CANCER CHEMOPREVENTION AND APOPTOSIS MECHANISMS INDUCED BY DIETARY POLYPHENOLICS

Giuseppe Galati[1], Shirley Teng[2], Majid Y. Moridani[2], Tom S. Chan[2] and Peter J. O'Brien[1,2*]

[1]Department of Pharmacology and [2]Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada M5S 2S2

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* Author for correspondence:
Dr. Peter J. O'Brien
Department of Pharmacology
University of Toronto
Toronto, Ontario, Canada M5S 2S2
e-mail: peter.obrien@utoronto.ca

SUMMARY

This review summarises current knowledge on the various molecular chemopreventive or therapeutic mechanisms that may be involved when the administration of flavonoids or polyphenols prevented chemical carcinogenesis in animal models. These mechanisms can be subdivided into the following: 1) the molecular mechanisms involved in preventing carcinogen metabolic activation, 2) the molecular mechanisms for preventing tumour cell proliferation by inactivation or downregulation of prooxidant enzymes or signal transduction enzymes, 3) the molecular cell death mechanisms for the induction of tumour cell death (apoptosis) and the molecular mechanisms for the inhibition of isolated mitochondria functions. Many of the flavonoids and polyphenols found in diets, supplements or herbal medicine were also ranked using "accelerated cytotoxic mechanism screening" by a combinatorial approach utilising isolated rat hepatocytes. A strong correlation of an early collapse of the mitochondrial membrane potential and cell death was found for most of the cytotoxic polyphenols but did not occur with non-toxic polyphenols. This screening could prove useful for eliminating polyphenols that have the potential for adverse health effects and for selecting safe and effective polyphenolic candidates for further development as supplements for preventing cancer or cardiovascular disease. Safety concerns of flavonoid/polyphenol supplements are also reviewed.

KEY WORDS

cancer chemoprevention, tumour cell apoptosis, bioflavonoids, hepatocytes, coumaric acids, antioxidants, stilbenes, curcumin, herbal medicine, flavones, capsaicin, safety

1. INTRODUCTION

Chemoprevention is one of the most promising new approaches in cancer research, i.e. the administration of agents to inhibit, delay or reverse the process of carcinogenesis. Epidemiological studies have suggested that frequent consumption of fruits and vegetables is associated with a decrease in cancer incidence (particularly colorectal)

and cardiovascular disease, whereas a high intake of fat or red meat may increase some types of cancer /1,2/. Because of this some food-derived products are now thought of as non-nutritious chemopreventive agents for major cancer targets including breast, prostate, colon and lung. *Nutraceuticals* are also the fastest growing segment of the food industry today. They are defined as naturally occurring ingredients that can be consumed as part of the daily diet which when ingested enhance or regulate a particular biological process or control a specific disease /3/.

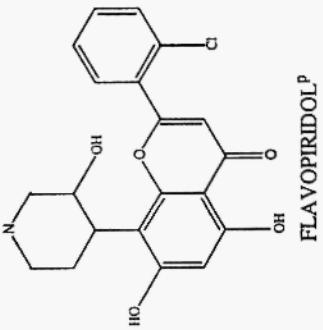
2. POLYPHENOLICS

Polyphenolics form a major part of the dietary antioxidant capacity of fruits and vegetables and have been identified by many researchers using several animal cancer models and cell cultures as chemopreventive agents. The polyphenolics include hydroxycinnamic acids (e.g. caffeic acid, ferulic acid), hydroxybenzoic acids (e.g. ellagic acid, gallic acid), hydroxystilbenes (e.g. resveratrol) and flavonoids. Of the flavonoids, flavonols and anthocyanins come predominately from the skins of fruit whereas catechins (flavan-3-ols) originate mainly from the seeds and stems. The structural formulae of the dietary polyphenolics/flavonoids discussed in this review are shown in Figure 1. The plant polyphenolics, e.g. tea polyphenols, soy isoflavones, curcumin, are a class of chemopreventive agents that are unusual in that they are able to antagonise any of the stages of carcinogenesis, i.e. the initiation stage or promotion/progression stages, as well as cause apoptosis of tumour cells already formed. The chemoprevention mechanisms of polyphenolics/flavonoids were previously reviewed by O'Brien in 1994 /4/ so this review is mostly concerned with recent literature.

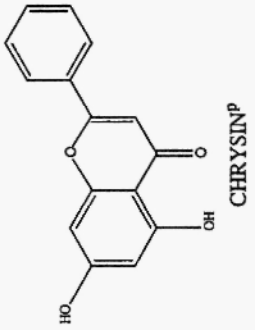
The daily intake of *flavonoids* has been estimated as between 3 and 70 mg, mostly *quercetin* (60-75%) which is a major flavonoid in tea, wine, onions, apples and berries. Clinical trials showed a protective role for flavonols in cardiovascular disease in most cohort studies /5/ in spite of the earlier belief that dietary flavonoids were not absorbed. However recent studies show that flavonoids and hydroxycinnamates can be relatively rapidly absorbed and metabolised in humans although their bioavailability requires more research /6/. Furthermore, even flavonoid glycosides can be absorbed without requiring bacterial

I Flavonoids

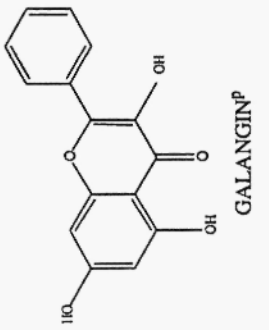
a) B ring without hydroxyl group



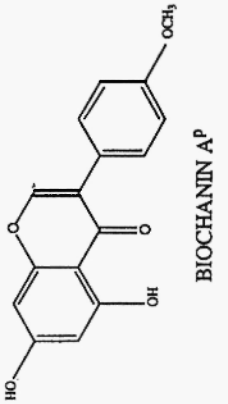
FLAVOPIRIDOL[P]



CHRYSIN[P]



GALANGIN[P]



BIOCHANIN A[P]

