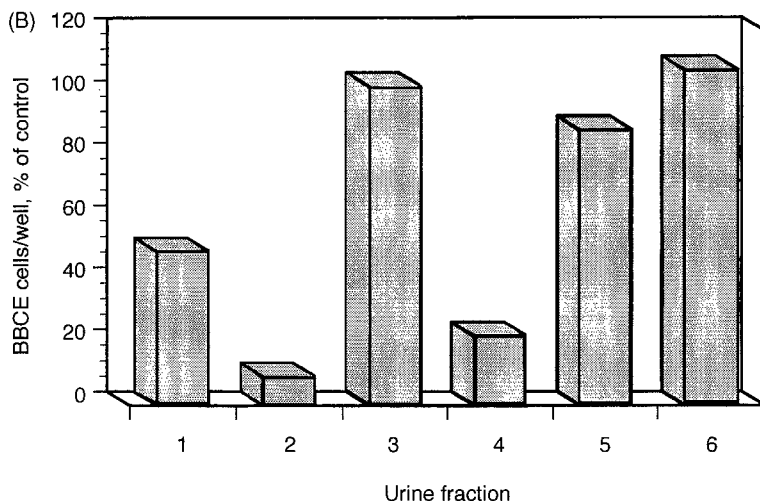**Figure 1(A).** For legend see opposite.

Figure 1. Fractionation of human urine and the effect of various semipure urine preparations on the proliferation of vascular endothelial cells. (A) Urine was fractionated as described (Fotsis, 1987; Fotsis and Adlercreutz, 1987; Adlercreutz et al, 1991a). The flow diagram shows the chromatographic steps, the fractions obtained (numbered 1 to 6) and the compound classes contained in each fraction. (B) Bovine brain-derived capillary endothelial cells were seeded at a density of 5000 cells/ml into each well and, every other day, received 10 µl aliquots containing bFGF (2.5 ng) plus 10 µl aliquots (containing an equivalent of 3.2 ml native urine) of the purified urine fractions (fractions 1–6 of Figure 1A) diluted in buffer or buffer only. The cells were counted after 5 days. Values are expressed as a percentage of the controls value, i.e. cells receiving bFGF and buffer only (approximately 100 000 cells per well). Figure 1A reproduced from Fotsis et al (1993), with the permission of the National Academy of Sciences, USA. Figure 1B reproduced from Fotsis et al (1995), with the permission of the American Society for Nutritional Sciences.

candidate compounds were then prepared, and definite identification was established by comparing their mass spectra with those of the unknown compounds. In this way, we were able to demonstrate the presence of the isoflavonoids genistein, daidzein and O-desmethylangolensin in fraction 1 and the lignans enterodiol, enterolactone and matairesinol as well as the isoflavonoid equol in fraction 3 (Adlercreutz et al, 1991b). Most isoflavonoids have weak oestrogenic activity. Indeed, high levels of circulating equol have been shown to be responsible for the infertility observed in Australian sheep grazing on a certain type of subterranean clover rich in isoflavonoids (Shutt, 1976). In this syndrome, known as clover disease of sheep, ewes exhibited cystic degeneration of the ovaries and insensitivity of the hypothalami to oestradiol (Shutt, 1976).

The synthetic analogues of the identified compounds were then tested with regard to their effect on the bFGF-stimulated proliferation of BBCE cells. Genistein had a potent and dose-dependent inhibitory effect on BBCE cell proliferation, with half-maximal and maximal effects at 5 and 50 µM concentrations respectively (Fotsis et al, 1993). In contrast, the remaining isoflavonoids, some of which are closely related to genistein, were considerably (5–20 times) less potent. Genistein also inhibited the proliferation of vascular endothelial cells derived from bovine adrenal cortex (ACE) or aorta (BAE).

GENISTEIN TARGETS PROLIFERATING BUT NOT QUIESCENT CELLS

Endothelial cells were not the only target of genistein. The proliferation of low-density cultures of various normal (NIH-3T3 mouse embryonic fibroblasts) and tumour (SH-EP and Kelly human neuroblastoma, SK-ES1 Ewing's sarcoma, RD and A-204 human rhabdomyosarcoma, and Y-79 human retinoblastoma) cells was also inhibited by genistein, with half-maximal concentrations varying between 10 and 45 μM (Schweigerer et al, 1992). Although endothelial cells are slightly more sensitive ($IC_{50} = 6$ μM) to the inhibitory effect of genistein, it appears that genistein has a broad inhibitory effect on proliferating cells.

In low-density cultures of proliferating endothelial cells, genistein induced marked morphological changes: at concentrations up to 25 μM, genistein induced a highly spread morphology compatible with growth arrest; cell densities always exceeded those determined at seeding (Fotsis et al, 1993), indicating that cell death was not involved. When exposed to genistein concentrations above 25 μM, the cells acquired an elongated morphology and eventually died. These results indicate that genistein is cytostatic up to concentrations of approximately 25 μM and that it becomes cytotoxic above this level. Reversibility experiments, in which low-density cultures of endothelial cells were exposed to bFGF (2.5 ng/ml) and increasing concentrations of genistein, and then to medium without genistein, confirmed this assumption (Figures 2 and 3A). The result was the same on uncoated and gelatin-coated substrata. In contrast, confluent, quiescent endothelial cells did not exhibit toxicity signs even at genistein concentrations up to 100 μM (Figure 3B). These data clearly suggest that genistein targets only proliferating cells, leaving quiescent, non-dividing cells unaffected. This property is important with respect to the possible use of this compound therapeutically as fewer side-effects are likely to be expected.

