3. Bioactive components in foods

3.1. Anticarcinogenic effects of flavonoids

Margaret M. Manson
Cancer Biomarkers and Prevention Group, University of Leicester, Leicester, UK

Introduction

Many thousands of different flavonoids are found in plant species, with major dietary sources including fruit, vegetables, tea, chocolate and soy. Total daily intake can range from 50–800 mg. Those flavonoids which have been studied in most detail exhibit many properties which could be protective against heart disease, ageing and cancer. These polyphenolic compounds are classified, according to structure, as flavonols (quercetin, kaempferol), flavones (luteolin, apigenin), flavanones (myricetin, naringin, hesperetin), isoflavones (genistein, daidzein), anthocyanins (cyanidin, pelargonidin, petunidin), catechins (epicatechin, epicatechin-3-gallate) and chalones (xanthohumol). Amongst their health-promoting properties are antioxidant, antiviral, anti-allergic, anti-inflammatory, and anticancer activities. Such chemopreventive agents can be effective at different stages of the carcinogenic process, both by blocking initiation and by suppressing the later stages involving promotion, progression, angiogenesis, invasion and metastasis. Several recent reviews have summarised the potential chemopreventive mechanisms for a number of flavonoids [1–4]. Some recent data for the well-studied flavonoids apigenin, epigallocatechin gallate (EGCG), genistein, resveratrol, quercetin, the chalone, xanthohumol and the novel flavonol, tricin, are summarised here.

Blocking mechanisms

Possible ways in which initiation of carcinogenesis can be blocked include prevention of reactive oxygen species attack on DNA, altered metabolism of procarcinogens in favour of conjugation and excretion of reactive metabolites, inhibition of carcinogen uptake into cells and enhanced DNA repair.

Many flavonoids possess antioxidant or free radical scavenging potential, which varies depending on the hydroxylation status of the benzene rings. Examples include quercetin (a flavonol in vegetables, apples and onions), xanthohumol (a chalone in hops and beer) and genistein (an isoflavone in soy). An early study by Duthie et al [5] reported that quercetin protected human lymphocytes from hydrogen peroxide-induced DNA damage. Similar findings were reported by Wilms et al [6], who also found that quercetin protected human lymphocyte DNA from bulky adduct formation following treatment with benzo[a]pyrene. Also in this study, volunteers consumed quercetin-rich blueberry/apple juice for 4 weeks, which led to a significant increase in antioxidant capacity of plasma.
Flavonoids can interact with the aryl hydrocarbon receptor (AhR) as agonists or antagonists, depending on structure and cell context. Such interactions influence the expression of drug metabolising enzymes such as cytochromes P450 [7]. They have also been shown to influence the multi-drug resistance phenotype acquired by many tumour cells. Quercetin and silymarin were found to inhibit MRP1/4/5-mediated drug transport from intact erythrocytes with high affinity, in a manner which suggested that they interact at the substrate-binding sites. Such interactions might influence bioavailability of anti-cancer drugs in vivo and could be considered for combination therapies [8]. In another recent study [9], the flavonols, quercetin and kaempferol, reduced P-glycoprotein expression and function in multi-drug resistant human cervical carcinoma KB-IV cells, while the isoflavones, genistein and daidzein, modulated intracellular drug levels by inhibiting function, without affecting expression.

Xanthohumol possesses several useful properties to block carcinogenesis including modulation of enzymes involved in carcinogen metabolism and detoxification (inhibition of Cyp1A, induction of quinone reductase activity), scavenging of ROS, including hydroxyl and peroxyl radicals, along with inhibition of superoxide anion radical formation and nitric oxide production [10].

**Suppressing mechanisms**

Mechanisms which result in suppression, or even better, elimination of tumour cells, include growth inhibition by induction of cell cycle arrest or apoptosis. A significant number of flavonoids, alone and in combination, have been shown to induce G2/M arrest in SW480 and CaCo2 human colon carcinoma cells [11]. Tricin, a novel flavonol in rice bran, was shown to inhibit the growth of breast tumour cells, causing G2/M arrest, but not apoptosis [12]. In a subsequent study by the same group [13], tricin decreased the number of intestinal adenomas in Apcmin mice by 33%, with inhibition of COX-1 and COX-2 activity. The latter led to a 34% reduction of PGE2 levels in small intestinal mucosa and blood. Xanthohumol was also found to inhibit COX-1 and COX-2 activities, and to be antiestrogenic [10]. The inhibitory effect of other flavonoids on COX-2 expression and activity has been reviewed by O’Leary et al. [14]. During later stages of carcinogenesis additional useful mechanisms include inhibition of angiogenesis, invasion and metastasis.

A range of tumour suppressing activities is shown for apigenin (Table 3.1.) and quercetin (Table 3.2.). Resveratrol, genistein and EGCG (reviewed in [2]) have a number of effects in common with those detailed here for apigenin and quercetin, namely inhibition of signalling through the EGFR family, NF-kB, and pAkt, induction of cell cycle arrest involving a decrease in cyclin D1 and phosphorylation of Rb, accompanied by upregulation of p21 and p27, and induction of apoptosis involving release of cytochrome c from mitochondria, activation of caspases 3 and 9 and downregulation of Bcl family members. However, depending on cell type and experimental conditions, flavonoids can both up- and down-regulate key molecules, including JNK, AP-1, p21, p27, cdc2, cyclin D1, p53, and PI3K.
### Table 3.1. Chemopreventive suppressing effects of apigenin

<table>
<thead>
<tr>
<th>Tissue/cell type</th>
<th>Mechanism</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat T cells</td>
<td>↓ chymotrypsin-like activity of 20S and 26S proteosomes; ↑ Bax; ↑ IκBα</td>
<td>Apoptosis</td>
<td>[15]</td>
</tr>
<tr>
<td>PWR-1E, LNCaP, PC-3, DU145 prostate tumour cells</td>
<td>↑ ROS; ↓ Bcl2; ↑ cleavage of caspases 3,7,8,9 and clAP-2</td>
<td>Apoptosis</td>
<td>[16]</td>
</tr>
<tr>
<td>Breast and prostate cancer cells</td>
<td>↓ fatty acid synthase activity</td>
<td>Growth inhibition and apoptosis</td>
<td>[17]</td>
</tr>
<tr>
<td>NUB-7, LAN-5, SK-N-BE neuroblastoma cells</td>
<td>↑ p53; ↑ p21; ↑ Bax; ↑ cleavage of caspase 3</td>
<td>Apoptosis (p53-dependent)</td>
<td>[18]</td>
</tr>
<tr>
<td>Breast cancer cells</td>
<td>↓ Her2; ↓ Akt; ↑ cleavage of caspase 3; ↑ DFF-45 cleavage; ↓ cyclin D1/D3 &amp; cdk4; ↑ p27</td>
<td>Apoptosis; ↓ colony formation</td>
<td>[19]</td>
</tr>
<tr>
<td>PC-3 prostate cancer cells</td>
<td>↓ p50; ↓ p65; ↓ NF-κB-DNA-binding; ↓ IκBα degradation and phosphorylation; ↓ IκKα activity; ↓ TNFα activation of NF-κB</td>
<td>Apoptosis; Bcl2, cyclin D1, COX-2, MMP9, NOS-2, VEGF</td>
<td>[20]</td>
</tr>
<tr>
<td>DU145 prostate cancer cells</td>
<td>↓ cyclin D1/2 &amp; E; ↓ CDK2/4/6; ↑ p21, p27, p16, p18; altered Bax:Bcl2 ratio; ↑ cyt c release; ↑ APAF-1; ↑ IκBα; ↓ NF-κB p50 &amp; p65</td>
<td>Growth inhibition, G1 arrest, apoptosis</td>
<td>[21]</td>
</tr>
<tr>
<td>HER2-overexpressing breast tumor cells</td>
<td>↓ PI3K &amp; Akt activity; ↓ PI3K-HER2 docking; ↓ HER2/neu phosphorylation; ↑ HER2 degradation</td>
<td>Apoptosis</td>
<td>[22]</td>
</tr>
<tr>
<td>A549 lung cancer cells in vitro and in nude mice</td>
<td>↓ Akt; ↓ p70S6K1; ↓ HIF-1α; ↓ VEGF</td>
<td>Growth and angiogenesis inhibition</td>
<td>[23]</td>
</tr>
<tr>
<td>OVCAR-3 and A2780/CP70 ovarian cancer cells</td>
<td>↓ Akt; ↓ p70S6K1; ↓ HIF-1α; ↓ VEGF; ↑ p53; ↓ HDM2</td>
<td>Angiogenesis inhibition</td>
<td>[24]</td>
</tr>
<tr>
<td>HCT 116 colon carcinoma cells</td>
<td>↑ ERK &amp; p38 phosphorylation and activity</td>
<td>↑ phosphorylation of Elk &amp; ATF2</td>
<td>[25]</td>
</tr>
<tr>
<td>HCT 116, HT-29 colon cancer cells</td>
<td>↓ CK2; ↓ TNFα-induced NF-κB activation</td>
<td>Apoptosis;</td>
<td>[26]</td>
</tr>
</tbody>
</table>
Table 3.2. Chemopreventive suppressing effects of quercetin

<table>
<thead>
<tr>
<th>Tissue/cell type</th>
<th>Mechanism</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60 human myeloid leukemia cells</td>
<td>↓Bax; ↑phosphoBcl2; ↓Pgp</td>
<td>Apoptosis</td>
<td>[27]</td>
</tr>
<tr>
<td>Jurkat T cells</td>
<td>Inhibiting chymotrypsin-like activity of 20S and 26S proteosomes; ↑Bax; ↑IκBα</td>
<td>Apoptosis</td>
<td>[15]</td>
</tr>
<tr>
<td>Breast and prostate cancer cells</td>
<td>↓fatty acid synthase activity</td>
<td>Growth inhibition and apoptosis</td>
<td>[17]</td>
</tr>
<tr>
<td>Colonic aberrant crypt foci</td>
<td>↑Bax; ↓Bcl2; ↑cleavage of caspase 9</td>
<td>Suppression by 4-fold; apoptosis ↑3-fold</td>
<td>[28]</td>
</tr>
<tr>
<td>MiaPaCa pancreatic tumour cells</td>
<td>↓phosphoFAK</td>
<td>Decreased invasion</td>
<td>[29]</td>
</tr>
<tr>
<td>MCF7 breast tumour cells</td>
<td>↑PTEN; ↑p27; ↓Akt</td>
<td>Growth inhibition and apoptosis</td>
<td>[30]</td>
</tr>
<tr>
<td>LNCaP, PC3 prostate tumour cells</td>
<td>↓Sp1 interaction with AR; ↑c-jun</td>
<td>Inhibition of androgen receptor activity</td>
<td>[31]</td>
</tr>
<tr>
<td>HT29, SW480 colon cancer cells</td>
<td>↓ErbB2/3; ↓Bcl2; ↓phosphoAkt</td>
<td>Growth inhibition and apoptosis</td>
<td>[32]</td>
</tr>
<tr>
<td>SW480 colon cancer cells</td>
<td>↓β-catenin/Tcf transcriptional activity</td>
<td>↓c-myc</td>
<td>[33]</td>
</tr>
<tr>
<td>A549, H1299 human lung carcinoma cells</td>
<td>↑cyclin B1; ↑phospho cdc2; ↑survivin; ↑p53; ↑p21</td>
<td>Growth inhibition; G2/M arrest</td>
<td>[34]</td>
</tr>
<tr>
<td>PC3 prostate cancer cells</td>
<td>↓HSP70</td>
<td>Apoptosis</td>
<td>[35]</td>
</tr>
</tbody>
</table>

One recent report by Fenton and Hord [36] has suggested a novel chemopreventive mechanism for flavonoids. In normal colon, epithelial cells migrate to the apex of the crypt, a process involving the APC gene, which is often mutated in colon cancer. These authors reported that apigenin, epicatechin, naringin and hesperidin induced a greater migratory response in APC^min/+ cells compared to those expressing wild type APC. Such flavonoid-induced migration was dependent on matrix metalloproteinase activity.