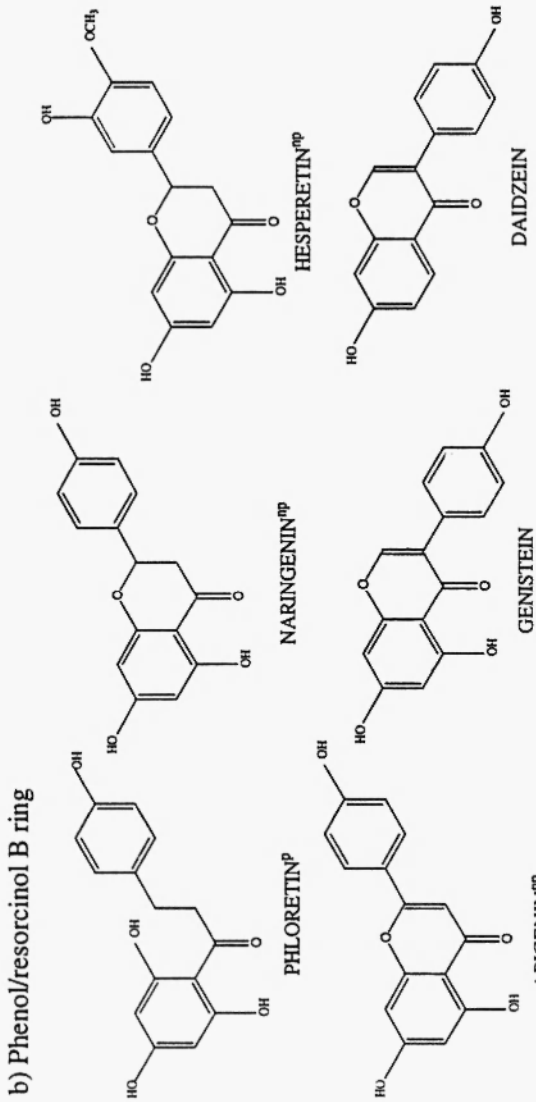
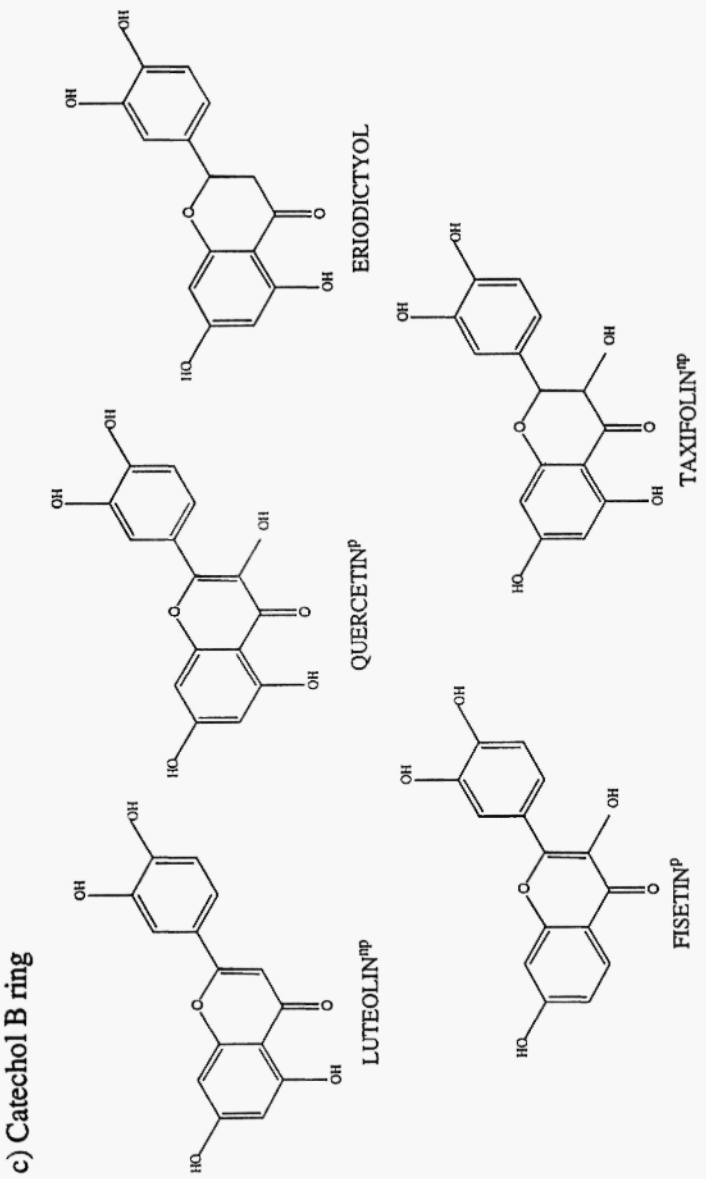


Fig. 1: Structural formulae of anticancer polyphenolics. **cont.**



d) Gallic acid A or B ring

MYRICETIN[nn]

BAICALEIN

EPIGALLOCATECHIN GALLATE

Fig. 1: Structural formulae of anticancer polyphenolics. **cont.**

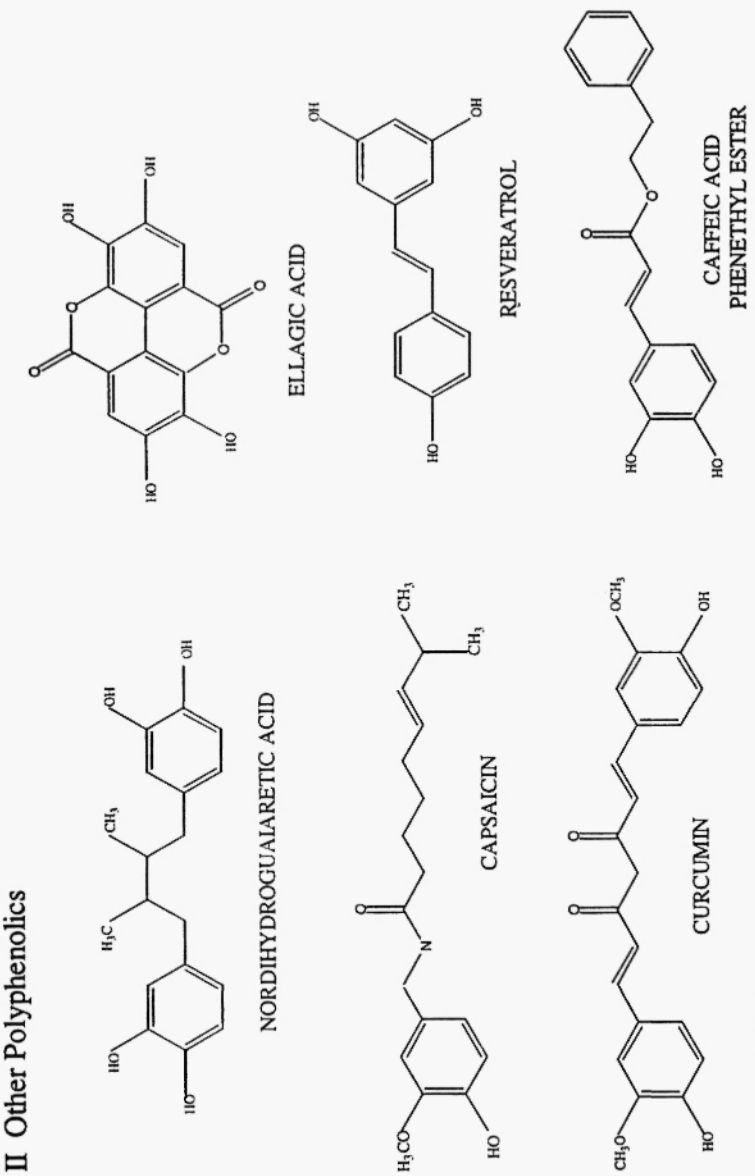


Fig. 1: Structural formulae of anticancer polyphenolics.

hydrolysis in the colon; human studies with ileostomy patients showed that 52% of quercetin glycosides and 24% of quercetin aglycone were absorbed /5/. In other human studies, 0.2-0.9% of orally administered tea polyphenols and their metabolites appeared in the plasma at 1 h /5/. Furthermore, oral administration to rats of a single meal containing 0.2% quercetin caused the appearance of quercetin/metabolites in the plasma 2-24 h later /7/. *Epicatechin*, a major component of tea polyphenols, orally administered to rats (172 μmol/kg) was also absorbed from the alimentary tract and caused the appearance of 23 μM epicatechin and its metabolites in the plasma one hour later which was still detectable at 8 h /8/. The liver also further metabolises absorbed flavonoids and their metabolites utilising hepatocyte enzymes including β-glucosidase, sulfotransferases, catechol methyltransferases, UDP-glucuronyl transferases and cytochrome P450 isozymes /5/.

3. STAGES OF CARCINOGENESIS INHIBITED BY DIETARY POLYPHENOLICS/FLAVONOIDS

The stages of carcinogenesis inhibited by dietary polyphenolics/ flavonoids and the molecular mechanisms suggested for their biological action are as follows:

3.1 Initiation stage: Preventing carcinogen metabolic activation

Phase I metabolising enzymes (e.g. cytochrome P450) catalyse the introduction of a polar reactive group into lipophilic carcinogens/ xenobiotics to form a potent electrophile which can react with cellular nucleophiles, e.g. DNA. The introduced group and/or the original xenobiotic is detoxified as a result of conjugation catalysed by **phase II metabolising enzymes** to sugars, amino acids, glutathione and sulphate. The conjugates formed are usually more polar, less toxic, more water soluble and are thus more readily eliminated from the body.