GENISTEIN INHIBITS ANGIOGENESIS IN VITRO

Having established the inhibitory effects of genistein on the proliferation of endothelial cells, it was interesting to investigate whether genistein had additional effects on other functions of endothelial cells important for angiogenesis. Angiogenesis is a complex process requiring the co-ordinated, sequential involvement of a number of cellular events in addition to proliferation. Indeed, the formation of new capillaries begins with a localized breakdown of the basement membrane of the parent vessel through the finely tuned elaboration of proteolytic enzymes and their inhibitors (Pepper and Montesano, 1990), followed by the migration of endothelial cells and invasion of the surrounding matrix. The initial sprout elongates further as a result of continued migration and endothelial cell replication. Once a lumen has been formed, the capillary fuses with the tip of another maturing sprout, forming a functional capillary loop (Folkman and Klagsbrun, 1987). Proteolytic degradation of the extracellular matrix by endothelial cells is
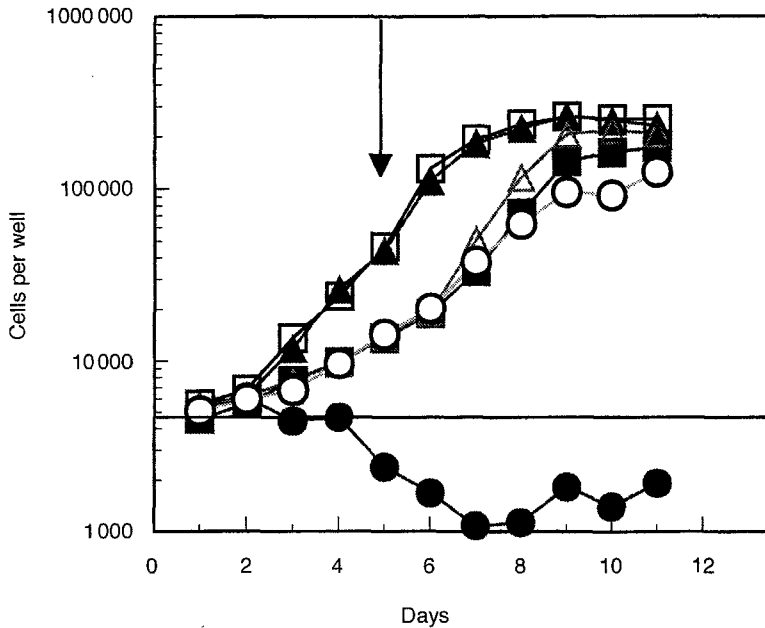
Figure 2. Time course and reversibility of the effect of genistein on the proliferation of vascular endothelial cells. Bovine brain-derived capillary endothelial cells were seeded at a density of 5000 cells per well and received either no additions (△, ○) or every other day bFGF (2.5 ng/ml) without (□) or with 1 (▲), 10 (■) or 100 (●) μM concentrations of genistein. On day 5 (indicated by the arrow), the cells were washed and received medium free of genistein containing buffer (○) or bFGF (2.5 ng/ml) (△, □, ▲, ■, ●) only. Duplicate wells were counted at the indicated times. The horizontal line indicates the cell seeding density. Reproduced from Fotsis et al (1995) with the permission of the American Society for Nutritional Sciences.

controlled by angiogenic factors, for example bFGF, which induce the production of urokinase-type plasminogen activator (Montesano et al, 1986) and its physiological inhibitor, plasminogen activator inhibitor-1 (Pepper and Montesano, 1990). Genistein markedly reduced both bFGF-stimulated and basal levels of both plasminogen activator and plasminogen activator inhibitor-1 activity in bovine microvascular endothelial cells (BME) (Fotsis et al, 1993). Moreover, genistein inhibited the bFGF-induced migration of endothelial cells in wounded confluent monolayers of endothelial cells (data not shown). Inhibition of the production of proteolytic enzymes and the migration of endothelial cells by genistein thus represents a more complex interference by the compound, with important early events of angiogenesis in addition to endothelial cell proliferation.