During the carcinogenic process, both hypermethylation of the promoter regions of tumour suppressor genes and hypomethylation of oncogenes can occur, resulting in under- or over-expression. Both EGCG [37] and genistein [38] have been shown to reactivate a number of key genes, such as the cell cycle inhibitor p16 and the retinoic
acid receptor (RARβ), in several different cancer cell types. The mechanism proposed was through inhibition of DNA methyltransferase, which, in the case of EGCG, involved direct interaction with the enzyme.

Combined effects

There is now accumulating evidence for the additive, synergistic or antagonistic effects of combinations of more than one chemopreventive agent. For example Mertens-Talcott et al. [39], using MOLT-4 human leukaemia cells, showed that quercetin and ellagic acid acted synergistically in inducing apoptosis. Ellagic acid potentiated the inducing effect of quercetin on levels of p21 and phosphorylation of p53 at serine 15. Phosphorylation of JNK1/2 and p38 was increased by the combination, while quercetin alone only induced p38 phosphorylation. Neither the generation of ROS, nor quercetin stability were affected by ellagic acid. Combinations of flavonoids were found to have an inhibitory effect on the breast cancer resistance protein (ABCG2), suggesting the potential use of ‘flavonoid cocktails’ to reverse multi-drug resistance in treatment of this cancer [40].

Conclusions

Flavonoids, like other types of dietary chemopreventive agents, exhibit a wide range of potentially useful activities for cancer prevention. Their blocking activities include antioxidant effects, modulation of drug metabolising enzymes and multidrug resistant genes. Suppressing activities include inhibition of signalling pathways responsible for cell proliferation and survival, and induction of apoptosis, mainly through intrinsic pathways involving Bcl family members, mitochondrial membrane depolarisation, cytochrome c release and activation of caspases. They can also induce cell cycle arrest by modulating key components of cell cycle regulation, including cyclins, cyclin dependent kinases and inhibitors.

References


7. Zhang S, Qin CH, Safe SH. Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell-context. Env Health Persp 2003;111:1877–82.


3.2. Biomarkers of dietary polyphenol intake for studying diet-cancer relationships

Jakob Linseisen and Sabine Rohrmann
Division of Clinical Epidemiology, German Cancer Research Centre, Heidelberg, Germany

Introduction

The primary aim of analytical cancer epidemiology is to detect associations between exposure variables and disease endpoints, associations that are accompanied and supported by basic research findings enabling one to identify and understand insofar as possible the steps contained in the causal chain of disease development. Nutritional epidemiology deals with dietary factors that are difficult to assess. Long-term dietary habits are usually explored in epidemiological studies by use of food frequency questionnaires (FFQ). Dietary measurements tend to suffer, however, from imprecision, particularly concerning dietary components provided by only certain kinds of foods, and components for which bioavailability is low, such as various secondary plant products. The use of biomarkers for such compounds should overcome some of the methodological problems in nutritional epidemiology just referred to. The analytical data obtained are objective and more precise, measurement error being independent of that contained in the corresponding questionnaire data. The validity and reproducibility of nutritional biomarker measurements, however, needs to be demonstrated in advance of their use in epidemiological and etiological studies [1].

Polyphenols

Polyphenols are provided by plant-derived foods, including beverages. The basic flavonoid structure and various examples of them are given in Figure 3.1. Some of the polyphenols are found in a rather wide variety of foods (kaempferol contained in many vegetables, for example), whereas others are limited to only a few kinds of food (such as apigenin found in parsley and celery). Flavonoids are classified in several different sub-groups, these including the flavonols, flavones, flavanols, flavanones, isoflavones, lignans, anthocyanins and proanthocyanins; phenolic acids consist of two main subgroups, the hydroxybenzoic and the hydroxycinnamic acids.

Estimates of dietary intake estimations are hampered by incomplete or missing data in food composition tables. Even more problematic, is the fact that the polyphenols markedly differ from one another in their bioavailability and intestinal metabolism. Current evidence from bioavailability studies suggests that the bioavailability varies from about 0.5 to 43% (based on urinary excretion) of the dose administered, reaching plasma concentrations of 0–4 µmol/l at an intake of 50 mg aglycone equivalents [2]. Bioavailability is determined by different factors, the sugar moiety of the compound in question and its further metabolism by the gut microflora, for example. Isoflavones and gallic acid are polyphenols that are absorbed to the highest extent, followed
by the catechins, flavanones and quercetin glucosides, although the kinetics involved differ considerably. The proanthocyanidins, the galloylated tea catechins and the anthocyanins are much less available. Thus far, data on the phenolic acids are very limited. Flavonoids undergo extensive first-pass phase II metabolism in the intestinal epithelial cells and the liver, their being substrates for methylation, sulfation, and glucuronidation. For many of the polyphenols, their half-life time in the plasma is short, concentrations of them reaching baseline levels within 24 hours [3]. The steady-state levels of the plasma polyphenols should only be achievable through regular intake of the foods in question. The plasma concentrations present in free-living (fasting-status) subjects are even lower than the abovementioned range obtained after intervention. Measurement of the compounds found in urine samples, ideally 24-hour samples, is a promising approach, due mainly due to the higher concentrations of polyphenols — at least after extraction and enrichment — as compared with plasma samples. The polyphenol concentrations contained in biological specimens are estimated in most studies after hydrolysis of the glycosides involved, the aglycones being quantified.

Fig. 3.1. The basic structure of flavonoids and the chemical structure of selected polyphenols.
Laboratory techniques

Two recent reviews provide an overview of the analytical methods used to determine the structure and content of the flavonoids and phenolic acids contained in foods and in food-based matrices [4,5]. Although in principle these methods can be applied to human specimens, the clean-up procedures for this type of samples may be more complicated than required for many food systems.

Hydrolysis

In foods, the flavonoids are usually glycosylated and the phenolic acids are ester-bound. Hydrolysis (acidic or enzymatic) is frequently used to simplify the analytical procedure, the respective aglycones and acids being subsequently detected and quantified. In biological specimens, flavonoids mainly undergo modification by means of phase II enzymes leading to methylated, sulfated and glucuronidated compounds. Accordingly, frequent use is made of enzymic hydrolysis by means of sulfatase and glucuronidase unless a study aims at determining the exact metabolites. Phenolic acids undergo further metabolism and degradation, although a part of them is excreted unmodified.

Clean-up procedures

For human specimens, such as plasma, serum, or urine samples, solid phase extraction (SPE)-columns provide the most convenient solution for removing from a sample any matrix compounds that would disturb the analysis. Some techniques such as liquid chromatography-mass spectrometry (LC-MS), do require only minor sample-preparation steps.

Separation and detection systems

The two major separation techniques for the quantification of polyphenolics are HPLC and GC, combined with different detection systems (Table 3.3.), although the use of LC-MS is becoming increasingly common. For the flavonoids, HPLC has become the method of choice, and the phenolic acids are generally quantified by means of GC after derivatisation. Identification of compounds by means of mass fragmentation is used as a gold standard. However, a single mass-selective detector often fails to fulfil the requirements for sensitivity. Thus, new developments include the creation of HPLC-ESI-MS-MS systems and similarly coupled devices. For the structural characterization of compounds, mass spectrometric techniques need to be used.

The availability of antibodies for isoflavones and lignans has enabled the antibody-based assays with a high degree of sensitivity to be developed [6]. For the purpose of quantification, the fluorescence emitted is recorded by means of a plate reader, with the option of time-resolved measurement. Thus far, the scientific literature contains no report on use of the metabonomics techniques (NMR, LC-MS) to characterise clusters of polyphenolic compounds that can potentially be used as biomarkers.
Table 3.3. Principal techniques and detection systems used for the identification and quantification of flavonoids and phenolic acids in human specimens (metabonomics/NMR excluded)

<table>
<thead>
<tr>
<th>Separation</th>
<th>Detection system</th>
<th>Substances class technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>UV/VIS spectroscopy (Diode array detection)</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td></td>
<td>Mass spectrometry</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td></td>
<td>Electrochemical</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td></td>
<td>Fluorometric</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td>GC</td>
<td>Mass spectrometry</td>
<td>Phenolic acids</td>
</tr>
<tr>
<td>LC</td>
<td>Mass spectrometry</td>
<td>Flavonoids, Phenolic acids</td>
</tr>
<tr>
<td>Immunoassays</td>
<td>Time-resolved fluorescence</td>
<td>Isoflavones, lignans</td>
</tr>
</tbody>
</table>

HPLC — high-performance liquid chromatography; GC — gas chromatography; LC — liquid chromatography.

Bioavailability studies and other short-term interventional studies

A report was published very recently summarizing all scientific studies (n = 97) that has been conducted thus far on the bioavailability of polyphenols [7]. Most of the studies concerned only one or some few compounds within a given subclass of polyphenols. Kinetic data from the experiments in question are summarized in Table 3.4. (according to [7]). Relatively low plasma concentrations were obtained in each case, even after the administration of polyphenol preparations or polyphenol-rich food corresponding to 50 mg aglycone equivalents. Differences between the various compounds and of classes of polyphenol are striking nevertheless.