Green and *black* tea extracts strongly inhibit neoplastic transformation in mammary organ cultures or epithelial cells, inhibited benzo(*a*)pyrene DNA adduct formation and induced the phase II metabolising enzymes **glutathione-*S*-transferase, quinone reductase (NQO)** /9/ and **UDP-glucuronyl transferase** /10/. Green tea polyphenols also induced the phase II enzymes **glutathione peroxidase**,

glutathione reductase, **superoxide dismutase** and **catalase** /11,12/. Phase II enzyme induction may explain the chemopreventive effect of tea in inhibiting heterocyclic amine (from cooked meat)-induced colonic aberrant crypt foci formation in the rat /13/. Green or black tea *in vivo* also induced the phase I metabolising enzyme CYP1A2.

The order of efficacy of flavonoids found for inducing quinone reductase in murine hepatoma cells was *galangin*, *kaempferol* > *quercetin* > *myricetin*, *apigenin*, with epicatechin, catechin and taxifolin being inactive. This suggests that the 2,3-double bond in the flavonoid C ring is essential for induction, and the presence of a 3-hydroxyl group also increases the flavonoid's ability to induce quinone reductase /14/. The synthetic flavonoid *4′-bromoflavone* was the most potent *in vivo* inducer of quinone reductase and glutathione synthesis enzymes, and prevented mammary carcinogenesis in rats induced by polycyclic aromatic hydrocarbons /15/.

Ellagic acid is a polyphenolic compound generated *in vivo* by hydrolysis of dietary ellagitannins present in nuts (e.g. walnuts) and fruit (e.g. raspberries, strawberries and grapes). Ellagic acid or quercetin in the drinking water increased mouse liver and lung GSH levels twofold, which was associated with a marked decrease in *N*-nitrosodiethylamine-induced lung tumourigenesis /16/. Ellagic acid also acted as a cancer chemopreventative agent in other animal chemical carcinogenesis models /17/ and induced phase II enzymes likely mediated by the Antioxidant Response Element (ARE) of each phase II gene /18, 19/. The phase I enzymes CYP2E1 and 1A1 were also inhibited /18/. Large doses of ellagic acid prevent lung tumourigenesis induced by the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) which was attributed to inhibition of lung cytochrome P450 /20/. *Caffeic acid phenethyl ester* (CAPE), a major constituent of propolis, is an *in vivo* inhibitor of tumour initiation/promotion and also induced NQO1 activity by stimulating the ARE of the NQO1 gene /21/.

Capsaicin is responsible for the pungent and hot sensation of hot chilli peppers and is widely used in spicy food. It is also a phenolic antioxidant with sensory neurotoxic effects and chemopreventive activity against chemical carcinogens and mutagens /22/. Its chemoprotective action against tumourigenesis and mutagenesis by vinyl carbamate or *N*-nitrosodimethylamine was attributed to its inhibition of the CYP2E1 responsible for activation of these carcinogens /23/.

CYP1A and 2B were also inhibited and could explain the inhibition of benzo(*a*)pyrene-induced skin carcinogenesis by capsaicin /22,23/.

Curcumin (diferuloylmethane) is the yellow pigment from the rhizome of *Curcuma longa*. The yellow powdered rhizome is commonly called turmeric and the spice is widely used for flavoring and colouring in foods, e.g. curry. It has also been widely used in India in the treatment of sprains and inflammation. The dietary administration of turmeric also protects against tumour induction by carcinogens of diverse structures. Curcumin was recently found to be a highly effective phase II enzyme inducer as only 7.3 μM was required to double the quinone reductase activity in Hepa1c1c7 murine hepatoma cells. This was attributed to its β-diketone moiety (which could act as a Michael reaction acceptor) and its hydroxyl group at the ortho-position on the aromatic rings /24/. Induction of phase II enzymes could partly explain the chemopreventive effects of curcumin against forestomach and duodenal colon carcinogenesis /25,26/.

Carnosol is thought to be the major antioxidant in the leaves of rosemary, a commonly used herb and flavoring agent. It is a catechol which has chemopreventive activity in mice against skin and mammary tumourigenesis, possibly because it induces phase II enzymes (GSH-*S*-transferase, quinone reductase) and inhibits CYP1A1 /27/.

The polyphenolic *resveratrol* (3,5,4′-trihydroxystilbene) is an active ingredient of "kojo-kon", the powdered root of *Polygonum cuspidatum*. This Chinese and Japanese folk medicine has been used since ancient times for the treatment of inflammatory and allergic diseases and to fight liver, skin and circulatory diseases. Resveratrol is a phytoalexin also found in peanuts and wine (particularly red wine) made from the grapes of vines affected by fungal infection, wounding or ultraviolet light irradiation whose role is likely to inhibit fungal infection progression. The reduced risk of coronary heart disease associated with moderate wine consumption (particularly red wine), known as the "French paradox", has been attributed by some investigators to the antioxidant/anti-inflammatory activity or COX-1 inhibitory activity of resveratrol. Resveratrol also has cancer chemopreventive activity and inhibits carcinogen-induced mouse skin or mammary tumourigenesis and preneoplastic lesions /28/. This was attributed to the induction of quinone reductase (NQO1) and inhibition of COX-1 (IC_{50} 15 μM), a phase I enzyme that metabolically activates some carcinogens /29/. Furthermore, resveratrol is

also a selective inhibitor of human CYP1A1 (IC_{50} 11 μM) which can activate carcinogenic polycyclic aromatic hydrocarbons /30/.

3.2 Promotion stage: Preventing tumour cell proliferation (cytostatic effects) by inactivation or downregulation of prooxidant enzymes/ signal transduction enzymes

3.2.a Prooxidant enzymes

It is generally believed that the formation of growth promoting oxidants (reactive oxygen species, [ROS]) is a major "catalyst" of the tumour promotion and progression stages which follow the initiation stages (carcinogen metabolic activation to mutagens). The prooxidant enzymes induced or activated by various tumour promoters, e.g. phorbol esters, include the arachidonate metabolizing enzymes, **cyclo-oxygenases (COX)** and **lipoxygenase (LOX)**. Nonsteroidal anti-inflammatory drugs or COX-2 inhibitors have shown potent chemo-preventive activity in animal colorectal carcinogenesis models /31/. Dietary polyphenolics such as curcumin, chlorogenic acid, caffeic acid, resveratrol or the flavonoid silymarin have also been shown *in vivo* to prevent colon carcinogenesis induced by azoxymethane (e.g. /26/) and skin carcinogenesis induced by phorbol esters, polycyclic aromatic hydrocarbons or UV (e.g. /32/).

A polyphenolic fraction isolated from green tea was shown to prevent phorbol ester-induced skin papilloma promotion in 7,12-dimethylbenzanthracene-initiated mouse skin tumours as well as prevent skin squamous cell carcinoma induced by benzoyl peroxide or 4-nitroquinoline N-oxide /33/. The active components of the polyphenolic fraction of green or black tea extracts are flavonoids and include epicatechin, epigallocatechin, epicatechin-3-gallate and epigallocatechin-3-gallate. A single cup of brewed green tea contains up to 200 mg of epigallocatechin gallate (EGCG). Daily i.p. injections of EGCG (50 mg/kg) also inhibited or prevented the growth of MCF-7 human breast cancer cells and LNCaP 104R human prostate cancer cells transplanted into athymic immunodeficient mice /34/. Furthermore, the oral administration of green tea or black tea or caffeine as the sole source of drinking fluid to SKH-1 mice prevented the formation and growth of skin cancer induced by ultraviolet B light /35/.