The combined effects of genistein on the proliferation, proteolytic enzyme production and migration of endothelial cells prompted us to investigate the effects of the compound on an experimental in vitro system that mimics angiogenesis in vivo. As previously shown (Montesano et al, 1986), BME cells seeded on the surface of collagen gels (Figure 4A) invade the gels when exposed to bFGF and form capillary-like tubes beneath the
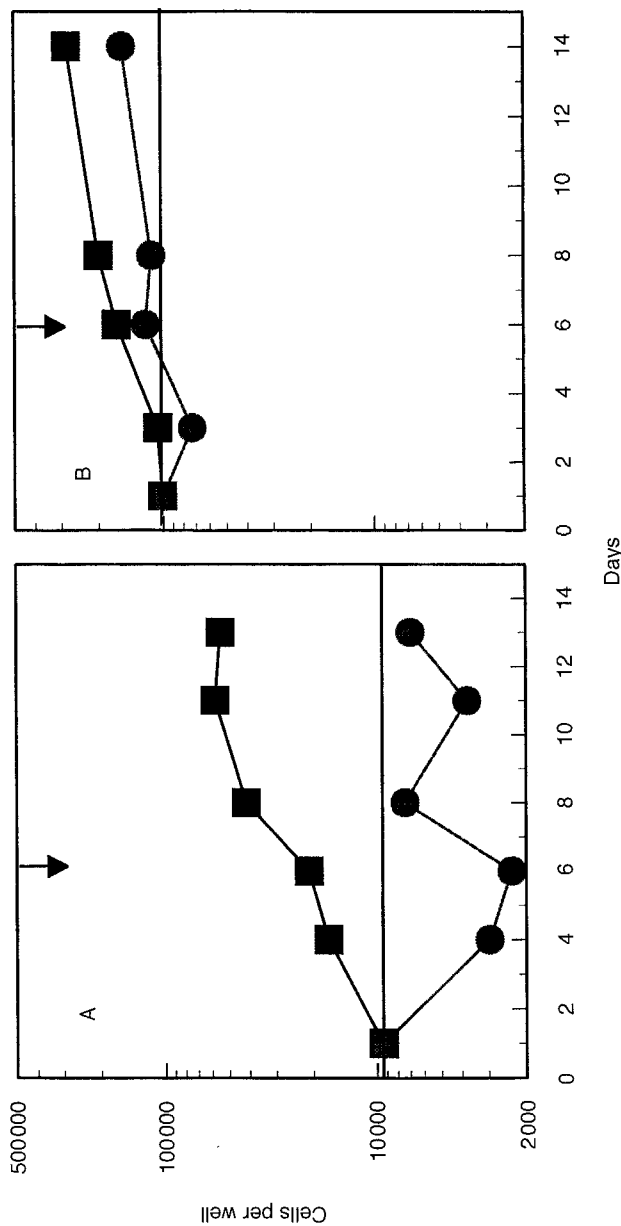
Figure 3. The effect of cell density on the cytotoxicity of genistein. Bovine microvascular endothelial cells (BME) were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum and seeded in 1 ml aliquots containing either 1×10^4 (A) or 1×10^5 (B) cells into 12-well dishes. BME cells seeded at a density of 1×10^4 per ml received bFGF (2.5 ng/ml) every other day plus either 10 (■) or 100 μM (●) concentrations of genistein (A). When seeded at a density of 1×10^5 per ml, BME cells were first allowed to grow to confluence and then received either 10(■) or 100 μM (●) concentrations of genistein (B). In both sets, the medium was changed at day 6 (indicated by the arrow), and thereafter the cells received only 2.5 ng/ml bFGF every second day. In all the experiments, substances were added in 10 μl aliquots. The horizontal line indicates the cell seeding density, and the values represent the means of duplicate determinations carried out at the indicated times. Reproduced from Fotsis et al (1995), with the permission of the American Society for Nutritional Sciences.

gel surface (Figure 4B). Genistein alone at concentration of 200 μM had no effect on confluent BME cultures (Figure 4C). However, when added together with bFGF, it inhibited their ability to invade the gels and generate capillary-like structures (Figure 4D). A quantitative analysis revealed that genistein inhibited the bFGF-induced invasion of BME cells, with a half-maximal effect at a concentration of approximately 10 μM (Fotsis et al, 1993, 1997).
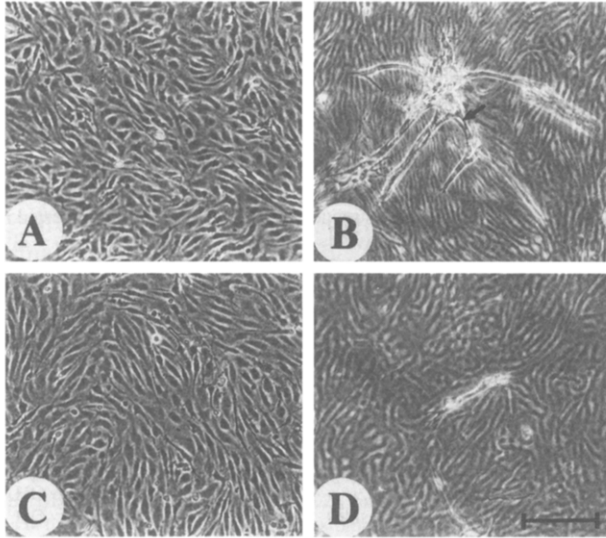

Figure 4. The effect of genistein on in vitro angiogenesis. Three-dimensional collagen gels were prepared in 18 mm tissue culture wells as described (Montesano and Orci, 1985). 5×10^4 bovine microvascular endothelial (BME) cells (in a volume of 0.5 ml) were seeded into each well, and the medium was changed every 2–3 days until the cells were confluent. After confluence, they received either no additions (A), bFGF (30 ng/ml) only (B), genistein (200 μM) only (C) or genistein (200 μM) 2 hours prior to bFGF (30 ng/ml) (D). After 2 days of incubation, the medium was changed and the cells exposed to the same conditions again. Representative pictures were taken after another 3 days. In (B), the arrow shows the lumen of a capillary-like tube, and in (D), genistein inhibits the formation of such tubes. Bar = 150 μM. Quantification was carried out by measuring the total additive length of all the cells that penetrated the underlying gel either as single cells or in the form of cell cords. The addition of genistein 2 hours prior to the addition of bFGF significantly ($*P < 0.025$; $**P < 0.0001$) inhibited bFGF-induced invasion. Reproduced from Fotsis et al (1993) with the permission of the National Academy of Sciences, USA.
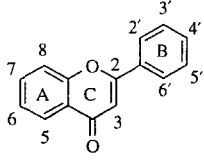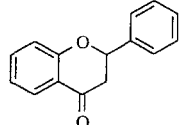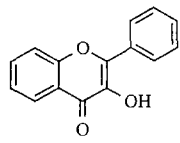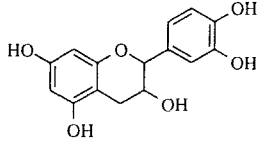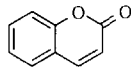
SEVERAL OTHER MEMBERS OF THE FLAVONOID FAMILY INHIBIT ENDOTHELIAL AND TUMOUR CELL PROLIFERATION

The promising results with the isoflavonoid genistein prompted us to extend these studies by investigating the anti-mitotic and anti-angiogenic effects of other members of the large flavonoid family. Many flavonoids

bind to the oestrogen receptor and possess oestrogenic activity (Miksicek, 1994, 1995). Flavonoid aglycones all consist of a benzene ring (A) fused with a six-member ring (C), which in the 2-position carries a phenyl ring (B) as a substituent (Table 1), whereas isoflavonoids carry the B ring in position 3. Flavonoids are widely distributed in the plant kingdom (Harborne, 1973; Harborne and Marby, 1982; Middleton, 1988), rendering them very attractive targets for further studies. Most of them possess vicinal hydroxyls in one of the phenyl rings, being therefore eluted in fraction 2 of the purification method (see Figure 1A above). Indeed, fraction 2 exhibited strong inhibitory activity on the proliferation of endothelial cells in vitro (see Figure 1B above), providing further support for the investigation of the anti-mitotic and anti-angiogenic properties of flavonoids.