Table 3.4. Pharmacokinetic data from 97 studies concerning the bioavailability of polyphenols1 (according to Manach et al. [7])

<table>
<thead>
<tr>
<th></th>
<th>Tmax (h)</th>
<th>Cmax (µmol/l)</th>
<th>Urinary excretion2 (% of intake)</th>
<th>Elimination half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Range</td>
<td>Mean(SEM)</td>
<td>Range</td>
</tr>
<tr>
<td>Daidzin</td>
<td>6.3 (0.6)</td>
<td>4.0–9.0</td>
<td>1.92 (0.25)</td>
<td>0.36–3.14</td>
</tr>
<tr>
<td>Daidzein</td>
<td>4.9 (1.0)</td>
<td>3.0–6.6</td>
<td>1.57 (0.52)</td>
<td>0.76–3.00</td>
</tr>
<tr>
<td>Genistin</td>
<td>6.5 (0.6)</td>
<td>4.4–9.3</td>
<td>1.84 (0.27)</td>
<td>0.46–4.04</td>
</tr>
<tr>
<td>Genistein</td>
<td>4.1 (0.6)</td>
<td>3.0–5.2</td>
<td>2.56 (1.00)</td>
<td>1.26–4.50</td>
</tr>
<tr>
<td>Glycitin</td>
<td>5.0</td>
<td>1.88 (0.38)</td>
<td>1.50–2.26</td>
<td>42.9 (12.0)</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>5.5 (0.1)</td>
<td>5.4–5.8</td>
<td>0.46 (0.21)</td>
<td>0.21–0.87</td>
</tr>
</tbody>
</table>
Table 3.4. Pharmacokinetic data from 97 studies concerning the bioavailability of polyphenols (according to Manach et al. [7]) — cont.

<table>
<thead>
<tr>
<th></th>
<th>$T_{max}$ (h) Mean (SEM)</th>
<th>$C_{max}$ (µmol/l) Mean(SEM)</th>
<th>Urinary excretion (%) of intake Mean(SEM)</th>
<th>Elimination half-life (h) Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringin</td>
<td>5.0 (0.1)</td>
<td>0.50 (0.33)</td>
<td>8.8 (3.17)</td>
<td>2.1 (0.4)</td>
</tr>
<tr>
<td>Quercetingluc.</td>
<td>1.1 (0.3)</td>
<td>1.46 (0.45)</td>
<td>18.5 (5.7)</td>
<td>2.5 (0.4)</td>
</tr>
<tr>
<td>Rutin</td>
<td>6.5 (0.7)</td>
<td>0.20 (0.06)</td>
<td>19.9 (8.1)</td>
<td>19.9 (8.1)</td>
</tr>
<tr>
<td>(Epi)catechin</td>
<td>1.8 (0.1)</td>
<td>0.40 (0.09)</td>
<td>18.5 (5.7)</td>
<td>2.5 (0.4)</td>
</tr>
<tr>
<td>EGC</td>
<td>1.4 (0.1)</td>
<td>1.10 (0.40)</td>
<td>11.1 (3.5)</td>
<td>2.3 (0.2)</td>
</tr>
<tr>
<td>EGCG</td>
<td>2.3 (0.2)</td>
<td>0.12 (0.03)</td>
<td>0.06 (0.03)</td>
<td>3.5 (0.3)</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>1.6 (0.2)</td>
<td>4.00 (0.57)</td>
<td>37.7 (1.0)</td>
<td>1.3 (0.1)</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>1.0</td>
<td>0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1.4 (0.6)</td>
<td>0.96 (0.26)</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>2.0</td>
<td>0.03</td>
<td>27.6 (17.6)</td>
<td></td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>1.5 (0.4)</td>
<td>0.03 (0.02)</td>
<td>0.4 (0.3)</td>
<td></td>
</tr>
<tr>
<td>Proanthocyanidins</td>
<td>2.0</td>
<td>0.02 (0.01)</td>
<td>0.008–0.03</td>
<td></td>
</tr>
</tbody>
</table>

1 All the data were converted so as to correspond to a supply of 50 mg aglycone equivalent.
2 Usually represent 24-h urine samples.

$T_{max}$ — time to reach $C_{max}$; AUC — area under the plasma concentration-time curve; EGC — epigallocatechin; EGCG — epigallocatechin gallate.

A review of short-term intervention studies (n = 93) in which polyphenols were administered (either as isolated compounds or in the form of foods or food extracts) to human subjects was published recently [8]. The authors examined evidence relating to the effects of these polyphenols on biomarkers used to test various pathophysiological hypotheses in vivo.

Observational studies: cross-sectional studies and studies related to cancer

Various studies have dealt with the suitability of using fasting plasma or urinary concentrations of polyphenols (mainly flavonols, flavanones or isoflavones) as biomarkers of polyphenol intake [9–16]. The results of these studies as a whole suggest biomarker measurements of this sort to reflect in an adequate way the degree of short-term intake of the polyphenols that were investigated, although one study in particular failed to support this conclusion [12]. The magnitude of the variation in the plasma polyphenol concentrations found between free-living subjects following their usual diet habits was
found to be rather high (Table 3.5.), the intra-individual variation also being high [14]. The correlation coefficients between estimates of the dietary intake of polyphenols of the day before blood sampling took place and fasting plasma concentrations polyphenols were as high as 0.75, but it should be emphasized that the validity of such correlations may be limited by a lack of precision in the estimates of dietary polyphenol intake, and that the correlations are much lower when intake calculations are based on data obtained either three or seven days prior to blood sampling. Due to the short half-life time of most polyphenols, close to of steady-state plasma concentrations of them can only be achieved if the substance in question is consumed regularly, a precondition most likely to be fulfilled by compounds such as kaempferol that are widely distributed in plant foods, and if the bioavailability of the compound is not particularly low.

Table 3.5. Concentrations (nmol/l) of selected flavonoids and phenolic acids in plasma samples of 41 men on their usual diet, participating in a cross-sectional study (according to Bolarinwa and Linseisen [16]).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Median</th>
<th>Mean (SD)</th>
<th>Min. (nmol/l)</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallocatechin</td>
<td>68.6</td>
<td>91.2 (17.1)</td>
<td>0.0</td>
<td>561.9</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>413.0</td>
<td>520.8 (77.2)</td>
<td>48.0</td>
<td>2641.9</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>742.8</td>
<td>1031.3 (153.4)</td>
<td>0.0</td>
<td>4528.3</td>
</tr>
<tr>
<td>Catechin</td>
<td>82.1</td>
<td>107.8 (15.6)</td>
<td>0.0</td>
<td>388.1</td>
</tr>
<tr>
<td>Gentisinic acid</td>
<td>1357.8</td>
<td>2160.6 (275.9)</td>
<td>0.0</td>
<td>6849.7</td>
</tr>
<tr>
<td>Epigallocatechingallate</td>
<td>158.9</td>
<td>267.7 (48.6)</td>
<td>0.0</td>
<td>1348.7</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>372.4</td>
<td>477.3 (67.2)</td>
<td>0.0</td>
<td>2290.8</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>159.5</td>
<td>260.3 (42.7)</td>
<td>0.0</td>
<td>1121.6</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>114.0</td>
<td>277.7 (78.5)</td>
<td>0.0</td>
<td>2542.9</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>478.3</td>
<td>908.1 (203.5)</td>
<td>0.0</td>
<td>7103.0</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>644.6</td>
<td>1304.6 (254.3)</td>
<td>0.0</td>
<td>6103.4</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>460.6</td>
<td>652.1 (94.6)</td>
<td>58.8</td>
<td>2904.4</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>339.1</td>
<td>6990.0 (30779)</td>
<td>26.2</td>
<td>104468.3</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>53.8</td>
<td>140.1 (41.4)</td>
<td>0.0</td>
<td>1253.4</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.0</td>
<td>10.1 (2.8)</td>
<td>0.0</td>
<td>59.9</td>
</tr>
<tr>
<td>Quercetin</td>
<td>78.5</td>
<td>108.7 (15.9)</td>
<td>19.7</td>
<td>563.8</td>
</tr>
</tbody>
</table>
The urinary excretion of polyphenols in subjects on habitual diets was also shown to be correlated significantly with estimates of the short-term of fruits and vegetables, the correlation coefficients for selected flavonols and flavanones being between 0.28 and 0.38 [13]. The urinary polyphenol excretion rates show a high degree of variability, just as was indicated already for the plasma concentrations. To give an example of the polyphenol concentrations found in 24-h urine samples of subjects on a habitual diet, Nielsen and coworkers [13] reported average (SD) concentrations of quercetin, kaempferol, naringenin, phloretin, and total flavonoids as being 25(23), 50(32), 701(659), 76(110), 1638(1316) µg/24 h, respectively.

Plasma and urinary polyphenol concentrations are not expected to reflect long-term or habitual dietary intake, although this has not been investigated tested extensively. One study reported correlation coefficients of between 0.24 and 0.74 for plasma isoflavone concentrations, the dietary intake estimates being based on FFQ data [11].

In large-scale epidemiologic (etiological) studies of disease-related effects of dietary polyphenol levels, little use has been made of biomarker measurements. Hertog and colleagues were the first to analyze commonly consumed foods in terms of their flavonol and flavone content by means of HPLC. Their work provided the basis for the estimation of dietary flavonol and flavone intake. In the following, several studies on associations with the risk of cardiovascular diseases and with cancer at different sites, some of which appear very promising, are taken up. Only few studies concerning the use of biomarkers of polyphenol intake were found to be available (Table 3.6), except as regards the intake of phytoestrogens (isoflavones and lignans).

### Table 3.5.
Concentrations (nmol/l) of selected flavonoids and phenolic acids in plasma samples of 41 men on their usual diet, participating in a cross-sectional study (according to Bolarinwa and Linseisen [16] — cont.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Median</th>
<th>Mean (SD)</th>
<th>Min. (nmol/l)</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringenin</td>
<td>79.7</td>
<td>122.5 (21.7)</td>
<td>0.0 a</td>
<td>533.1</td>
</tr>
<tr>
<td>Luteolin</td>
<td>388.0</td>
<td>545.8 (80.8)</td>
<td>0.0 a</td>
<td>2555.2</td>
</tr>
<tr>
<td>Genistein</td>
<td>108.1</td>
<td>1571 (24.1)</td>
<td>0.0 a</td>
<td>639.7</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>27.6</td>
<td>372 (4.9)</td>
<td>0.0 a</td>
<td>151.7</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>36.8</td>
<td>126.1 (40.4)</td>
<td>0.0 a</td>
<td>1356.8</td>
</tr>
<tr>
<td>Apigenin</td>
<td>5.3</td>
<td>9.3 (1.9)</td>
<td>0.0 a</td>
<td>52.5</td>
</tr>
<tr>
<td>Isoflavone</td>
<td>14.2</td>
<td>31.0 (7.3)</td>
<td>0.0 a</td>
<td>204.8</td>
</tr>
</tbody>
</table>

* Below the detection limit.
Table 3.6. Epidemiologic (observational) studies of the risk of cancer in which biomarkers of dietary polyphenol intake (other than that of isoflavones and lignans) were employed

<table>
<thead>
<tr>
<th>Author</th>
<th>Type</th>
<th>N, cases/controls</th>
<th>Specimen; Flavonoid</th>
<th>Cancer site</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dai et al. 2002 [17]</td>
<td>CCS</td>
<td>250/250</td>
<td>Urine; citrus flavonoids</td>
<td>Breast</td>
<td>NS</td>
</tr>
<tr>
<td>Zheng et al. 1999 [18]</td>
<td>CCS</td>
<td>60/60</td>
<td>Urine; total phenols</td>
<td>Breast</td>
<td>NS</td>
</tr>
<tr>
<td>Sun et al. 2002 [19]</td>
<td>Cohort</td>
<td>232/772</td>
<td>Urine; tea polyphenols</td>
<td>Gastric and esophageal</td>
<td>Significant inverse association</td>
</tr>
</tbody>
</table>

CCS — case-control study; NS — not significant.