Propolis is a gum produced from the bark of conifer trees (*Populus* spp) that is gathered by bees. It is masticated with salivary enzymes,

mixed with wax and used by honeybees to seal holes in their hives as well as to protect the entrance against intruders. It is widely used in folk medicine (at least from 300 BC) for its anti-inflammatory activity (recommended dosage 200 mg/day). The anti-inflammatory activity of propolis has largely been attributed to its content of *caffeic acid phenethyl ester* (CAPE), a phenolic antioxidant which causes a non-selective inhibition of COX-1/COX-2 and the suppression of 12,O-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 mRNA expression /36/. Previously CAPE was shown to inhibit azoxymethane-induced colon aberrant crypt formation /37/ and prevented TPA promoted skin cancer in animal models *in vivo* /38/. CAPE also inhibits H_2O_2 formation by activated polymorphonuclear neutrophils that infiltrate the skin following TPA application /39/.

The molecular mechanism of cancer chemoprevention by the flavonoids may involve the inhibition of the prooxidant processes that cause tumour promotion. Flavonoids and caffeic acid analogues are particularly effective at inhibiting the prooxidant enzymes xanthine oxidase /40,41/, COX /42/ or LOX /42/.

One of the Kampo traditional herbal medicines in Japan is known as Sho-saiko-to (TJ-9) and is obtained from the root of *Scutellaria baicalensis* Gerrgi. This medicine is used to treat patients with chronic hepatitis or liver cancer because of its anti-inflammatory and anti-fibrogenetic effects. It is believed that the flavonoids are the active ingredients, i.e. baicalein, baicalin, wogonin and wagonoside. *Baicalein* (Fig. 1) is 5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one and *wogonin,* 5,7-dihydroxy-8-methoxy-3-phenyl-4H-1-benzopyran-4-one, has a similar structure. Baicalin and wagonoside are their respective glycosides. Baicalein inhibits tumour cell proliferation *in vivo* and *in vitro* and can induce apoptosis /43/ partly because of its efficacy at inhibiting COX-1, 12-lipoxygenase and lipid peroxidation /44/. Resveratrol is also effective at inhibiting COX /29/ and LOX /45/. Chemoprevention by flavonoids or resveratrol could therefore result from the decrease in the 5-lipoxygenase metabolite of arachidonate, 5-hydroxyeicosatetraenoic acid (5-HETE), or from the increase in arachidonate levels. This is because 5-HETE stimulates tumour cell proliferation whereas arachidonate induces inhibition of cell growth and massive apoptosis in chronic myeloid leukemia cells or human prostate cancer cells as a result of its role as a second messenger in cell functions /46,47/. This would also help explain why

diets high in fat are associated with an increased risk of prostate cancer /47/.

Nordihydroguaiaretic acid (NDGA) is a major constituent (12% of plant dry weight) of the resinous exudates from the leaves and stem of the evergreen creosote bush *Larrea tridentada* found all over the world and was used at levels of 0.01-0.02% as an antioxidant to preserve fat-containing food products. An aqueous extract of the creosote bush has also been used to regress malignant melanomas. NDGA prevents TPA- or benzoyl peroxide-induced tumour promotion and ornithine decarboxylase in mouse skin, probably by inhibiting lipoxygenase /48/.

Tumour promoters, e.g. phorbol esters, also induce the migration and activation of polymorphonuclear leukocytes resulting in a respiratory burst and the formation of ROS, prooxidants that stimulate cell proliferation. Chemoprevention by *piceatannol* or *resveratrol* has been attributed to the inhibition of ROS formation by activated polymorphonuclear leukocytes /49/, likely by inhibiting protein tyrosine kinase /50/.

3.2.b Ornithine decarboxylase and polyamine synthesis

Tumour promoters readily induce **ornithine decarboxylase**, a rate limiting enzyme in polyamine biosynthesis which has been correlated with the rate of DNA synthesis and cell proliferation in several tissues. The flavonoid *apigenin* strongly suppressed tetradecanoyl-phorbol-acetate-mediated tumour promotion in mouse skin carcinogenesis and inhibited epidermal ornithine decarboxylase /51/. Dietary supplementation with flavonoids, e.g. *quercetin*, *diosmin* and *hesperidin*, caused an *in vivo* decrease in polyamine levels and inhibited the development of oral neoplasms in rats induced by 4-nitroquinoline 1-oxide /52,53/ as well as colon carcinogenesis in rats induced by azoxymethane /54/. Inhibition of polyamine biosynthesis could therefore be a contributing mechanism to the chemopreventive properties of flavonoids.

Silymarin is an antioxidant flavonoid derived from the milk thistle (*Silybum marianum*), a member of the aster family, which has been used for almost 2000 years as a herbal remedy for diseases of the liver and biliary tract. Preliminary clinical trials suggest it may improve the clinical course of viral, drug, toxin and alcoholic hepatitis without causing adverse side effects /55/. Silymarin also has chemopreventive

properties as it prevented photocarcinogenesis in a mouse skin model /56/. It also inhibited phorbol ester-induced epidermal ornithine decarboxylase activity and mRNA expression in SENCAR mice /57/. Topical applications of *catechin* derivatives also inhibited phorbol ester-induced ornithine decarboxylase *in vivo* and subsequent H_2O_2 formation and DNA/protein synthesis /58/.

Dietary *curcumin* prevented azoxymethane-induced colon carcinogenesis in rats and decreased colonic phospholipase A_2 and COX activity /59/. Dietary curcumin inhibited azoxymethane-induced ornithine decarboxylase in the rat colon /26/. The mechanism is not known but may involve inhibition of ornithine decarboxylase mRNA expression, e.g. by suppression of transcription, as occurs with rotenoids.

The hydroxycinnamic acids are the major polyphenolics of coffee, wine, beer and rice and include *caffeic acid*, *chlorogenic acid*, *ferulic acid* and *protocatechuic acid*. These polyphenolics given singly in the diet at 20 ppm-0.025% for 24-32 weeks after the administration of 4-nitroquinoline-1-oxide (oral carcinogen), methylazoxymethanol (intestinal carcinogen) or azoxymethane (large bowel carcinogen) prevented the increase in polyamine levels/ornithine decarboxylase and decreased tumour formation /60,61/.

Rotenoids are promising new chemopreventive or anticancer agents (e.g. deguelin) which inhibit phorbol ester-induced ornithine decarboxylase activity /62,63/ and prevent liver hyperplasia *in vivo* /64/. They also contain flavonoid-like structures, e.g. *rotenone*, and are the most commonly used inhibitors of the proton-translocating mitochondrial **NADH-ubiquinone oxidoreductase** as are some other flavonoids /65/.

3.2.c Signal transduction enzymes – Polyphenolics as ATP-site directed inhibitors

Flavonoids are also effective at inhibiting the protein kinases which are involved in the regulation of cell proliferation, differentiation and transformation. **Protein tyrosine kinase** (PTK) catalysed protein phosphorylation is also closely associated with the regulation of cellular proliferation. Tyrosine kinase may play a role in transformation or in maintaining the transformed state of cells, and inhibition of PTK may reverse or suppress carcinogenic processes. The isoflavone *genistein* and the catechol *tyrphostin A23* are the most

widely used inhibitors of this kinase as they are relatively ineffective against other kinases /66/. The flavonoids *quercetin* and *luteolin* are also effective inhibitors. All of these polyphenolic inhibitors are believed to act by binding to the ATP binding site /67/.

In contrast to the high incidence of breast cancer in the Western world, Asian women consuming a traditional diet high in soybean have a low breast cancer incidence. The low cancer incidence is retained when they migrate to the U.S.A., but markedly increases in the children who are born and raised in the Western world. It is therefore hypothesised that exposure to soy isoflavones early in life programmes "resistance" against breast cancer. Genistein and daidzein are the major isoflavones of soybean and a dietary intake of soybean is associated with a decreased incidence of hormonally dependent and independent cancers. It was also shown that a single dose of soybean milk administered to human volunteers caused the appearance of 2 μM genistein and daidzein in the plasma after 6.5 h /5/. However, even though soy isoflavones are phyto-oestrogens and can bind to the oestrogen receptor, their inhibition of tumour growth is not oestrogen receptor dependent. The ability of *genistein* to inhibit PTK could explain the inhibition of tumour cell proliferation /66/. Genistein also induces apoptosis in tumour cells, possibly by stabilising the transient DNA:DNA topoisomerase complex which induces DNA strand breaks during replication /66,68/.