Towards this purpose, a series of flavonoid metabolites were tested with regard to their effect on the bFGF-stimulated proliferation of BBCE cells.

Table 1. Substances tested. The chemical structures of the various substances tested in vitro for their anti-proliferative and anti-carcinogenic activities

Chemical formula	Name	Substitution					
		5	7	2′	3′	4′	5′
Flavones	Flavone	H	H	H	H	H	H
	5-Methoxyflavone	OCH$_3$	H	H	H	H	H
	Crysin	OH	OH	H	H	H	H
	2′, 3′-Dihydroxyflavone	H	H	OH	OH	H	H
	3′, 4′-Dihydroxyflavone	H	H	H	OH	OH	H
	Apigenin	OH	OH	H	H	OH	H
	Luteolin	OH	OH	H	OH	OH	H
	Luteolin-7-glucoside	OH	OGLC	H	OH	OH	H
Flavanones	Hesperetin	OH	OH	H	OH	OCH$_3$	H
	Eriodictyol	OH	OH	H	OH	OH	H
Flavonols	3-Hydroxyflavone	H	OH	H	H	H	H
	Fisetin	H	OH	H	OH	OH	H
	Quercetin	OH	OH	H	OH	OH	H
	Myrisetin	OH	OH	H	OH	OH	OH

Catechin   Coumarin

The list of the flavonoids was selected to cover a large range of structures in order to facilitate potential structure–activity relationships (Table 1). To this list, coumarin was added as a control because of its closely related chemical structure. This initial experiment showed that several flavonoid metabolites could inhibit the in vitro proliferation of BBCE at half-maximal concentrations in the lower micromolar range (Table 2). To the best of our knowledge, there are no previous reports on the effects of flavonoids on endothelial cells, the target cells in angiogenesis. At least three flavonoids, 3′, 4′-dihydroxyflavone, luteolin and 3-hydroxyflavone, inhibited the bFGF-induced proliferation of BBCE cells (Table 2 and Figure 5) at a lower half-maximal inhibitory concentration (1.4, 1.9, and 2.7 μmol/l respectively) than that of genistein (5–6 μmol/l) (Fotsis et al, 1993). There was no specificity towards any endothelial subtype, BAE, ACE and human umbilical vein endothelial (HUVE) all being inhibited by the most potent flavonoids in a manner similar to that of BBCE cells (data not shown).

Next we investigated the anti-proliferative effects of flavonoids on various normal and tumour cells. The rationale was twofold: first, to investigate the possible direct anti-tumour effects of flavonoids, and second, to observe whether there is any selectivity in their anti-mitotic activity. Towards this end, several normal and tumour cells were employed, all of human origin. The normal cells included fibroblasts (HFK2) and

Table 2. Anti-proliferative effects of flavonoids. The half-maximal concentration (μmol/1) of the inhibitory effect of the various substances tested on the in vitro proliferation of bovine capillary endothelial cells (BBCE), human fibroblasts (HFK2), human keratinocytes (HaCaT), breast cancer adenocarcinoma cells (MCF7) and human neuroblastoma cells (SHEP and WAC2).

|	BBCE	HFK2	HaCaT	MCF7	SHEP	WAC2
Flavones						
Flavone	8.5					
5-Methoxyflavone	12.0					
3′,4′-Dihydroxyflavone	1.4	9.5	1.5	0.7	5.0	0.8
Chrysin	9.0	25.0	20.0	7.0	5.0	
Apigenin	6.5	10.0	6.0	3.5	4.0	
Luteolin	1.9	7.6	4.0	6.5	4.0	1.1
Luteolin-7-glucoside	18.0		6.0	6.0	4.0	
Flavonoles						
3-Hydroxyflavone	2.7	2.8		2.1		2.4
Fisetin	8.6	20.1	20.0	10.6	7.0	6.6
Quercetin	5.35	36.2	>50.0	5.55	8.0	6.6
Myricetin	28.0	40.0	40.0	n.e.		
Flavanones						
Eriodictyol	7.0	>50.0	15.0	18.0	10.0	
Hesperetin	30.0	n.e.	n.e.	40.0	>50.0	
Flavan-3-ol						
Catechin	>50.0	>50.0	n.e.	n.e.	n.e.	
Coumarins						
Coumarin	n.e.	n.e.	n.e.	n.e.	n.e.	