One of the major reasons for the frequent use of biomarkers of phytoestrogen intake in epidemiological studies (see Table 3.7.) may be the ready availability of appropriate immunoassays suitable for measurements on large numbers of samples, enabling the attainment of the requirement of sufficient statistical power.

Table 3.7. Epidemiological studies of the risk of breast cancer risk in which biomarkers of dietary isoflavone and mammalian lignans intake were employed

<table>
<thead>
<tr>
<th>Author</th>
<th>Type</th>
<th>N, cases/controls</th>
<th>Specimen; Phytoestrogen</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingram et al. [21]</td>
<td>CCS</td>
<td>144/144</td>
<td>Urine; equol, enterolactone</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>Zheng et al. [18]</td>
<td>CCS</td>
<td>60/60</td>
<td>Urine; isoflavones</td>
<td>NS</td>
</tr>
<tr>
<td>Murkies et al. [22]</td>
<td>CCS</td>
<td>18/20</td>
<td>Urine; isoflavones</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>Dai et al. [17]</td>
<td>CCS</td>
<td>250/250</td>
<td>Urine; isoflavones, lignans</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>Piller et al. [23]</td>
<td>CCS</td>
<td>220/237</td>
<td>Plasma; enterolactone</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>Pietinen et al. [24]</td>
<td>CCS</td>
<td>194/208</td>
<td>Serum; enterolactone</td>
<td>Significant inverse association</td>
</tr>
<tr>
<td>den Tonkelaar et al. [25]</td>
<td>Cohort: nested CCS</td>
<td>88/268</td>
<td>Urine; genistein, enterolactone</td>
<td>NS</td>
</tr>
<tr>
<td>Hulten et al. [26]</td>
<td>Cohort: nested CCS</td>
<td>248/492</td>
<td>Plasma; enterolactone for highest and lowest categories</td>
<td>Significant positive association</td>
</tr>
<tr>
<td>Grace et al. [27]</td>
<td>Cohort: nested CCS</td>
<td>97/187</td>
<td>Serum, Urine; isoflavones, lignans</td>
<td>Higher risk when isoflavone estimates are higher</td>
</tr>
</tbody>
</table>

CCS — case-control study; NS — not significant.
In view of the usually rather low sample volumes available in epidemiologic studies, the analysis of polyphenol content is restricted in most cases to only a few compounds or classes of compounds. Accounts of analytical procedures that permit a wide variety of polyphenols to be determined in a single run were published recently [16,20].

**Specificity and sensitivity**

In applying mass-selective detection methods at least to standard mixtures or biological specimens spiked with the compound in question, the concentrations were found to be high enough to enable the characteristic mass fragments to be identified. This differs from other methods, such as HPLC-UV/VIS, ECD (electron capture detection), or use of fluorescence detectors, in which peak identity cannot be confirmed with sufficient certainty. Antibody-based assays are also subject to failures in specificity due to cross-reactions with other matrix compounds. For all these methods, confirmation of their results by use of MS-based techniques is necessary. MS-based systems also have their problems in connection with concentrations close to the detection limit, however, where the characteristic fragments may be absent.

Sensitivity is a major issue with regard to analytical methods for determining polyphenols in biological specimens. In epidemiological studies in particular, in which the sample volumes available are usually very small, the degree of sensitivity a method provides can be decisive for whether it can be used or not (along with other factors, such as analytical time and costs). Detection limits very close to 1 nmol/l of polyphenols have been found for techniques involving HPLC-ECD, HPLC-MS-MS, and TR-FIA (immunoassay). The sensitivity achievable with HPLC-MS, LC-MS, GC-MS and HPLC with use of fluorometric detection is slightly lower.

**Reproducibility, validity and reliability**

For each well-developed method, satisfactory figures concerning the analytical precision and accuracy are available. The higher values here are those for the MS-based techniques (coefficient of variation < 5–7%, recovery 90–105%), the immuno-assays being located at the other end of the scale, having coefficients of variation close to or above 10% and recovery rates frequently at < 90%. However, working at the detection limit of a method is always a challenge, and reports on the quality of the method in question are usually obtained clearly above the detection limit. As already mentioned, MS-based systems also have their problems when the concentrations involved are very low. To the best of our knowledge, except for some few small studies [14], no systematic findings on repeated within-subject samples or on losses during sample storage are available.
References


3.3. Anticarcinogenic compounds of olive oil and related biomarkers

Theodore G. Sotiroudis and Soterios A. Kyrtopoulos
Institute of Biological Research and Biotechnology, The National Hellenic Research Foundation, Athens, Greece

Olive oil is an important ingredient of the Mediterranean diet. Epidemiological studies demonstrate rather conclusively that populations within Europe consuming this diet have a particularly low incidence of a number of common cancers [1,2]. A plethora of minor constituents in olive oil have been identified as being effective agents mitigating against the initiation, promotion and progression of multistage carcinogenesis. These include toco-pherol and carotenoid antioxidants, that have been thoroughly studied, a number of simple and bound phenolics (tyrosol, hydroxytyrosol, secoiridoids and lignans), the triterpene hydrocarbon squalene and the phytosterol β-sitosterol [1–3]. The occurrence of these constituents also calls for the development of specific nutritional biomarkers that reflect the nutritional status of these dietary constituents with respect to their intake or metabolism and that can provide information useful for nutritional epidemiology regarding the effects of disease processes that can occur [4]. A brief overview is presented of recent findings concerning the bioavailability of certain minor but important olive oil minor components (polyphenols, lignans, squalene and β-sitosterol), considered as putative nutritional biomarkers in relation to cancer the incidence of cancer.

Phenolic compounds

HPLC chromatography of the methanol extract of virgin olive oil reveals seven major polyphenol peaks corresponding to hydroxytyrosol, tyrosol, oleuropein, the aglycone of ligstroside, two secoiridoids (dialdehydes related to oleuropein and ligstroside but lacking the carboxymethyl group at C4), and a peak containing the lignans (+)-1-acetoxypinoresinol and (+)-pinoresinol [1–3] (Table 3.8., Figure 3.2.). Oleuropein and its metabolites tyrosol and hydroxytyrosol, which represent major antioxidants in olive oil, are dose-dependently absorbed in humans after the ingestion of realistic doses of virgin olive oil. When olive oil samples containing increasing amounts of a phenolic extract of olive oil were administered to human volunteers, a dose-dependent decrease in the urinary excretion of the F2-isoprostane 8-iso-PGF$_{2\alpha}$, a biomarker of in vivo lipid peroxidation processes, was observed. This indicates olive oil phenolics to maintain their antioxidant activities in vivo. It has also been shown that olive oil phenolics are excreted in the urine as glucuronide conjugates and that the urinary free tyrosol concentration is responsive to the dietary intake of virgin olive oil. In addition, a statistically significant negative correlation has been found between homovanillyl alcohol (HValk, a major metabolite of hydroxytyrosol, together with homovanillic acid — HVA) and isoprostane excretion, the excretion of both HValk and HVA also being significantly correlated with the dose of administered hydroxytyrosol. Thus, HValk in urine reflects the in vivo
concentration of hydroxytyrosol [5–11]. After the ingestion of olive oil of low phenolic content plasma glutathione peroxidase activity was found to decrease postprandially, but this was not observed after the intake of olive oils of moderate to high phenolic content [12]. An HPLC method for the simultaneous determination of oleuropein and of its metabolites hydroxytyrosol and tyrosol in human plasma has been developed [13,14].

Table 3.8. Concentrations of the major phenolic compounds found in virgin olive oil

<table>
<thead>
<tr>
<th>Compounds</th>
<th>mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosol</td>
<td>27.45 [2], 2.65–4.75 [3]</td>
</tr>
<tr>
<td>Oleuropein aglycone</td>
<td>103–205 [3]</td>
</tr>
<tr>
<td>Total secoiridoids</td>
<td>27.72 [2]</td>
</tr>
<tr>
<td>Lignans</td>
<td>41.53 [2], 38–65 [3]</td>
</tr>
</tbody>
</table>

References [2] and [3].

Fig. 3.2. Structures of certain phenolic compounds detected in olive oil. Hydroxytyrosol (A), oleuropein aglycone (B), (+)-pinoresinol (C).

Recent findings suggest that olive oil may also affect the bioavailability of other food bioactive components with a chemopreventive potential. It was observed in this respect, that the concentration in human plasma of lycopene, a biomarker of the intake of tomato-rich food and hypothesized to be responsible for reducing the risk of various
cancers, increased dramatically after the consumption of tomatoes cooked in olive oil, as compared to the consumption of tomatoes cooked without olive oil [15]. The consumption of tomato products prepared together with olive oil, but not with sunflower oil, was found to improve the antioxidant activity of plasma [16].

Lignans are plant compounds metabolized in the gut to produce the phytoestrogens enterolactone and enterodiol. Phytoestrogens have an anticarcinogenic potential through the anti-estrogenic, anti-angiogenic, proapoptotic and anti-oxidant mechanisms established for them [17,18]. Recent findings suggest that enterolactone is more rapidly metabolized in human colon epithelial cells and/or excreted by them than enterodiol is, that the phase II metabolism of enterolactone and enterodiol already may take place during their uptake in the colon, and that the epithelial cells in the colon may be responsible for this metabolism [19]. Mean residence times and elimination half-lives that have been obtained indicate that enterolignans accumulate in the plasma when consumed 2–3 times a day, their reaching a steady state. Plasma enterolignan concentrations can thus be considered to be good biomarkers of dietary lignan exposure and be used to evaluate the effects of lignans [20]. A number of in vitro and animal studies support a role for lignan-rich foods and of purified lignans in the modulation of cancer events in the breast, the prostate and the colon, whereas the findings of epidemiological studies are controversial [18]. Nevertheless, a tendency for a lower risk of breast cancer to be associated with higher plasma concentrations of enterolactone, restricted almost entirely to estrogen-receptor alpha negative breast cancer has been found, suggesting that dietary lignans may be important in the etiology of breast cancer, particularly in premenopausal women [21].

Squalene

It has been suggested that the lower risk of cancers of various types associated with high olive oil consumption (as compared with other human foods) may be due to the presence of squalene (reviewed in [1]). This triterpene hydrocarbon is found mainly in nonedible shark liver oil, while virgin olive oil is a major source of phytosqualene, its content ranging from 800 to 12,000 mg/kg. If virgin olive oil were the sole source of dietary fat, the squalene intake would be more than 200 mg/d [22]. Nevertheless, very little is known concerning the postprandial metabolism of squalene. It has been observed that postprandial squalene metabolism is age dependent [23], and that the content of squalene in the whole plasma and in the lipoprotein fractions (where its ratio to cholesterol is highest in the VLDL and the intermediate density lipoproteins [24]) varies directly with the triglyceride content and is increased in hypertriglyceridemia, which expands the plasma pool of this metabolically active hydrocarbon [25]. Experiments in vitro and animal models suggest squalene to play a tumour-inhibiting role, which is most probably based on its strong inhibitory action on the catalytic activity of beta-hydroxy-beta-methylglutaryl-CoA reductase, leading to a reduced farnesyl pyrophosphate availability for prenylation of the ras oncogene [26]. Although animal studies have enhanced our understanding of the possible
action of squalene in decreasing carcinogenesis, one should be cautious in extrapolating findings there to humans, both because of possible species differences and because the long-term effects of greater consumption of squalene are unknown. Several factors must be taken into account when examining the evidence for squalene’s inhibition of carcinogenesis factors, such as the effective dose used and exposure time [27]. At present, therefore, its use as a nutritional biomarker is hardly to be considered.