Baicalein, *quercetin* and the citrus flavonoids *hesperetin* and *naringenin*, found in oranges and grapefruit respectively, were found to be much more effective than genistein at inhibiting the proliferation of MDA-MB-435 human breast cancer cells /69/; this was attributed to the inhibition of kinases including PTK. The inhibition of PTK by *piceatannol* and *resveratrol* has been implicated in the ability of these polyphenols to prevent polymorphonuclear leukocyte activation, i.e. spreading and releasing H_2O_2 /50/.

Epidermal growth factor receptor (EGFR)-mediated tyrosine phosphorylation, which is activated by phorbol ester, UV or oxidative stress, signals cell growth and proliferation in skin tumour promotion. *Silymarin* likely inhibits skin tumour promotion by inhibiting EGFR /70/. The inhibitory effects of the flavonoids *luteolin* and *quercetin* on skin tumour cell lines overexpressing EGFR were attributed to their inhibition of EGFR /67/. However, other investigators have shown that inhibition of EGFR did not explain the inhibitory effect of geni-

stein or quercetin on tumour cell growth /66,71/. Furthermore, the isoflavones *daidzein* and *genistein* also induce cell death in mouse erythroleukemia cells and are not PTK inhibitors /72/. Instead, the induction of apoptosis by a p53 independent mechanism is favored /73/.

cAMP-dependent protein kinase (PKA) is activated by cyclic AMP, an important intracellular second messenger to hormonal signals (glucagon, catecholamines) that phosphorylates and activates enzymes involved in catabolism (e.g. glycogen degradation, glycolysis, lipolysis), gluconeogenesis, fatty acid/cholesterol synthesis, smooth muscle relaxation (vasorelaxation and bronchodilatation). Protein synthesis may be increased as there are cAMP response elements (CRE) in the promoter regions of many genes. Increasing cAMP levels in tumour cells can inhibit growth inhibition/ differentiation and modulate apoptosis /74/. Adenylate cyclase converts ATP to cAMP and **cAMP phosphodiesterase** then catalyses cAMP hydrolysis to AMP. Both phosphodiesterase and adenylate cyclase can be activated by phosphorylation by PTK so that PTK flavonoid inhibitors, e.g. genistein (but not daidzein), can increase cAMP levels /75/. Flavonoids, e.g. *butein* (IC_{50} 10 μM) /76/ and genistein (IC_{50} 4 μM) /77/, also readily inhibit phosphodiesterase IV directly. On the other hand, *in vitro* experiments show that liver PKA is also readily inhibited by most flavonoids (IC_{50} 1-32 μM) /78/.

Phosphoinositide 3-kinases (PIP_3 kinases) specifically catalyse inositol lipid phosphorylation in the D3 position of the inositol ring generating the lipid messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3). The latter play an essential role as signal transducers in the regulation of cell proliferation, differentiation, apoptosis, cytoskeleton organisation and membrane traffic /79/. A *quercetin* analogue LY294002 or wortmannin are the most widely used inhibitors and *myricetin* is the most potent flavonoid inhibitor /80,81/. These inhibitors likely compete with ATP by binding to the ATP binding site of the kinase. The antiproliferative action of quercetin in K562 human leukemia cells has been reported to result from the downregulation of the c-*myc* and ki-*ras* oncogenes as well as the inhibition of the phosphatidyinositol cascade, leading to a decrease in IP_3 levels /82/. The latter was also found in a human HL-60 promyelocytic leukemia cell line treated with quercetin. Cytosolic protein kinase C and membrane

PTK were also inhibited and likely implicated in growth inhibition by quercetin /83/.

Protein kinase C (PKC) is another ubiquitous phospholipid-dependent family of enzymes (at least 12 isoenzymes) involved in signal transduction mechanisms associated with cellular growth and differentiation. Most of the PKC resides in the cytosol in the inactive state. Binding of phorbol esters (PE) or hormones to their surface membrane receptor activates G-protein dependent phospholipases to form DAG and IP_3. The latter causes the release of endogenous Ca^{2+} that binds to cytosolic PKC which translocates to the membrane where it is activated by DAG. The activated PKC induces nuclear proto-oncogene expression associated with cell proliferation. Apigenin inhibits PKC by competing with the ATP substrate at the ATP binding site. Apigenin also inhibited PE-stimulated protein phosphorylation and PE-induced c-*jun* and c-*fos* expression in NIH3T3 fibroblast cells. Of 15 flavonoids, only apigenin, kaempferol and genistein (25 μM) inhibited anchorage-independent colony formation and reverted the transformed phenotypes of V-H-*ras* transformed NIH3T3 cells /84/. However, other studies with brain PKC isozymes show that fisetin, quercetin, myricetin and luteolin are also effective inhibitors of PKC /80,81/, which suggests that the susceptibility of PKC to flavonoids depends on the isozyme studied.

Mitogen-activating protein kinases (MAPK) are protein kinases activated by receptors for extracellular growth factors, mitogens and cytokines. Activation of MAPK by green tea polyphenol stimulates the transcription of phase II detoxifying enzymes through the antioxidant-responsive element of the phase II enzyme genes /85/.

Cyclin-dependent kinases (CDKs) are a diverse family of proteins that play a central role in the timing of the cell cycle. A number of cancers are also associated with mutation of the CDK genes or CDK inhibitor genes. The levels of CDKs oscillate during the cell cycle and they are activated by phosphorylation on a specific threonine residue by CDK-activating kinase. The apigenin analogue *flavopiridol* is a potent CDK inhibitor which explains its antiproliferative activity towards some tumour cell lines in which cell cycle progression is blocked in both G_1 and G_2 phases. Flavopiridol, unlike apigenin, is planar and the aromatic portion of flavopiridol has been shown to bind to the hydrophobic adenine-binding pocket of the ATP site of CDK2 /86/.

The **$p34^{cdcz}$ kinase** complex promotes chromosome condensation, cytoskeletal reorganization and nuclear envelope breakdown that occurs in the G_2 to M phase of the cell cycle. Apigenin induces a reversible G_2/M arrest in colon carcinoma cell lines which is associated with $p34^{cdcz}$ kinase inhibition /87/.

3.2.d Inhibition of DNA synthesis enzymes

Resveratrol triggers partial arrest of G_1/S transition in prostate cancer cell lines and induces apoptosis /88/. The mechanisms for this include inhibition of **ribonucleotide reductase** /89/ and DNA polymerase or topoisomerase II /90/. Ribonucleotide reductase is a rate limiting enzyme for DNA synthesis as it catalyses the reduction of ribonucleotides to deoxyribonucleotides, the precursors for DNA synthesis.

3.3 Tumour development stage: Induction of tumour cell death (apoptosis) by polyphenolics

Chemoprevention by flavonoids or polyphenolics could also result from tumour cell death (apoptosis) caused by the cytotoxic effect of flavonoids/polyphenolics.

3.3.a Topoisomerase mediated apoptosis

DNA topoisomerases play a role in DNA replication, transcription, recombination, integration and transposition by introducing transient breaks in linear DNA sequences. Some polyphenolics inhibit DNA topoisomerase which results in DNA fragmentation /66/. *Genistein* is more planar than *myricetin* and stabilises the transient DNA:DNA topoisomerase complex /90/ while myricetin prevents enzymatic turnover /68/. The ATP binding domain of topoisomerase II may serve as the binding site for genistein, myricetin and quercetin resulting in the inhibition of the ATPase component of the topoisomerization reaction. ATP supplies the energy for returning topoisomerase II to its original conformation after it has released its product so that it can catalyse another supercoiling reaction. The flavonoids myricetin, quercetin, fisetin and kaempferol were more effective than genistein at inhibiting topoisomerase I relaxing and topoisomerase II unknotting activities. Morin, phloretin and genistein were also

effective as inhibitors /68/. Topoisomerase II inhibition by flavonoids induces cell cycle G_2/M arrest in murine fibroblasts but not in p53 knockout fibroblasts, suggesting that p53 is also involved in G_2/M arrest /91/. However genistein-induced tumour cell apoptosis was found in tumour cells (e.g. H460 non-small cell lung cancer) containing mutated inactive p53, indicating the existence of p53-independent pathways /92/. Genistein also induced apoptosis and G_2/M arrest in human breast carcinoma cells that possessed mutant p53 and lacked oestrogen receptor /73/.