n.e. = no effect.
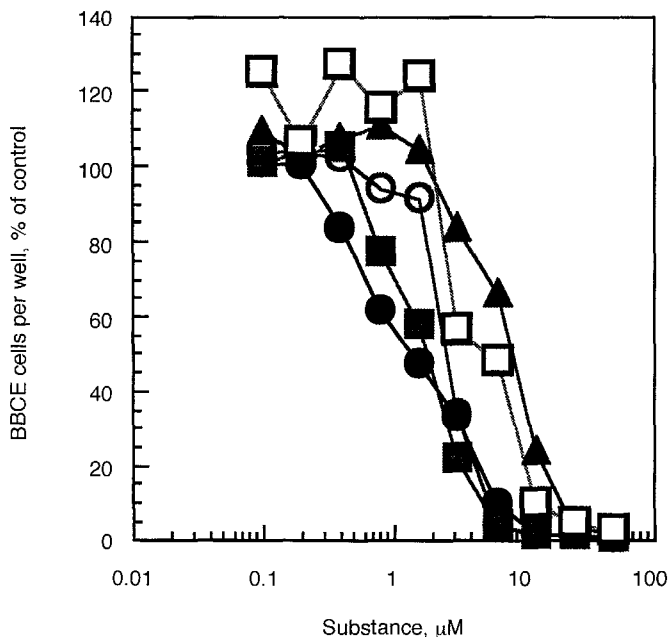
Figure 5. The effect of increasing concentrations of flavonoids on the proliferation of bovine capillary endothelial (BBCE) cells. BBCE cells were seeded at a density of 5000 cells per well and received bFGF (2.5 ng/ml) every other day. In addition, they received, every other day, 5 μl aliquots of solvent only or of solvent containing the indicated concentrations of 3′,4′-dihydroxyflavone (●), luteolin (■), 3-hydroxyflavone (○), fisetin (▲) or quercetin (□). The cells were counted after 5 days. The values are expressed as a percentage of that for controls, i.e. cells receiving solvent only. Reproduced from Fotsis et al (1997), with the permission of the American Association for Cancer Research.

keratinocytes (HaCaT). The tumour cells were also of different tissue origins: MCF7 cells from breast adenocarcinoma, and SHEP and WAC2 neuroblastoma cells of neuroectodermal origin. WAC2 cells are stable transfectants of SHEP cells that express the MYCN oncogene and thus have an increased proliferative potential on the same genetic background as the SHEP cells (Schweigerer et al, 1990). 3′, 4′-Dihydroxyflavone, luteolin, and 3-hydroxyflavone were again among the most potent anti-proliferative metabolites (Table 2). As for endothelial cells, several of the flavonoids exhibited a potent anti-mitotic activity, especially on tumour cells, whereas others had minimal or no effect. Among the normal cell types, there appeared to be a greater inhibitory effect on endothelial cells than on fibroblasts and keratinocytes (Table 2), indicating a certain degree of selectivity towards endothelial cells. In contrast, the inhibitory effect of flavonoids on tumour cells was comparable to that of endothelial cells (Table 2). It is possible that the rapid proliferation of the latter in response to bFGF stimulation accounts for the similar inhibitory profiles seen with endothelial and tumour cells. These results point to a selective targeting of rapidly proliferating cells, a conclusion that is supported by the results obtained with the isoflavonoid genistein (Fotsis et al, 1995).

SELECTED FLAVONOIDS INHIBIT ANGIOGENESIS IN VITRO IN A MORE POTENT MANNER COMPARED TO GENISTEIN

In order to examine the potential of flavonoids as inhibitors of angiogenesis, we tested selected flavonoids in the in vitro angiogenesis assay on three-dimensional collagen gels, described above for genistein. When 10 μm concentrations of the most potent anti-mitotic flavonoids were added together with a synergistic combination of bFGF and VEGF (Pepper et al, 1992), they inhibited the ability of BME cells to invade the gels and generate capillary-like structures. A quantitative analysis revealed that several flavonoids inhibited the bFGF/VEGF-induced invasion of BME cells to a greater extent than did the same concentration of genistein (Figure 6). A more extensive evaluation, carried out in a dose–response manner, confirmed these findings and revealed a half-maximal concentration for the inhibition of in vitro angiogenesis that closely correlates with that of the anti-mitotic effects (data not shown). It is noteworthy that the order of potency of the various flavonoids with respect to anti-mitotic effects and the inhibition of in vitro angiogenesis did not strictly correlate. However, the requirement for a non-hydroxylated ring C with an oxo function at position 4 and a C2–C3 double bond is also valid for the inhibition of in vitro angiogenesis, as catechin is once again totally inactive.