**Phytosterols**

Phytosterols are plant sterols that are structurally similar to cholesterol and that possess anticarcinogenic properties [27]. Together with squalene, they represent markers of cholesterol synthesis and absorption and are transported together with cholesterol in serum lipoproteins [24]. β-Sitosterol, one of the most common phytosterols and the main olive oil sterol [1], together with campesterol are the two predominant phytosterols in the blood. It has been suggested that the high reproducibility and high reliability over time (consistency of the plasma phytosterol level over time) of the plasma measurements of these sterols makes them suitable for clinical and population-based studies of cancer prevention [28]. In recent years, functional foods high in phytosterol-ester content for lowering the cholesterol level have been developed. Although phytosterols act as immune modulators and anticancer agents *in vitro* [29], the protection (if any) that high concentrations of phytosterol provide against the development of cancer in humans has not been adequately examined, further study of this being needed.

**Conclusions**

Since the phenolic content of the olive oil consumed may account for the postprandial antioxidant activity *in vivo* after the ingestion olive oils of moderate to high phenolic content, we suggest that these biomolecules, or certain polyphenol metabolites in human plasma and urine, can serve as practical biomarkers for olive oil consumption and as an alternative biomarker for future epidemiological studies in dietary cancer prevention and health promotion.

**References**

3.4. Anticarcinogenic effects of glucosinolate breakdown products

John D. Hayes¹, Michael O. Kelleher¹ and Ian M. Eggleston²

¹ Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY
² Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom

Glucosinolates and their association with cancer chemoprevention

Epidemiological studies have revealed that regular consumption of cruciferous vegetables, such as broccoli, Brussels sprouts, cabbage, cauliflower, kale, swede and turnip, is associated with a reduced incidence of cancer [1,2]. Furthermore, greater health benefit may be obtained from raw as opposed to cooked vegetables [3]. The types of neoplastic disease in man that these vegetables appear to protect against include colorectal cancer [4], lung cancer [5], and possibly prostate cancer [6]. Feeding experiments in animals have also suggested broccoli can protect against liver cancer [7]. Cruciferous vegetables uniquely contain glucosinolates at approximately 20 µmol/g dry mass of vegetable [8,9], and it is thought that these phytochemicals are primarily responsible for the putative cancer chemoprevention conferred by eating diets that contain significant quantities of these vegetables [10,11].

Glucosinolates are substituted β-thioglucoside N-hydroxysulfates, formed by the plant from any one of eight amino acids, namely, alanine, valine, leucine, isoleucine, phenylalanine, methionine, tyrosine and tryptophan [2]. Over 115 naturally occurring glucosinolates have been identified. Each cruciferous vegetable contains a mixture of glucosinolates that varies according to the strain of the plant [8,12–15]. The glucosinolate content is primarily under genetic control, though it can be influenced by environmental factors [16,17]. Much of the diversity amongst glucosinolates arises from the addition of different sized alkyl groups to the side chain of those amino acids, principally valine, phenylalanine and methionine, used in their biosynthesis; this variable elongation of amino acid side chains entails repetitive additions of methyl groups through a series of transamination, condensation, isomerisation and decarboxylation reactions [18]. As shown in Figure 3.3., the synthesis of glucosinolates proceeds through the conversion of elongated amino acids to their oxime derivatives, catalysed by members of the cytochrome P450 (CYP) 79 family [19]. Subsequently, the oxime is metabolised to a thiohydroximate, which is in turn conjugated with glucuronic acid to form a desulfoglucosinolate before finally being sulfated to yield the glucosinolate [2].

The task of establishing a link between the ingestion of particular glucosinolates and their possible health benefits is not straightforward. This endeavour is simplified to some extent by the fact that relatively few glucosinolates are present in the human diet. The most common of these are the methylsulfinylalkyl glucosinolates glucoiberin and glucoraphanin, the olefinic glucosinolates sinigrin, gluconapin, glucobrassicanapin and progoitrin, and the aromatic glucosinolate gluconasturtiin (Table 3.9.) [9,20]. Glucoraphanin has been reported to be abundant in broccoli [9], though certain broccoli
strains also contain substantial amounts of glucoiberin [21]. Sinigrin has been reported to be the predominant glucosinolate in Brussels sprouts, cabbage, cauliflower and kale [9]; gluconapin is also found in high levels in Brussels sprouts [9]. Substantial amounts of progoitrin are present in many cruciferous vegetables [9]. The aromatic glucosinolate gluconasturtiin is present in watercress. The indolyl glucosinolate glucobrassicin is present in Savoy cabbage, Brussels sprouts and cauliflower [9,22], and whilst not abundant it can elicit distinct pharmacological effects.

Table 3.9. Trivial names of some glucosinolates with the corresponding side-chain (R) composition

<table>
<thead>
<tr>
<th>Name</th>
<th>R side-chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinigrin</td>
<td>2-Propenyl</td>
</tr>
<tr>
<td>Gluconapin</td>
<td>3-Butenyl</td>
</tr>
<tr>
<td>Glucobrassicin</td>
<td>3-Indolylmethyl</td>
</tr>
<tr>
<td>Glucobrassicinanin</td>
<td>4-Pentenyl</td>
</tr>
<tr>
<td>Progoitrin</td>
<td>2-Hydroxy-3-butenyl</td>
</tr>
<tr>
<td>Glucoiberin</td>
<td>3-Methylsulfinylpropyl</td>
</tr>
<tr>
<td>Gluconapoleiferin</td>
<td>2-Hydroxy-4-pentenyl</td>
</tr>
<tr>
<td>Glucocheirolin</td>
<td>3-Methylsulfonylpropyl</td>
</tr>
<tr>
<td>Glucoerucin</td>
<td>4-Methylthiobutyl</td>
</tr>
<tr>
<td>Glucoberteroin</td>
<td>5-Methylthiopentyl</td>
</tr>
</tbody>
</table>

Fig. 3.3. Synthesis of glucosinolates. The R group is derived from the original amino acid and is highly variable.
Production of isothiocyanates, thiocyanates, nitriles, cyano-epithioalkanes and oxazolidine-2-thiones from glucosinolates

Evidence suggests that inhibition of carcinogenesis by glucosinolates is not primarily attributable to this class of compound, but rather it appears to be due to certain of their breakdown products. Hydrolysis of these phytochemicals is catalysed by myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.147), an enzyme that is physically segregated from glucosinolates within the intact plant by virtue of the fact that it is sequestered in specialised “myrosin” cells [23]. Upon wounding of the vegetable, for example during harvesting, during freeze-thawing, during food preparation, or during chewing whilst eating, myrosinase is released from the “myrosin” cells and is able to hydrolyse glucosinolates within the damaged plant. In addition, myrosinase activity may be present in human colonic microflora, suggesting that glucosinolates can be hydrolysed in the gastrointestinal tract during digestion of food [24,25]. Myrosinase cleaves glucosinolates at the thioglycoside linkage to produce glucose and an unstable aglycone thiohydroximate-O-sulfonate that spontaneously rearranges to yield several breakdown products. The outcome of the reaction with myrosinase depends on the nature of the aglycone, as well as the reaction temperature, the pH and the presence of ferrous ions (Figure 3.4A. and 3.4B.).

![Diagram of glucosinolate hydrolysis](image)

**Fig. 3.4A.** Hydrolysis of glucosinolates. At high or neutral pH the formation of isothiocyanates is favoured while at low pH the formation of nitriles is favoured. Epithiospecifier protein (ESP) in the presence of Fe²⁺ ions interacts with myrosinase to promote the transfer of the sulfur to the alkenyl group from the S-Glucose of the terminally unsaturated glucosinolate, resulting in the formation of an epithioalkane.
Fig. 3.4B. Hydrolysis of sinigrin. Following damage to the plant tissue the glucosinolate sinigrin is hydrolysed by myrosinase resulting in the formation of four distinct compounds. On the right-hand side of the figure, an arrow shows that allyl thiocyanate, formed from sinigrin, can convert spontaneously to form allyl isothiocyanate [26].

The thiohydroximate-O-sulfates formed from methylsulfinylalkyl, olefinic and aromatic glucosinolates undergo a Lossen rearrangement, with the elimination of sulfate, to form their respective isothiocyanates (ITCs), thiocyanates or nitriles [10,23]. Certain thiocyanates that are formed during a Lossen rearrangement, such as allyl-ITC, are unstable and can undergo a relatively slow spontaneous conversion to their respective isothiocyanate [26]. Elemental sulfur is also formed in certain circumstances. At neutral pH, hydrolysis of glucosinolates with aliphatic or aromatic side chains gives rise primarily to isothiocyanates (ITCs). The glucosinolates glucoiberin, gluconapin, glucoraphanin, glucobrassicanapin and sinigrin yield 3-methylsulfinylpropyl-ITC, 3-butenyl-ITC, 4-methylsulfinylbutyl-ITC (sulforaphane), 4-pentenyl-ITC and 2-propenyl-ITC (allyl-ITC), respectively.

At low pH, the thiohydroximate-O-sulfates formed by myrosinase from glucosinolates with a side chain containing a double bond (e.g. sinigrin, gluconapin and glucobrassicanapin) may, in the presence of an epithiospecifier protein (ESP) and ferrous ions, give rise to a cyano-epithioalkane [27]. In this case, ESP interacts with myrosinase to promote sulfur transfer from the S-glycosyl unit to the alkenyl chain derived from the amino acid part of the aglycone [28]. Thus, at pH 4 and in the presence of Fe$^{2+}$ ions,
myrosinase and ESP convert sinigrin to 1-cyano-2,3-epithiopropane [29]. Gluconapin can similarly be converted by the combined actions of myrosinase and ESP to 1-cyano-3,4-epithiobutane [30,31]. Likewise, glucobrassicanapin can be hydrolysed to 1-cyano-4,5-epithiopentane [31]. Progoitrin, a (2R)-hydroxy-3-butenyl glucosinolate, is also converted in the presence of myrosinase, ESP and Fe$^{2+}$ ions to an epithionitrile [32] and in the case of epi-progoitrin ((2S)-hydroxy-3-butenyl glucosinolate), it can be hydrolysed by myrosinase to crambene (1-cyano-2-hydroxy-3-butene) [33,34]. Two cDNAs for ESP have recently been cloned from Arabidopsis and broccoli and the purified proteins characterized following their heterologous expression in E. coli [35,36].