The liver cancer-preventive effects of the herbal medicine Shosaiko-to (TJ-9) may partly be attributed to the flavonoids, baicalein, baicalin and wogonin. *Baicalein* suppressed the proliferation of human hepatocellular carcinoma cells and inhibited topoisomerase II. However, the mode of cell death depended on the cell line, which suggested that topoisomerase inhibition was not sufficient to cause apoptosis /93/. The *genistein*-induced topoisomerase II-mediated DNA cleavage in colon cancer cells was associated with a cell cycle arrest at the G_2/M phase. Aclarubicin, a topoisomerase II antagonist, prevented DNA cleavage but not apoptosis, suggesting another apoptosis mechanism was involved /94/.

3.3.b Mitochondrial toxin mediated apoptosis

The comparative potency of *flavonoids* (60 μM) at inducing apoptosis in HL-60 leukemic cells in 12 h was apigenin > quercetin > myricetin > kaempferol >> genistein or epigallocatechin, but not epicatechin-3-gallate or epigallocatechin-3-gallate /95/. However, the DNA fragmentation observed at 6-12 h was associated with DNA ladders and poly-(ADP-ribose) polymerase fragmentation and occurred after caspase-3 and -9 activation, a characteristic of apoptosis. Mitochondrial toxicity included a loss of membrane potential, cytochrome *c* release and ROS formation /95/. This suggested that the apoptosis was initiated by flavonoid-induced **mitochondrial toxicity** /65/.

Research on apoptosis has recently shifted from the paradigm that the nucleus controls cell death to the idea that mitochondria are the life/death regulator in apoptosis and necrosis. **Mitochondria** play the role as the central executioner of apoptosis by releasing the intermembrane apoptosis inducing factor (AIF), cytochrome *c* and caspases (caspase-2, -3 and -9). This occurs as a result of toxin-inducing

membrane permeabilization which often includes the opening of a **permeability transition pore complex** (MPT) that interacts with pro- and anti-apoptotic members of the Bcl-2 family localized in the mitochondrial membranes. Toxins that induce MPT include mitochondrial toxins, e.g. rotenone /96/, antimycin A and oligomycin /97/, which dissipate the inner transmembrane potential, cause MPT, form ROS and induce mitochondrial swelling in the cell, resulting in apoptosis.

Cytochrome *c* upon release from mitochondria forms part of and activates the so-called apoptosome by interacting with Apaf-1, mitochondrial heat shock proteins and procaspase-9. The caspases are a class of cysteine proteases which when activated cause the execution phase of apoptosis. The activated apoptosome proteolytically activates procaspase-3 which initiates specific caspase cascades resulting in the apoptotic repackaging of cellular proteins. The release of cytochrome *c* also interrupts mitochondrial electron transport, which causes ROS formation with deleterious consequences for cellular energy, redox homeostasis and mitochondrial biogenesis. The released AIF translocates from the mitochondria to the nucleus and induces DNA loss, chromatin condensation and digestion (nuclear apoptosis). Cell death may therefore be triggered by the loss of proteins from the mitochondria and their ectopic presence in extramitochondrial compartments /98/. Receptor-mediated apoptosis, however, involves a distinct caspase-8 cascade that does not involve mitochondrial toxicity /99/.

Ascites tumour cell growth inhibition and cytotoxicity induced by NDGA was also associated with the inhibition of mitochondrial respiration at the NADH dehydrogenase-ubiquinone level (complex I) resulting in decreased cellular ATP levels /100/.

Capsaicin-induced inhibition of the growth of HL-60 or melanoma cells, however, was attributed to an inhibition of the plasma membrane NADH oxidase /101/. *Quercetin* and other flavonoids induced ascites tumour cell growth inhibition, and cytotoxicity was associated with an inhibition of lactate formation, whereas galangin increased lactate formation. This could indicate that quercetin inhibits mitochondrial H^+-ATPase whereas galangin uncouples oxidative phosphorylation /102/.

Flavopiridol, a flavone analogue widely used in traditional medicine, is now in phase II clinical trials as it causes apoptosis in human chronic lymphocytic leukemia cells. The cytotoxic mechanism involves the activation of caspase-3 which cleaves poly-(ADP-ribose)

polymerase /103/ but it is not yet known whether the cytotoxic mechanism involves mitochondrial toxicity.

The cytotoxic efficacy of dietary *polyphenols* and *flavonoids* towards isolated rat hepatocytes has been correlated with mitochondrial membrane potential measurements, shown in Table 1. Surprisingly, the cytotoxicity of these dietary polyphenol/flavonoid antioxidants varied at least 100-fold, with the most toxic being flavonoids containing a B ring without hydroxyl groups, e.g. galangin, chrysin and biochanin A. The cytotoxicity of *galangin/chrysin* can be attributed to an early collapse of mitochondrial membrane potential (Table 1) which was likely due to an inhibition of oxidative phosphorylation causing cytotoxicity induced by a mitochondrial permeability transition (shown for pinocembrin /104/). Galangin also inhibits tumour cell aerobic glycolysis /102/.

Galangin, *chrysin* and *pinocembrin* are the major flavonoids in honey and propolis (bee glue) with a concentration reported of 30 mg/kg in honey and 97 g/l in propolis, with CAPE at a lower concentration /105/. Galangin has recently been promoted by several investigators as a promising candidate for *in vivo* chemoprevention as it is an antioxidant which induces tumour cell apoptosis/proliferation (Table 1) /102/ and prevents *in vivo* benzo(*a*)pyrene or *N*-methyl-N-nitrosourea-induced micronuclei in reticulocytes of mice /106,107/. This has been attributed to the efficacy of galangin at inhibiting CYP1A1 activity and induction due to its AhR antagonist activity /108/ and its effectiveness as an inducer of the phase II enzyme quinone reductase /14/. The scavenging of genotoxic alkyl radicals or diazonium ions by galangin may also contribute /107/. However, in view of the marked cellular toxicity of galangin (Table 1), there could be safety concerns involved in the ingestion of high doses of propolis or galangin supplements.

The polyphenolics *NDGA*, *capsaicin* and *resveratrol* were also cytotoxic towards isolated hepatocytes in this order of cytotoxicity. Cytotoxicity was also preceded by an early collapse of the mitochondrial membrane potential. Because the toxic molecular mechanisms for collapsing the membrane potential can be caused in different ways by inhibitors of mitochondrial respiration or oxidative phosphorylation, the phenolics such as *rotenone* (from the roots of several leguminous plants) and pentachlorophenol are included for comparison, as rotenone is a specific mitochondrial NADH dehydrogenase

TABLE 1

Hepatocyte LD_{50} (2 h) and mitochondrial susceptibility towards dietary plant polyphenolics

COMPOUNDS	SOURCE (major)	CLASS	Tumor ED_{50} μM HeLa Cells[a] 3 d	Hepat. LD_{50} μM 2 h[b]	% Mitoch. Membrane Potential Ψ[b] 1 h	log P[c]	PTA[d]	Isolated Mitochondria NADH Oxidase ED_{50} μM[e]	F_0F_1 ATPase ED_{50} μM[f]	UOP[g] max. state 4 μM
MITOCH. TOXIN										
Oligomycin	fungi	ATPase inhibitor		2 ± 0.2	22 ± 2				5	
Rotenone	derris root, insecticide	NADH dh. inhibitor		50 ± 5	29 ± 5			< 1		
Pentachlorophenol	wood preservative	uncoupler		50 ± 4	27 ± 3					50
DIETARY POLYPHENOLIC										
Galangin	propolis, honey, HM[h]	flavonol	23	105 ± 11	31 ± 3	2.86	planar			
Chrysin	propolis, honey, HM	flavone	221	110 ± 12	17 ± 2	2.67	planar	250		500
NDGA	HM	bicatechol		150 ± 15	24 ± 3	1.76				17[i]
Biochanin A	soy, clover, HM	isoflavone		175 ± 16	33 ± 4	3.39	planar		65	50
Capsaicin	hot chili peppers, HM	methoxy- phenol		400 ± 35	7 ± 1			< 163[j]		
Resveratrol	red wine, HM	trihydroxy-stilbene		470 ± 39	18 ± 2	1.87			19	
Phloretin	apples	dihydroxy-chalcone		500 ± 51	100	1.53	planar[k]		87	
Fisetin	wine, HM	flavonol	35	575 ± 62	68 ± 7	2.2	planar	15		300

cont.