STRUCTURE–ACTIVITY RELATIONSHIP ISSUES

Although anti-proliferative effects of flavonoids have been previously reported (Kandaswami et al, 1991; Huang et al, 1994; Yit and Das, 1994; Hirano et al, 1995), most of these studies were confined to the effects of single or a few metabolites on one or two cell types. This has made it difficult to draw any conclusion regarding a potential structure–activity correlation. Our studies allowed a more comprehensive approach by employing the testing of a set of various flavonoid structures on several normal and tumour cells of different origin. Our results clearly show that a non-hydroxylated ring C with an oxo function at position 4 and a C2–C3 double bond is required for maximal biological activity. Catechin, which lacks both the C4 oxo group and the C2–C3 double bond, is completely devoid of anti-proliferative activity. Eriodictyol, the flavanone derived from reduction of the C2–C3 double bond of the flavone luteolin, is at least three times weaker in inhibiting all the cells tested. The presence of a hydroxyl function in position 3, as occurs in flavonols, seems to modify the anti-mitotic activity in an unpredictable manner. Thus, 3-hydroxyflavone, the 3-hydroxylated flavonol derived from flavone, has an anti-proliferative activity which is fourfold greater. In contrast, quercetin, the flavonol derived from luteolin, is 2.5-fold weaker in its inhibition of cell proliferation. The catechol structure alone is insufficient for the inhibition of proliferation. This is clearly demonstrated by catechin, which, despite the presence of aromatic vicinal hydroxyls at positions 3′ and 4′, does not have an anti-mitotic effect.
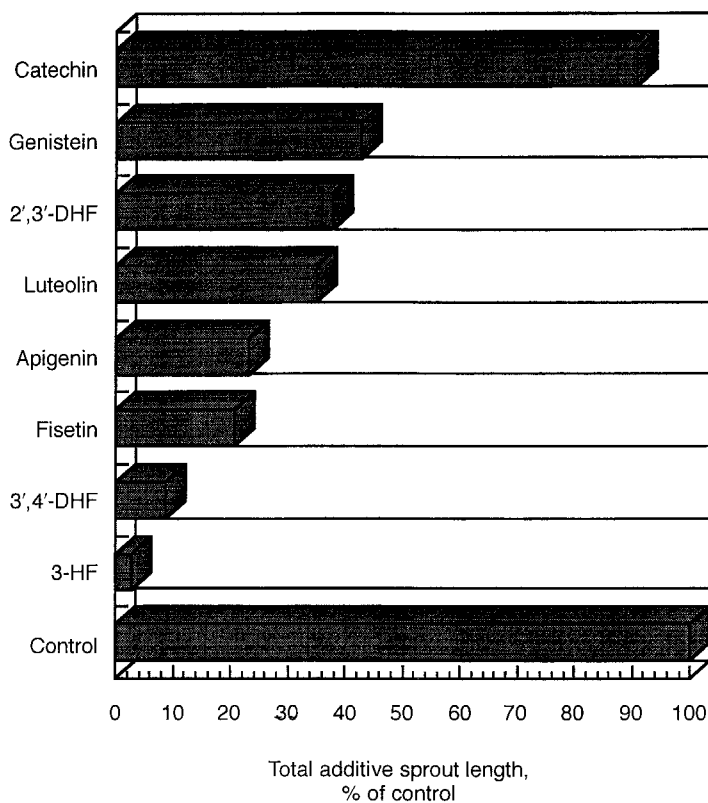
Figure 6. The effect of flavonoids on in vitro angiogenesis. Bovine microvascular endothelial cells (BME) were grown to confluence on three-dimensional collagen gels as described (Montesano and Orci, 1985). After confluence, they received solvent or recombinant human bFGF (10 ng/ml) plus vascular endothelial growth factor (VEGF) (30 ng/ml) with or without 10 µmol/l concentrations of the substances to be tested. After 2 days of incubation, the medium was changed and the cells exposed to the same conditions again. Quantitative analysis was carried out on BME cells treated for 5 days as described above. Fields measuring 1 mm × 1.4 mm were then randomly selected and photographed, and the BME cell invasion was quantified by measuring the total length of all the cells that penetrated the underlying gel either as single cells or in the form of cell cords. The results are expressed as a percentage of that for controls, i.e. cells receiving solvent and recombinant human bFGF plus VEGF. DHF = dihydroxyflavone; HF = hydroxyflavone. Reproduced from Fotsis et al (1997), with the permission of the American Association for Cancer Research.

Also, 3-hydroxyflavone, which has no catechol functions, has anti-proliferative activity comparable to that of the catecholic metabolites luteolin and 3′, 4′-dihydroxyflavone. On the other hand, hesperetin, derived from methylation of the catechol hydroxyl at C-4′ of eriodictyol, inhibits BBCE cells with a half-maximal concentration of 30 µmol/l, which is fourfold weaker than the anti-mitotic activity of the catecholic eriodictyol (7 µmol/l).

Although several of the flavonoid metabolites tested exhibited stronger half-maximal inhibitory concentrations on endothelial cell proliferation

compared with the isoflavonoid genistein, this difference does not warrant the conclusion that flavonoid metabolites are more potent than isomeric isoflavonoids. Indeed, apigenin, the corresponding flavonoid isomer of genistein with hydroxylations at positions 5, 7 and 4', exhibits a comparable half-maximal inhibitory concentration (6.5 μmol/l).

MECHANISM OF ACTION

Flavonoids exhibit several interesting biochemical properties (Havsteen, 1983). The inhibition of tyrosine kinases (Graziani et al, 1983; Cunningham et al, 1992) and protein kinase C (PKC) (Ferriola et al, 1989) are, however, of particular importance with regard to cellular processes studied in our investigations. Flavonoids are competitive inhibitors with respect to the adenosine triphosphate (ATP) binding site (Graziani et al, 1983) on a variety of enzymes, a region of considerable homology between kinases. Indeed, genistein has been shown to be a competitive inhibitor of ATP binding to the catalytic domain of tyrosine kinases (Akiyama et al, 1987) and was found to inhibit epithelial growth factor (EGF) receptor (Akiyama et al, 1987) and platelet-derived growth factor (PDGF) receptor (Hill et al, 1990) tyrosine kinase activities both in intact cells and in vitro. This appears as an attractive hypothesis, since high-affinity fibroblast growth factor (FGF) receptors are tyrosine kinases (Ullrich and Schlesinger, 1990) and since vanadate, an inhibitor of phosphotyrosine phosphatases, induces angiogenesis in the collagen gel assay (Montesano et al, 1988). In this context, apigenin was found to inhibit both PKC and TPA-stimulation of the FGF receptor (Huang et al, 1996).

A further important target of flavonoids appears to be 1-phospatidyl-inositol kinase (PI kinase) (Singhal et al, 1995), a key enzyme in signal transduction leading to the production of second messengers, inositol 1,4,5-trisphosphate (IP3) and diacyglycerol (DAG). Quercetin in breast carcinoma cells reduced the concentration of PI kinase and IP3 within 60 minutes to 5% and 6% respectively (IC$_{50}$: 6 μmol/l). However, no other flavonoids were tested in this study (Singhal et al, 1995).

Synthetic analogues of flavonoids are potent inhibitors of cell-division-cycle 2 (cdc2) (Losiewicz et al, 1994) and cyclin-dependent (CDK) kinases (Carlson et al, 1996; De Azevedo et al, 1996), thereby inhibiting cell cycle progression. At least in the case of cdc2, the inhibition was competitive with respect to ATP binding (Losiewicz et al, 1994). Although the inhibition of kinases appears to be the most likely target, selected flavonoids have been reported to inhibit the DNA binding or the DNA re-ligation step of eukaryotic topoisomerase I (Boege et al, 1996). The strong scavenging of free radicals (Havsteen, 1983) does not appear to be the mechanism of anti-mitotic and anti-angiogenic activity of flavonoids. Indeed, myricetin and catechin, some of the strongest protectors against single-stranded breaks induced by singlet molecular oxygen (Devaśagayam et al, 1995), are devoid of anti-mitotic or anti-angiogenic activity.