If the aglycone generated by myrosinase is from a glucosinolate with a side chain lacking a double bond, the sulfur atom may be lost and a nitrile formed [37–39]. This reaction may involve ESP, and is diminished by heating [40]. A few glucosinolates produce thiocyanates though the mechanism involved is unclear [23]. Upon hydrolysis by myrosinase, those aglycones from glucosinolates that contain β-hydroxylated side-chains form oxazolidine-2-thiones, as a consequence of spontaneous cyclization. Examples of these include progoitrin, glucoconringiin and gluconapoleiferin [2].

**Production of indoles from glucosinolates**

The indolyl glucosinolates glucobrassicin and neoglucobrassicin are synthesised by the plant from tryptophan. The best studied of these is glucobrassicin. At neutral pH, hydrolysis of glucobrassicin by myrosinase does not generate an ITC, but rather gives rise to indole-3-carbinol and a thiocyanate ion (Figure 3.5.); this reaction probably proceeds

![Fig. 3.5. Production of indoles from glucosinolates. At neutral pH the hydrolysis of glucobrassicin by myrosinase leads to the formation of an unstable isothiocyanate intermediate which degrades to form indole-3-carbinol and a thiocyanate ion.](image-url)
through a Lossen rearrangement generating an unstable ITC intermediate [22]. At acidic pH, hydrolysis of glucobrassicin yields indole-3-acetonitrile, hydrogen sulfide and elemental sulfur [22]. In the acidic environment of the stomach, indole-3-carbinol condenses to form various compounds including indolo[3,2-b]carbazole and 3,3′-diindolylmethane, both of which have potent pharmacological effects [41]. It can also combine with ascorbic acid to form ascorbigen [42] (Figure 3.6.).

![Fig. 3.6. Structures of indoles produced from glucosinolates — 3,3′-Diindolylmethane (DIM), indolo [3,2-b] carbazole (ICZ) and ascorbigen (ASG).](image)

**Chemopreventive mechanisms stimulated by glucosinolate hydrolysis products**

In view of the diverse spectrum of chemicals generated from glucosinolates by the actions of myrosinase and ESP, it is not surprising that a number of distinct cancer chemopreventive mechanisms have been proposed to account for the putative anti-cancer properties of cruciferous vegetables. These include induction of antioxidant and detoxifying genes, inhibition of CYP enzyme activity, cell cycle arrest, and stimulation of apoptosis. There is a dose-dependency in these responses: generally, induction of cytoprotective genes and inhibition of CYP activity occurs at relatively low concentrations of phytochemical, whereas activation of cell cycle arrest and apoptosis occurs at higher levels of phytochemical [43,44]. A major problem exists in interpreting experiments utilizing vegetable extracts because of uncertainty about attributing biological effects to specific phytochemicals. A challenge in evaluating the literature arises from the emphasis placed on certain glucosinolate breakdown products and the dearth of data relating to others. Thus, there is a relative abundance of information about isothiocyanates and indoles, when compared with the data about thiocyanates, nitriles, cyanoepithioalkanes, and oxazolidine-2-thiones.

**Induction of gene expression mediated by Nrf2**

Isothiocyanates increase the expression of antioxidant and detoxication proteins, such as glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1), both in vivo and in vitro [45,46]. Agents such as ITC that increase GST and NQO1
enzymes without increasing arylhydrocarbon hydroxylase activity, catalysed by the phase I drug-metabolising enzyme cytochrome P450 (CYP) 1A1, are sometimes called mono-functional inducers [47]. Sulforaphane, 3-methylsulfinylpropyl-ITC, allyl-ITC, 7-methylsulfanylheptyl-ITC, 8-methylsulfinyloctyl-ITC, benzyl-ITC and phenethyl-ITC, induce NQO1 in the mouse Hepa-1c1c7 hepatoma cell line [48,49] (Figure 3.7.). Many of these compounds induce GST P1-1 in the rat liver RL-34 cells [50]. The mouse nqo1 gene contains a functional antioxidant response element (ARE) in its 5′-upstream region [51], as does the rat GSTP1 gene (called GPEI) [52]. The induction of these two genes by ITCs is mediated by the Nrf2 (Nuclear Factor-Erythroid 2 p45-related factor 2) bZIP (basic-region leucine zipper) transcription factor that is recruited to the ARE as a heterodimer with small Maf protein [51]. Indeed, as far as is known, all genes that are induced by ITCs contain an ARE in their promoters and are regulated by Nrf2.

Fig. 3.7. Structures of isothiocyanates which induce NQO1: 1) Allyl-ITC; 2) Phenethyl-ITC; 3) Benzyl-ITC; 4) 3-methylsulfinylpropyl-ITC; 5) 7-methylsulfanylheptyl-ITC; 6) 8-methylsulfinyloctyl-ITC.

Examination of nrf2−/− and wild-type mice, suggests AREs are present in the promoters of at least 100 genes [53–55]. The battery of genes regulated by Nrf2 includes those encoding aldo-keto reductase (AKR), carboxyl esterase, ferritin, glutamate cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits, GST, heme oxygenase 1, NQO1, metallothionein, microsomal epoxide hydrolase, multidrug resistance-associated protein, thioredoxin, thioredoxin reductase, UDP-glucuronosyl transferase. Many of these genes are induced by sulforaphane in vivo in an Nrf2-dependent fashion in the stomach, small intestine and liver of rodents [53,54,55–58]. Importantly, feeding broccoli seed to mice increased the levels of GCLC, GST and NQO1 in the gastrointestinal tract in an Nrf2-dependent fashion [21]. It is thought that ITCs possess the ability to induce ARE-driven gene expression because they are thiol-active [59]. Through this characteristic, ITCs modify cysteine residues in the Cullin 3:Rbx1 E3 ubiquitin ligase substrate adaptor Keap1, leading to its inhibition and inability to serve as a substrate adaptor.
required for the ubiquitination of Nrf2 under homeostatic conditions [60–62]. Consistent with this view, exposure of RL-34 cells or HepG2 cells to sulforaphane causes stabilisation and rapid accumulation of Nrf2 [63,64]. Surprisingly, allyl-ITC does not increase Nrf2 stability [64] and there may therefore be other factors involved in enzyme induction by these phytochemicals. Treatment of cells with benzyl-ITC causes a rapid increase in the level of reactive oxygen species, and this may also contribute to gene induction [46].

It has been found that administration of cranbene to Fischer 344 rats causes an elevation in hepatic GST and NQO1 enzyme activities, but not CYP1A1, suggesting it is a mono-functional inducer [65]. By comparison with sulforaphane, cranbene was found to be an approximately equally potent inducer in the rat [66]. However, it was not a particularly effective inducer of NQO1 activity in Hepa-1c1c7 cells suggesting that the relatively high potency of induction observed in vivo is due to bio-transformation of cranbene to a thiol-active metabolite. The identity of this metabolite is not known.

The indole-containing glucobrassicin breakdown products can activate gene expression by several mechanisms. Indole-3-carbinol is a modest inducer of Nrf2-dependent ARE-driven gene expression in the liver and small intestine of mice [52,67]. Although indole-3-acetonitrile has not been studied in the Nrf2 knockout mouse, it is a good inducer of GST enzyme activity in mouse liver and small intestine [68], a fact that implies it works through Nrf2.

**Induction of gene expression mediated by AhR**

The major effect that indole-3-carbinol has on gene expression occurs because it can condense in acid conditions to form indolo[3,2-b]carbazole and 3,3′-diindolylmethane. Both these condensation products induce CYP1A1 genes via the xenobiotic response element (XRE) in their promoter regions because they are ligands for the Ah receptor. Examination of the dose of indole required to double XRE-driven reporter gene expression shows that indolo[3,2-b]carbazole is a much more potent inducing agent than 3,3′-diindolylmethane, indole-3-carbinol or ascorbigen [43,69]. Amongst genes for drug-metabolising enzymes, mouse, rat and human CYP1A1 are prototypic XRE-regulated genes. This cytochrome has O-deethylase activity towards ethoxyresorufin, and there is abundant evidence from enzyme assays, western and northern blotting that CYP1A1 is inducible by indolo[3,2-b]carbazole and 3,3′-diindolylmethane [2]. The promoters of other genes including rat and human NQO1, rat ALDH-3, rat GSTA2, rat UGT1A1, and rat UGT1A6 contain an XRE, as does the mouse BAX gene, and it is therefore anticipated that activation of AhR by glucobrassicin-derived indoles will influence significantly the metabolism of xenobiotics (for a review, see [70]). Interestingly, the mouse nrf2 gene promoter also contains an XRE [71], indicating that AhR ligands could lead to an increased production of Nrf2 mRNA; this may not necessarily lead to an increase in Nrf2 protein levels. Whether indolo[3,2-b]carbazole and 3,3′-diindolylmethane can increase the stability of Nrf2 protein is not known, but unless they are metabolised by CYPs, and thereby generate reactive oxygen species, this seems unlikely [72].
In addition to induction of \( CYP1A1 \) by indoles, other cytochromes are inducible, such as \( CYP1B1 \) and \( CYP19 \) [73], as are the drug metabolising enzymes AKR, GST T1-1, sulfotransferase and UGT1 [74]. Furthermore, \( 3,3' \)-diindolylmethane can induce expression of the transcription factors ATF3, c-jun and NF-IL6 as well as genes involved in cell growth, such as growth arrest and DNA damage (GADD) GADD34, GADD45 and GADD153 [75,76]. Also, p21 is induced by indole-3-carbinole [77]. It is not however clear whether the genes for ATF3, c-Jun, NF-IL6 and the GADD proteins are regulated through XREs and whether the process is mediated by AhR.

It has been argued that induction of \( CYP1A1 \) by indoles is potentially deleterious to the cell because the cytochrome can activate polycyclic aromatic hydrocarbons to ultimate carcinogens. This viewpoint is probably an oversimplification and does not take into account the multiple changes in gene expression that indoles affect. Bonnesen et al [43] have reported that treatment of human colon LS-174 cells with indolo[3,2-\( b \)]carbazole before exposure to benzo[a]pyrene provides a small measure of protection against DNA damage as measured by the comet assay. Most importantly, prior treatment of the LS-174 cells with both indolo[3,2-\( b \)]carbazole and sulforaphane before exposure to benzo[a]pyrene was found to confer substantial protection against genotoxicity, and this protection was greater than was achieved by either phytochemical alone [43].

**Inhibition of carcinogen activation by glucosinolate breakdown products**

Certain isothiocyanates can block the activation of several carcinogens to their ultimate carcinogenic forms. Tumorigenesis caused by the carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N-nitrosobis-(2-oxopropyl)amine can be prevented by phenethyl-ITC through a process that involves inhibition of activation of pro-carcinogens by CYP isoenzymes [78,79]. Inhibition of CYP can also be achieved by sulforaphane [80,81]. In the case of the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, the ITCs with highest lipophilicity and low reactivity of their NCS group had the greatest ability to inhibit lung tumorigenesis [82].