Table 1 cont.

DIETARY POLYPHENOLIC	SOURCE (major)	CLASS	Tumor ED_{50} µM HeLa Cell[a] 3 d	Hepat. LD_{50} µM 2 d[b]	% Vis.h. Membrane Potential[b] 1 h	log P[c]	PTA[d]	Isolated Mitochondria NADH Oxidase ED_{50} µM[e]	F_0F_1 ATPase ED_{50} µM[f]	UOP[g] max. size 4 µM
Luteolin	millet, bell pepper, celery, HM	flavone	14	575 ± 58	68 ± 7	2.05	16.3°	48		
Baicalein	HM	flavone		600 ± 61	81 ± 7	1.31		77		100
Quercetin	onions, HM	flavonol	56	620 ± 66	100	2.26	planar	145	50	300
Myricetin	tea, wine, cranberry, HM	flavonol	60	640 ± 68	100	1.71	-27°	35		
Apigenin	parsley, celery, HM	flavone	> 50[h]	710 ± 72	46 ± 6	2.62	16.5°	920	105	500
Naringenin	grapefruit	flavanone		740 ± 77	6 ± 1	2.05	-42.7°			
Hesperetin	oranges	flavanone	26	750 ± 78	6 ± 1		-42°			
Eriodictyol	oranges, HM	flavanone	53	1,000±112	36 ± 4	1.55				
Kaempferol	tea, kale, Ginkgo, HM	flavonol	30	1,000±18	98	2.72			55	
Genistein	tofu, soy milk	isoflavone		1,500±161	28 ± 3	2.58		365	55	150
Ellagic Acid	berries, nuts	OH benzoate		> 1,500	100					
Daidzein	soy milk	isoflavone		> 2,000	100					> 500
Morin	yellow Brazil wood	flavonol	200	> 2,000	100	0.66	-38°	430	60	
Taxifolin	wine, HM	flavanonol	161	> 2,000	100	0.25	-27°	173		

Caffeic acid	coffee, honey	OH cinnamate		> 7,000	100	1.24			
Epicatechin	tea	flavan-3-ol	198	> 9 000		0.04			
Catechin	tea, chocolate	flavan-3-ol	>344	> 10,000	95	0.08	35.6[e]	1800	
Chlorogenic acid	coffee	OH cinnamate		> 10,000	100	0.60			
Ferulic acid	beer, tomato, rice	OH cinnamate		> 10,000	100				
Gallic acid	rhubarb, tea	OH benzoate		> 10,000	100				
Curcumin	turmeric (curry), HM	β diketone: OH cinnamate		10,000	100				40

[a] LD_{50} 3 days for HeLa tumour cells /129/.

[b] The LD_{50} (2 h) was determined for isolated rat hepatocytes using trypan blue exclusion as a measure of cell viability /130/. The hepatocyte mitochondrial membrane potential ($\Delta\Psi$) was determined with rhodamine 3 as described /130/.

[c] The partition coefficient (log P) was measured spectrophotometrically between MOPS buffer (3-[N-morpholino]propanesulfonic acid) (0.1 M), pH 7.4 containing 1 mM diethelenetriaminopenta-acetic acid and 1-octanol by monitoring the change in the absorbance at λmax. Both solvents were mutually presaturated with the other one prior to partition coefficient measurements.

[d] Planarity torsion angle (PTA) /131/.

[e] Concentration (μM) that inhibits 50% NADH oxidase of beef heart submitochondrial particles /65/.

[f] Concentration (μM) that inhibits 50% rat liver F_0F_1-ATPase /111/.

[g] Uncoupling of oxidative phosphorylation (UOP) estimated concentration (μM) for maximum increased state 4 respiration of mitochondria from rat liver /112/, plant /114/.

[h] Herbal medicine (HM); [i] /111/; [j] /109/; [k] Phlorizin is planar /132/; [l] /95/.

inhibitor whereas pentachlorophenol uncouples oxidative phosphorylation. Oligomycin is included as it is commonly used as an inhibitor of oxidative phosphorylation. The collapse of the hepatocyte mitochondrial membrane potential induced by *capsaicin* (Table 1) is best explained by the inhibition of NADH-ubiquinone reductase observed when isolated mitochondria were incubated with capsaicin /22,109/, i.e. like *rotenone*. *NDGA* was also toxic to isolated heart mitochondria, likely due to its *o*-quinone autoxidation product, as glutathione (GSH) prevented the NDGA induced decrease in mitochondrial GSH and inhibition of succinate cytochrome *c* reductase /100,110/, i.e like antimycin. It is not known whether NDGA toxicity towards hepatocytes involves metabolic oxidation to the *o*-quinone but hepatocyte respiration was inhibited by NDGA (results not shown).

The collapse of the hepatocyte mitochondrial membrane potential induced by *resveratrol* (Table 1) is best explained by the inhibition of the **H^+-transporting ATPase** (presumably by binding one of the F_0 or F_1 subunits). Oligomycin, on the other hand, inhibits phosphorylation by binding the oligomycin-sensitive conferring protein (OSCP) of the δ subunit that forms part of the ATPase stator stalk. The ranking potency (IC_{50} in parentheses) found for inhibiting rat liver or brain F_0F_1-ATPase was as follows: *piceatannol* (8 μM) > *resveratrol* (19 μM), epigallocatechin gallate (17 μM) > curcumin (45 μM) > genistein, biochanin A, quercetin, kaempferol, morin (55-65 μM) > phloretin, apigenin, daidzein (100 μM), with little effect of catechin /111/.

By contrast, although the isoflavones *biochanin A* and *genistein* collapsed the mitochondrial membrane potential well before cytotoxicity ensued (Table 1), they also increased hepatocyte respiration when added to hepatocytes (results not shown). This can be attributed to mitochondrial uncoupling because, as shown in Table 1, isolated rat liver mitochondria were readily uncoupled (increased state 4 respiration) by the addition of biochanin A or genistein /112/, i.e. like pentachlorophenol. Another isoflavone, *daidzein,* was not cytotoxic and did not collapse the mitochondrial membrane potential even at 2 mM. State 3 respiration of rat liver mitochondria was also inhibited by biochanin A or genistein, whereas daidzein was much less effective /112/. It is therefore likely that the isoflavones biochanin A and genistein act as proton ionophores which requires that they are lipid soluble and have weakly acidic phenolic hydroxyl groups with a pKa

between 4.5 and 6.5, i.e. like pentachlorophenol or dinitrophenol. As shown in Table 1, the partition coefficient of biochanin A is much higher than genistein (presumably because of the 4′-OCH_3 group) and could explain the higher cytotoxicity of biochanin A. These molecules may thus shuttle protons across the mitochondrial membrane and destroy the proton gradient required for the chemi-osmotic hypothesis. Alternatively, they may bind to membrane protein factors that are required for oxidative phosphorylation, e.g. resulting in the activation of H^+-transporting ATPase (F_0F_1-ATPase) and inhibition of ATP synthesis.