FUTURE PROSPECTS

Flavonoids comprise a large group of naturally occuring, low-molecular weight substances that are present in fruits, vegetables, nuts, seeds, stems, leaves, flowers, bark and the roots of most plants as well as tea, coffee and wine (Harborne, 1973; Harborne and Marby, 1982; Middleton, 1988). The average Western-type diet is estimated to contain approximately 23 mg per day of quercetin, kaempferol, myricetin, apigenin and luteolin (Hertog et al, 1993), the total content of all naturally occurring flavonoids not being known. There is also a lack of information about the content of flavonoids in the diet of vegetarians. However, as the concentration of genistein in the urine of vegetarians is increased 30-fold compared with omnivores (Adlercreutz et al, 1991a, 1991b, 1995), it is possible that similar values might be also anticipated for flavonoids. The development of sensitive methods for the determination of flavonoids in biological materials, a task that our group has already undertaken, is expected to provide valuable information in this respect. Considering the biological functions of flavonoids presented in this and previous studies, it is quite possible that flavonoids might contribute to the preventive effects of a vegetarian diet on the incidence of and mortality from cancer. In this respect, animal experiments have already shown that flavonoids exert inhibitory effects on carcinogenicity (Elangovan et al, 1994; Das et al, 1995). Further studies should focus on the in vivo effects of flavonoids on angiogenesis and tumourigenesis models.

Acknowledgements

REFERENCES

Adlercreutz H (1990) Western diet and western diseases: some hormonal and biochemical mechanisms and associations. *Scandinavian Journal of Clinical and Laboratory Investigation* **50** (supplement 201): 3–23.

Adlercreutz H, Fotsis T, Bannwart C et al (1991a) Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine including identification of genistein. *Clinica Chimica Acta* **199**: 263–278.

*Adlercreutz H, Honjo H, Higashi A et al (1991b) Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming traditional Japanese diet. *American Journal of Clinical Nutrition* **54**: 1093–1100.

Adlercreutz H, Van der Wildt J, Kinzel J et al (1995) Lignan and isoflavonoid conjugates in human urine. *Journal of Steroid Biochemistry and Molecular Biology* **52**: 97–103.

*Akiyama T, Ishida J, Nakagawa S et al (1987) Genistein a specific inhibitor of tyrosine-specific protein kinases. *Journal of Biological Chemistry* **262**: 5592–5595.

De Azevedo WF, Mueller-Dieckmann HJ, Schulze-Gahman U et al (1996) Structural basis for specificity and potency of a flavonoid inhibitor of human CDK2 a cell cycle kinase. *Proceedings of the National Academy of Sciences of the USA* **93**: 2735–2740.

Boege F, Straub T, Kehr A et al (1996) Selected novel flavones inhibit the DNA binding or the DNA religation step of eukaryotic topoisomerase I. *Journal of Biological Chemistry* **271**: 2262–2270.

Carlson BA, Bubay MM, Sausville EA et al (1996) Flavopiridol induces G1 arrest with inhibition of cyclin-dependent kinase (CDK)2 and CDK4 in human breast carcinoma cells. *Cancer Research* **56:** 2473–2478.

Crum R, Szabo S & Folkman J (1985) A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science* **230:** 1375–1378.

*Cunningham BDM, Threadgill MD, Groundwater PW et al (1992) Synthesis and biological evaluation of a series of flavones designed as inhibitors of protein tyrosine kinases. *Anti-Cancer Drug Design* **7:** 365–384.

Das A, Wang JH & Lien EJ (1995) Carcinogenicity mutagenicity and cancer preventing activities of flavonoids: a structure–system–activity relationship (SSAR) analysis. In Jucker E (ed.) *Progress in Drug Research*, pp 133–166. Basel: Birkhaeuser Verlag.

Denekamp J (1990) Vascular attack as a therapeutic strategy for cancer. *Cancer Metabolism Review* **9:** 267–282.

Devasagayam TP, Subramanian M, Singh BB et al (1995) Protection of plasmid pBR322 DNA by flavonoids against single-stranded breaks induced by singlet molecular oxygen. *Journal of Photochemistry and Photobiology* **30:** 97–103.

Elangovan V, Sekar N & Govindasamy S (1994) Chemopreventive potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumorigenesis. *Cancer Letters* **87:** 107–113.

*Ferriola PC, Cody V & Middleton EJ (1989) Protein kinase C inhibition by plant flavonoids. *Biochemical Pharmacology* **38:** 1617–1624.

Folkman J (1985) Tumor angiogenesis. *Advances in Cancer Research* **43:** 175–203.

Folkman J & Cotran RS (1976) Relation of vascular proliferation to tumor growth. *International Review of Experimental Pathology* **16:** 207–248.

Folkman J & Klagsbrun M (1987) Angiogenic factors. *Science* **235:** 442–447.

Fotsis T (1987) The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS-II. Fractionation and quantitation of the main group of estrogen conjugates. *Journal of Steroid Biochemistry* **28:** 215–226.

*Fotsis T & Adlercreutz H (1987) The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS-I. Quantitation of estrogens after initial hydrolysis of conjugates. *Journal of Steroid Biochemistry* **28:** 203–213.

*Fotsis T, Pepper M, Adlercreutz H et al (1993) Genistein a dietary-derived inhibitor of *in vitro* angiogenesis. *Proceedings of the National Academy of Sciences of the USA* **90:** 2690–2694.