**Inhibition of histone deacetylase by isothiocyanates**

The isothiocyanate sulforaphane has been shown to inhibit histone deacetylase (HDAC) [83,84]. This function is likely to alter gene expression substantially. It may also have profound implications for cell fate as a change in the balance between histone acetyl transferase (HAT) and HDAC could alter tumourigenesis. Indeed, recognition of this possibility has lead to considerable recent interest in the ability of HDAC inhibitors to act as both chemopreventive and chemotherapeutic agents.

Within the cell, DNA is tightly coiled around an octamer of histone proteins in a structure known as a nucleosome, the basic structural unit of chromatin. Each of the histone proteins contains an evolutionary conserved amino tail protruding from
the nucleosome, which can determine the accessibility of the DNA to transcription factors. The tail is also subject to many post-translational modifications including acetylation. The addition of an acetyl group to the histone tail results in a conformational change which enables the tail to move away from the DNA, allowing transcription factors access to the regulatory regions of genes. Conversely, removal of acetyl groups causes the tail to wrap tightly around the DNA thereby preventing interaction with the transcription machinery. The addition and removal of the acetyl groups is carried out by HAT and HDAC, respectively. In pre-cancerous and cancerous cells, tumour suppressor genes are associated with deacetylated histones resulting in the inactivation of these genes. Inhibition of HDAC may prevent the removal of acetyl moieties from histones thus allowing transcription of the tumour suppressor genes.

Sulforaphane has been shown to diminish HDAC activity with a concomitant rise in histone acetylation in prostate cancer cells [84], human embryonic kidney cells [83] and human colorectal cancer cells [83]. The link between inhibition of HDAC activity and the resultant increase in transcription of tumour suppressor genes has been reported for p21 [84,85], p53 [86] and Bax [84,85]. Equally, mammalian HDAC is capable of down-regulating p53 function, by deacetylation of histones associated with the p53 gene, resulting in a reduction in its transcriptional activity. In addition, sulforaphane has been found to cause a G2/M phase delay with an increase in apoptotic cell fraction in a time- and dose- dependent fashion [87].

Thus the ability of sulforaphane, along with other dietary HDAC inhibitors such as diallyl disulfide [88], to affect chemoprevention via its ability to alter chromatin structure is of acute importance to the welfare of the cell.

Stimulation of cell cycle arrest and apoptosis by isothiocyanates

Many of the naturally occurring ITCs can suppress the growth of cultured tumor cells by modulating multiple targets that influence cell cycle arrest, apoptosis and differentiation [89]. However, the majority of the studies into mechanisms by which this class of chemical inhibit cell growth have focussed on sulforaphane and phenethyl-ITC. For example, ApcMin/+ mice treated with sulforaphane at either 300 ppm or 600 ppm in their diet have been reported to develop fewer and smaller polyps in their small intestine than ApcMin/+ mice on a control diet; this was associated with a higher level of apoptosis and lower proliferation in animals on the ITC containing diet [90].

In human PC-3 prostate cancer cells, treatment with sulforaphane or phenethyl-ITC causes an arrest in G2/M phase of the cell cycle that is associated with a decrease in levels of cyclin B1 and cell division cycle (Cdc) 25B and Cdc25C proteins [91,92]. The loss of Cdc25C was reported to be due to proteasomal activity, and was accompanied by its translocation from the nucleus to the cytoplasm [92]. Relocation of Cdc25C was found to be controlled by its phosphorylation at Ser-216, mediated through activation of checkpoint kinase 2 (Chk2) [84]. Cell proliferation by ITCs may also be achieved by disrupting cytoskeletal structure and tubulin polymerisation [93,94].
Administration of ITCs to cells at growth suppressive concentrations results in the rapid generation of reactive oxygen species (ROS), within 1 h of exposure, which appears to be necessary for cell death [92,95]. The generation of ROS by ITCs is accompanied by depletion of intracellular GSH and is achieved through the rapid export of ITC-glutathione and ITC-cysteinylglycine conjugates via MRP1 and Pgp-1 efflux pumps [96]. Consistent with the view that production of ROS is necessary for apoptosis, overexpression of catalase suppresses ITC-initiated programmed cell death, as does pre-treatment with N-acetylcysteine [92]. Furthermore, addition of GSH subsequent to ITC treatment can block apoptosis [44,97]. Treatment with ITCs leads to a loss of mitochondrial membrane potential and release of cytochrome c from mitochondria [98]. There is evidence that ITCs can activate both the intrinsic and extrinsic caspase cascades, though this may be cell specific. For example, in PC-3 cells sulforaphane can increase Fas protein levels and activate caspase-8 whilst simultaneously targeting mitochondria and activating caspase-9 [92]. In human bladder cancer UM-UC-3 cells, benzyl-ITC and phenethyl-ITC are more effective at activating caspase-9 than caspase-8 [99]. By contrast, in human leukaemia HL60 cells, caspase-8 plays a major role in apoptosis stimulated by phenethyl-ITC [100]. The levels of pro-apoptotic proteins Bak and Bax, which neutralize the antiapoptotic effects of Bcl-2, are increased by phenethyl-ITC and sulforaphane in PC-3 prostate cancer cells, and this may lead to induction of Apaf-1 [91,92,101]. Furthermore, the pro-apoptotic proteins Bok and Bim EL are also induced by ITCs, and this is thought to amplify the effects of Bak and Bax [92]. Besides increasing the levels of these pro-apoptotic proteins, ITCs down-regulate the anti-apoptotic proteins Mcl-1 and Bcl-xL, though the effect is cell-specific [101]. Various ITCs have been shown to activate c-Jun N-terminal kinase (JNK) [102,103], and this is mediated by extracellular signal-regulated kinases, ERK1/2 [104]. The use of inhibitors indicated that JNK is essential for phenethyl-ITC to cause cytochrome c release and caspase-3 activation in human HT-29 colon adenocarcinoma cells [105]. However, the mechanism by which JNK activates caspases remains unclear.

Stimulation of cell cycle arrest and apoptosis by indoles

Treatment of human MCF-7 breast cancer cells with 100 µM indole-3-carbinol inhibits proliferation through affecting a G1 cell cycle arrest [106]. This may in part be due to 3,3’-diindolylmethane rather than indole-3-carbinol as significant quantities of the indole spontaneously condense to the dimer in culture conditions [107]. Cell cycle arrest at G1 occurs as a consequence of indole-3-carbinol inhibiting both cyclin-dependent kinase (CDK) 2 and CDK6. In the case of CDK6, expression of the gene is reduced because indole-3-carbinol attenuates recruitment of the Sp1 transcription factor to the CDK6 promoter [108]. Furthermore, in HaCaT keratinocytes, treatment with 400 µM indole-3-carbinol induces the CDK4/6 inhibitor p15INK4b mRNA and protein causing hypophosphorylation of Rb protein [109]. It therefore appears that cyclin D-CDK6 activity can be inhibited by dual mechanisms. In the case of CDK2, indole-3-carbinol has been
Bioactive components in foods: Anticarcinogenic effects of glucosinolate breakdown products

reported to decrease the kinase activity in MCF-7 cells and inhibit phosphorylation of Rb protein [77]. The reduction in CDK2 activity is attributed to a selective alteration in the size of the complex in which it is contained, from an active form within a 90 kDa complex to a lower activity form within a 200 kDa complex [110]; the 90 kDa and 200 kDa complexes include forms of cyclin E that differ in size, and the larger complex also contains an additional 75 kDa cyclin E immunoreactive protein. Furthermore, the reduction in CDK2 activity is accompanied by redistribution of the kinase in the 200 kDa complex from the nucleus to the cytoplasm, suggesting that indole-3-carbinol can influence the nucleocyttoplasmic shuttling of the kinase [110].

In cells that contain wild-type p53, such as MCF-10A, treatment with 300 µM indole-3-carbinol or 30 µM 3,3′-diindolylmethane has been found to result in activation of the ATM signalling pathway, an increase in p53 protein levels, induction of p21 [111]. These changes result in prevention of the CDK2-mediated G1/S transition [111].

Indole-3-carbinol, but not 3,3′-diindolylmethane, was found to inhibit the expression of the androgen receptor in human lymph node carcinoma of prostate (LNCaP) cells as well as the probable downstream target gene prostate specific antigen [112]. It is possible that down-regulation of this receptor represents an antiproliferative mechanism in prostate cells.

Indoles can affect apoptosis in breast and prostate cancer cells. Treatment of PC-3 prostate cancer cells with 60 µM indole-3-carbinol inhibits the EGF-induced autophosphorylation of PI3K and Akt [113]. Thus, the Akt/PI3K cell survival pathway appears to be targeted by indole-3-carbinol. Also, nuclear translocation of NF-κB is inhibited by 3,3′-diindolylmethane through a reduction in phosphorylation of IκBα [114,115].

In HCT-116 human colon cells, indole-3-carbinol can induce nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1), a TGF-β family member associated with pro-apoptotic activities [116]. This may also mediate the anti-tumour effects of indoles.

Concluding comments

It is becoming clear that glucosinolate breakdown products can influence the initiation and progression of carcinogenesis. They also appear to influence apoptotic responses to chemotherapeutic agents, such as tamoxifen [77]. A major impediment to our understanding of the chemopreventative mechanisms stimulated by glucosinolates is that relatively little is known about the biological effects of glucosinolate breakdown products other than isothiocyanates and the indole-containing derivatives. Specifically, there are few data about chemopreventative activities of thiocyanates, nitriles, cyanoepithioalkanes and oxazolidine-2-thiones. It is unclear whether formation of thiocyanates, nitriles, cyanoepithioalkanes and oxazolidine-2-thiones from glucosinolates, at the expense of forming isothiocyanates, is undesirable from a cancer chemoprevention perspective. It is unclear whether the activity of ESP, which reduces the formation of isothiocyanates from glucosinolates, is undesirable. If so, ESP should possibly
be eliminated by genetic means from commercial crops. Furthermore, relatively little is known about the pharmacokinetic properties of glucosinolate breakdown products in the human, and without this information it is difficult to relate responses of cells in culture to certain concentrations of phytochemical to what happens in the in vivo situation. These are areas that warrant further examination.

Mammalian cells display marked dose responsiveness to phytochemicals: at low doses of phytochemical, cytoprotective adaptive responses are activated, whereas at higher doses cell cycle arrest and apoptosis occurs. It is presently unclear how these different types of response are co-ordinated by the cell and how decisions about whether adaptation, growth arrest or apoptosis is chosen as the appropriate response are determined. Identification of mechanisms that control such outcomes will be useful.

References


42. Preobrazhenskaya MN, Bukhman VM, Korolev AM, Efimov SA. Ascorbigen and other indole-derived compounds from Brassica vegetables and their analogs as anticarcinogenic and immunomodulating agents. Pharmacol Ther 1993;60:301–3.

43. Bonnesen C, Eggleston IM, Hayes JD. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. Cancer Res 2001;61:6120–30.