At higher concentrations the flavonoids *luteolin*, *baicalein*, *fisetin*, *naringenin*, *hesperetin* and *apigenin* also collapsed the mitochondrial potential before cytotoxicity ensued (Table 1), which suggests that flavonoids containing phenol B rings uncouple or inhibit oxidative phosphorylation more effectively than flavonoids containing catechol B rings. Luteolin and 4,4′-dihydroxychalcone were the most effective flavonoids found for inhibiting the growth of human leukemic cells and this was attributed to their efficacy at inducing ATP depletion /113/. Isolated plant mitochondria were also uncoupled by baicalein (100 μM), luteolin (200 μM), fisetin (200 μM) or apigenin (500 μM) /114/. *Eriodictyol*, the catechol B ring-containing P450 metabolite of naringenin and hesperetin /115/, was less cytotoxic than naringenin or hesperetin, suggesting that P450 metabolic activation was not required. Similarly, *kaempferol* and *apigenin*, the P450 metabolites respectively of galangin and chrysin /115/, were much less cytotoxic than galangin or chrysin. The low cytotoxicity of kaempferol and taxifolin may be partly due to their 3-OH group.

The most cytotoxic flavonoids found (galangin, biochanin A, phloretin, luteolin, fisetin, quercetin, apigenin) were planar (or had a torsion angle < 17°), lipophilic and collapsed the hepatocyte mitochondrial membrane potential early on, well before hepatocyte lysis occurred. In general, these polyphenolics were also shown to inhibit the functional activities of isolated mitochondria (Table 1). On the other hand, the least cytotoxic flavonoids (catechin, taxifolin, morin) were nonplanar (torsion angle > 25°), more hydrophilic (lower partition coefficients) and were ineffective at collapsing the hepatocyte mitochondrial membrane potential. Hepatocytes were also more resistant to the flavonoids than cultured tumour cells, although the hepatocyte incubation time used was much shorter (Table 1). How-

ever, even though the cell types and cytotoxic end point were different (hepatocyte lysis versus tumour cell growth for a variety of tumour cell lines), there was a surprising similarity in the relative cytotoxic order of efficacy for the different flavonoids/polyphenolics.

The dietary hydroxycinnamates, such as *curcumin*, *ferulic acid*, *chlorogenic acid* and *caffeic acid*, were also found to be non-toxic at the highest concentrations used. As discussed above, however, these polyphenolics are chemopreventive agents which are major constituents of curry and the beverages tea, wine, beer and coffee. The non-toxic polyphenolics were also less effective at inhibiting the functional activities of isolated mitochondria (Table 1). Hepatocyte cytotoxicity by flavonoids and other polyphenolics therefore correlates best with their efficacy at decreasing the mitochondrial membrane potential. This suggests that mitochondrial toxicity is a major cause of cell death induced by most polyphenols.

3.3.c Oxidative stress induced apoptosis

The rate of autoxidation of flavonoids at pH 7.4 to form H_2O_2 decreases in the order *myricetin* > *baicalein* > *quercetin* > epicatechin > catechin > fisetin, 7,8-dihydroxyflavone, indicating that pyrogallol-type flavonoids generate more H_2O_2 than the catechol-type and are more effective antioxidants /116/. *Myricetin*, *quercetin* and *quercetagetin* incubated with isolated heart mitochondria also inhibited the mitochondrial respiratory chain (at the NADH-ubiquinone reductase site) and induced cyanide resistant respiration, likely as a result of autoxidation /117/ and redox cycling. Furthermore, hepatocyte cyanide resistant respiration was markedly increased by *myricetin* and *baicalein* (results not shown). However, as shown in Table 1, hepatocyte cytotoxicity induced by flavonoids did not correlate with the published autoxidation rate of the various flavonoids /116/ and surprisingly the mitochondrial membrane potential was more resistant to collapse before hepatocyte lysis occurred (Table 1). Cytotoxicity induced by these ROS-forming flavonoids may not therefore result from the collapse of the mitochondrial membrane potential.

Oxidative stress or ROS-induced or -modulated apoptosis has recently been reviewed /118/. ROS-induced apoptosis is characterised by mitochondrial cytochrome *c* release and activation of caspase-3 and -9 /119/. Tumour cell apoptosis induced by the flavonoids apigenin, quercetin, myricetin /96/ or curcumin /120/ has been suggested to be

ROS mediated, although the source of the ROS is not known. The prevention of hydrogen peroxide-induced apoptosis of human erythroleukemia K562 cells by resveratrol was attributed to inhibition of LOX and COX /45/.

As discussed above, the polyphenolics readily inactivate arachidonate metabolising enzymes resulting in increased arachidonate levels (and decreased HETE levels if 5-lipoxygenase is inhibited). Arachidonate or phospholipase A_2 activators (melitten) trigger massive apoptosis in human prostate cancer cells /121/ and human retinoblastoma Y79 cells /122/. The cellular mechanism seems to involve aldehyde oxidative carbonyl stress mediated by aldehydic decomposition products of lipid peroxidation. These reactive carbonyls cause GSH depletion and loss of mitochondrial membrane potential. Caspase-3 and -6 activation, resulting in poly-(ADP-ribose)-polymerase inhibition and lamin B cleavage respectively, then ensues, leading to characteristic apoptosis morphology /122/.

3.3.d Other mechanisms for inducing apoptosis

The induction of apoptosis in breast cancer cells by genistein was associated with upregulation of **Bax** and **p21** and downregulation of **Bcl-2** and **p53** which may also initiate apoptosis /123/. Elevating intracellular **cAMP** to high levels with cAMP analogues, adenylate cyclase activators (forskolin) or phosphodiesterase inhibitors (isobutyl methyl xanthine) also induces apoptosis in primary granulosa cells /124/. As described above, polyphenolics also increase intracellular cAMP to high cytotoxic levels by inhibiting phosphodiesterase /74-77/. The apoptosis mechanism is not known but may involve increased intracellular Ca^{2+} (activates endonuclease) and wild-type p53 overexpression /124/.

4. CONCLUSIONS: SAFETY OF POLYPHENOLIC/FLAVONOID SUPPLEMENT THERAPY

Flavonoids are generally regarded as having very low toxicity and are therefore often prescribed, usually for disorders of the peripheral circulation, although their therapeutic usefulness is far from conclusive. However, the therapeutic use of 1-1.5 g/day of the flavonoid drug cianidanol (catechin) has been associated with various side

effects, including acute renal failure, hemolytic anemia, thrombocytopenia, hepatitis, fever and skin reactions /125/. Furthermore, 44 cases of proven or suspected cianidanol-induced hemolysis were reported during the period 1979-1987. These adverse reactions were likely immune mediated /123/. No carcinogenicity was evident in rats fed 5% quercetin in the diet for two years /126/. However, the National Toxicology Program found an increased incidence of renal tubular cell carcinoma (mostly adenomas) in male rats fed 40,000 ppm quercetin /127/. The potential health impacts of excessive flavonoid intake have recently been reviewed /128/. It should be noted that the polyphenolics commonly found in beverages, fruits and vegetables were not toxic or had low toxicity in the hepatocyte "accelerated cytotoxicity mechanism screening technique" shown in Table 1. However, polyphenolic supplements or herbal medicine are likely to contain more of the cytotoxic polyphenolics identified in Table 1 and at much higher doses than would be present in the average daily intake of fruits and vegetables. Some of the supplements and herbal medicine could therefore have adverse health effects if taken on a long-term basis. On the other hand, the non-toxic polyphenolics/flavonoids identified in Table 1 with good cancer chemopreventive activity could be selected as appropriate candidates for further development and preclinical or clinical trials.

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