Fotsis T, Pepper M, Adlercreutz H et al (1995) Genistein a dietary ingested isoflavonoid inhibits cell proliferation and in vitro angiogenesis. *Journal of Nutrition* **125 (supplement):** 790S–797S.

*Fotsis T, Pepper MS, Aktas E et al (1997) Flavonoids dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. *Cancer Research* **57:** 2916–2921.

*Graziani Y, Erikson E & Erikson RL (1983) The effect of quercetin on the phosphorylation activity of the Rous sarcoma virus transforming gene product in vitro and in vivo. *European Journal of Biochemistry* **135:** 583–589.

*Hanahan D & Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86:** 353–364.

*Harborne JB (1973) Flavonoids. In Miller LP (ed.) *Phytochemistry*, pp 344–380. New York: Van Nostrand Reinhold.

Harborne JB & Marby TJ (1982) *The Flavonoids: Advances in Research*. London: Chapman & Hall.

*Havsteen B (1983) Flavonoids a class of natural products of high pharmacological potency. *Biochemical Pharmacology* **32:** 1141–1148.

Hertog MG, Hollman PC, Katan MB & Kromhout D (1993) Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutrition in Cancer* **20:** 21–29.

Hill TD, Dean NM, Mordan LJ et al (1990) PDGF-induced activation of phospholipase C is not required for induction of DNA synthesis. *Science* **248:** 1660–1663.

Hirano T, Abe K, Gotoh M & Oka K (1995) Citrus flavone tangeretin inhibits leukemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. *British Journal of Cancer* **72:** 1380–1388.

Huang HC, Wang HR & Hsieh LM (1994) Antiproliferative effect of baicalein a flavonoid from a Chinese herb on vascular smooth muscle cell. *European Journal of Pharmacology* **251:** 91–93.

Huang YT, Kuo ML, Liu JY et al (1996) Inhibitions of protein kinase C and proto-oncogene expressions in NIH 3T3 cells by apigenin. *European Journal of Cancer* **32A:** 146–151.

Ingber DE, Fujita T, Kishimoto S et al (1990) Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature* **348:** 555–557.

Kandaswami C, Perkins E, Soloniuk DS et al (1991) Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma in vitro. *Cancer Letters* **56:** 147–152.

Klagsbrun M & Folkman J (1990) Angiogenesis. In Sporn MB and Roberts AB (eds) *Peptide Growth Factors and their Receptors, II*, pp 549–586. Berlin: Springer Verlag.

Kolonel LN (1988) Variability in diet and its relation to risk in ethnic and migrant groups. *Basic Life Science* **43:** 129–135.

Lee HP, Courley L & Duffy SW (1991) Dietary effects on breast cancer risk in Singapore. *Lancet* **337:** 1197–1200.

Liotta LA, Steeg PS & Sterler-Stevenson WG (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* **64:** 327–336.

Losiewicz MD, Carlson BA, Kaur G et al (1994) Potent inhibition of CDC2 kinase activity by the flavonoid L86-8275. *Biochemical and Biophysical Research Communications* **201:** 589–595.

Middleton E Jr (1988) Some biological properties of plant flavonoids. *Annals of Allergy* **61:** 53–57.

Miksicek RS (1994) Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. *Journal of Steroid Biochemistry and Molecular Biology* **43:** 153–160.

Miksicek RJ (1995) Estrogenic flavonoids: structural requirements for biological activity. *Proceedings of the Society of Experimental Biology and Medicine* **208:** 44–50.

Miller AB (1990) Diet and cancer—a review. *Review of Oncology* **3:** 87–95.

*Montesano R & Orci L (1985) Tumor-promoting phorbol esters induce angiogenesis in vitro. *Cell* **42:** 469–477.

Montesano R, Vassalli J-D, Baird A et al (1986) Basic fibroblast growth factor induces angiogenesis in vitro. *Proceedings of the National Academy of Sciences of the USA* **83:** 7297–7301.

Montesano R, Pepper MS, Belin D et al (1988) Induction of angiogenesis in vitro by vanadate, an inhibitor of phosphotyrosine phosphatases. *Journal of Cell Physiology* **134:** 460–466.

Pepper MS & Montesano R (1990) Proteolytic balance and capillary morphogenesis. *Cell Differentiation and Development* **32:** 319–328.

Pepper MS, Ferrara N, Orci L & Montesano R (1992) Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochemical and Biophysical Research Communication* **189:** 824–831.

Rose DP, Boyar AP & Wynder EL (1986) International comparison of mortality rates for cancer of the breast ovary prostate and colon and per capita fat consumption. *Cancer* **58:** 2363–2371.

Schweigerer L, Breit S, Wenzel A et al (1990) Augmented MYCN expression advances the malignant phenotype of human neuroblastoma cells: evidence for induction of autocrine growth activity. *Cancer Research* **50:** 4411–4416.

Schweigerer L, Christeleit K, Fleischmann G et al (1992) Identification in human urine of a natural growth inhibitor for cells derived from solid pediatric tumors. *European Journal of Clinical Investigation* **22:** 260–264.

Shutt DA (1976) The effects of plant estrogens on animal reproduction. *Endeavour* **35:** 110–113.

Singhal RL, Yeh YA, Praja N (1995) Quercetin down-regulates signal transduction in human breast carcinoma cells. *Biochemical and Biophysical Research Communications* **208:** 425–431.

Ullrich A & Schlesinger J (1990) Signal transduction by receptors tyrosine kinase activity. *Cell* **61:** 203–212.

Yit CC & Das NP (1994) Cytotoxic effect of butein on human colon adenocarcinoma cell proliferation. *Cancer Letters* **82:** 65–72.