3.5. Combined action of different dietary compounds preventive of cancer

Theo M. de Kok and Simone G. van Breda
Department of Health Risk Analysis and Toxicology, University of Maastricht, Maastricht, The Netherlands

Introduction

The relatively consistent epidemiological finding that the consumption of whole foods of different types such as fruits, vegetables and whole grains is strongly associated with reduced risk of cancer and of other chronic diseases has led to the hypothesis that particular phytochemicals are responsible for the preventive effects observed. In the research conducted, numerous bioactive compounds have been isolated and identified, and their potential health-promoting effects evaluated extensively, both *in vitro* and *in vivo*. One of the key problems of research in this field, however, is that purified phytochemicals do not necessarily have the same beneficial health effect as these compounds do when their source is a food or even a complete diet. There is a growing body of evidence that the actions of phytochemicals administered as dietary supplements fail to provide the health benefits that have been observed for diets rich in fruits, vegetables, whole grains, and the like. Although relatively high doses of single bioactive agents may show potent anticarcinogenic effects, the cancer-preventive effects that certain whole foods and diets are shown to have can perhaps better be explained in terms of the chemopreventive properties that interactions between the different dietary ingredients involved create. In this chapter, evidence that bioactive compounds act synergistically is reviewed.

Evaluating the effects of phytochemicals that act synergistically

Carcinogenesis is an extremely complex multistep process in which numerous molecular mechanisms play an important role. Cancer-preventive dietary compounds may interfere at a variety of different levels with these processes. Table 3.10 summarizes various mechanisms by which phytochemicals can modulate the risk of cancer [1,2]. The combinations of phytochemicals that natural foods contain can reduce the risk of cancer by affecting different overlapping and complementary mechanisms. Isolated and purified compounds, in contrast, may lose their biological activity or fail to behave in the same way as in the complex matrix that the original item of food represents. This can be illustrated by the effects of increased intake of carotenoids and vitamin C in diets high in fruits and green and yellow vegetables, which are believed to have cancer-preventive effects. The intended positive effects of an increased intake of β-carotene or vitamin C as dietary supplements, in contrast, are far from certain. Some studies show no reduced incidence of cancer as a result of taking supplements of vitamin C [3] or β-carotene [4], and there are even reports of increased occurrence of lung cancer in smokers receiving dietary β-carotene supplements [5,6]. In addition to characterizing the chemopreventive effects of individual compounds, therefore, evaluation of the effects of synergistically acting phytochemicals is needed.
Table 3.10. Proposed mechanisms by which dietary phytochemicals can prevent cancer

<table>
<thead>
<tr>
<th>Category</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant activity</td>
<td>Scavenging of free radicals and reduction of oxidative stress</td>
</tr>
<tr>
<td>Inhibition of cell proliferation</td>
<td></td>
</tr>
<tr>
<td>Induction of cell differentiation</td>
<td></td>
</tr>
<tr>
<td>Inhibition of oncogene expression</td>
<td></td>
</tr>
<tr>
<td>Induction of tumour-suppressor gene expression</td>
<td></td>
</tr>
<tr>
<td>Induction of cell-cycle arrest</td>
<td></td>
</tr>
<tr>
<td>Induction of apoptosis</td>
<td></td>
</tr>
<tr>
<td>Inhibition of signal transduction pathways</td>
<td></td>
</tr>
<tr>
<td>Enzyme induction and enhancing detoxification</td>
<td></td>
</tr>
<tr>
<td>Phase II enzymes</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td></td>
</tr>
<tr>
<td>Enzyme inhibition</td>
<td></td>
</tr>
<tr>
<td>Phase I enzyme (blocking the activation of carcinogens)</td>
<td></td>
</tr>
<tr>
<td>Cyclooxygenase-2</td>
<td></td>
</tr>
<tr>
<td>Inducible nitric oxide synthase</td>
<td></td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td></td>
</tr>
<tr>
<td>Enhancement of immune functions and surveillance</td>
<td></td>
</tr>
<tr>
<td>Antiangiogenesis</td>
<td></td>
</tr>
<tr>
<td>Inhibition of cell adhesion and invasion</td>
<td></td>
</tr>
<tr>
<td>Inhibition of nitrosation and nitration</td>
<td></td>
</tr>
<tr>
<td>Prevention of DNA adduct formation or DNA intercalation</td>
<td></td>
</tr>
<tr>
<td>Regulation of steroid hormone metabolism</td>
<td></td>
</tr>
<tr>
<td>Regulation of estrogen metabolism</td>
<td></td>
</tr>
<tr>
<td>Antibacterial and antiviral effects</td>
<td></td>
</tr>
</tbody>
</table>

Modified from Liu et al. [2].

**Synergistic effects of combinations of various polyphenols**

A number of studies report enhanced chemopreventive effects of mixtures of polyphenols from green tea or other dietary sources. Table 3.11. presents a selection of relevant studies concerning such synergistic effects. Suganuma et al. [7] reported that the effects of tritium-labelled epigallocatechin gallate (EGCG) being incorporated into human lung cancer cells were enhanced by epicatechin (EC), another green tea polyphenol, one without a galloyl moiety. Epicatechin was found to promote the occurrence
of EGCG-induced apoptosis and to inhibit not only growth of PC-9 lung tumour cells but also the release of tumour necrosis factor-α. These effects, induced by a tea polyphenol having a galloyl moiety, were also enhanced in a dose-dependent way by EC. The study showed that the synergistic effects of two green tea polyphenols could result in the tea as a whole becoming a more efficient anticarcinogenic mixture than if supplementation of EGCG alone had been provided.

Table 3.11. Selection of studies of the synergistic effects of mixtures of polyphenols and of combinations of these with other types of phytochemicals

<table>
<thead>
<tr>
<th>Combination of compounds</th>
<th>Synergistic effect</th>
<th>Mechanisms involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG and EC, sulindac or tamoxifen</td>
<td>Inhibition of growth of human lung cancer cells</td>
<td>Enhanced cellular uptake of EGCG, enhanced apoptosis, reduced release of TNF-α</td>
<td>Suganuma et al. [7]</td>
</tr>
<tr>
<td>EGCG and EC</td>
<td>Inhibition of cell growth and induction of apoptosis in gastric carcinoma cells</td>
<td>Increased production of caspases-3, -8 and -9; Extracellular production of oxygen species</td>
<td>Horie et al. [8]</td>
</tr>
<tr>
<td>EGCG, EC, EGC and ECG</td>
<td>Modulation of CYP1A1 expression in human hepatocytes</td>
<td>Antagonism of TCDD-induced transcription of human CYP1A1 via interaction with the Ah-receptor</td>
<td>Williams et al. [10,11]</td>
</tr>
<tr>
<td>Green/black tea and soy (SPC)*</td>
<td>Inhibition of prostate tumours, tumour weight and metastasis</td>
<td>Reduction in serum levels of testosterone and DHT</td>
<td>Zhou et al. [12]</td>
</tr>
<tr>
<td>Green/black tea and soy (SPC)</td>
<td>Inhibition of breast tumour cell growth</td>
<td>Inhibition of tumour angiogenesis, reduced levels of estrogen receptor-α protein and of serum IGF-I.</td>
<td>Zhou et al. [13]</td>
</tr>
<tr>
<td>Green tea infusions and grape or grape skin extracts</td>
<td>Reduced tumour cell growth</td>
<td>Inhibition of tNOX, induction of apoptosis,</td>
<td>Morré and Morré [16]</td>
</tr>
<tr>
<td>Polyphenols, vitamin E, A and β-carotene</td>
<td>Reduced oxidative stress</td>
<td>Reduced formation of lipid hydroperoxides and malondialdehydes; reduced co-oxidation of vitamins E, C and β-carotene</td>
<td>Gorelik et al. [18]</td>
</tr>
<tr>
<td>EGCG, EC, EGC and ECG or gallic acid and α-tocopherol</td>
<td>Reduced oxidative stress in micelles and human LDL</td>
<td>Reduction in α-tocopheryl radicals, trapping of lipid peroxyl radicals and regeneration of vitamin E</td>
<td>Zhou et al. [19], Liu et al. [22]</td>
</tr>
</tbody>
</table>
Synergistic effects of green tea catechins, both on the inhibition of cell growth and the induction of apoptosis, were also found in gastric carcinoma cells [8]. Various gastric cell lines were shown to differ in their susceptibility to EGCG treatment. EC alone had virtually no effect on carcinomic cell growth or induction of apoptosis, but it had a significant synergistic effect on the induction of apoptosis when combined with other catechins. After this combined treatment, the activity levels of caspases 3, 8 and 9 became elevated, indicating them to be involved in catechin-induced apoptosis. Interestingly, catalase was found to block the synergistic effects of EC and EGCG, suggesting that the reactive oxidative species and production of hydrogen peroxide play a part in the synergistic mechanisms here [8].

Since the cytochrome P450 (CYP) enzymes are responsible for the metabolism of many environmental carcinogens, the modulation of their expression and activity by phytochemicals is a potential mechanism by which the risk of cancer can be reduced. Some of the cytochrome P450 genes are expressed constitutively, whereas others are inducible by xenobiotic compounds or by phytochemicals. Enzyme induction usually enhances detoxification, but under some circumstances substrates may be activated to become mutagens, carcinogens or cytotoxic substances [9]. The induction of CYP1A enzymes by PAH and by dioxins such as TCDD occurs at the transcription level and is mediated by the cytosolic aryl hydrocarbon receptor (AhR) [9]. Williams et al. [10] demonstrated that complex green tea extracts exert mixed agonist/antagonist activity on the Ah-receptors, whereas EGCG acts as a strict AhR antagonist. The authors concluded that the modulation of human CYP1A1 expression by green tea extracts cannot be attributed to the action of a single tea catechin, being due instead to the effects of the complex mixture involved. Co-treatment of human hepatocytes with TCDD and different tea catechin mixtures inhibited synergistically both TCDD-induced CYP1A-promoter-driven luciferase reporter activity (in the HepG2 cells) and CYP1A1 expression (in the HepG2 cells and the primary human hepatocytes). The optimal degree of synergy was achieved by a combination of the four major tea catechins — EC, EGCG, epigallocatechin (EGC), and epicatechin gallate (ECG) — and it was not found to be improved further by the addition of other compounds [11].

Table 3.11. Selection of studies of the synergistic effects of mixtures of polyphenols and of combinations of these with other types of phytochemicals — cont.

<table>
<thead>
<tr>
<th>Combination of compounds</th>
<th>Synergistic effect</th>
<th>Mechanisms involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 red wine polyphenols</td>
<td>Increased antioxidant potential</td>
<td>Regeneration of phenoxy radicals by phenolic compounds</td>
<td>De Beer et al. [25] Jørgensen et al. [27]</td>
</tr>
<tr>
<td>Different types of vegetables (cauliflower, carrots, peas and unions)</td>
<td>Up- and down-regulation of genes involved in carcinogenesis, interpreted mainly in preventive terms</td>
<td>No indication of specific synergetic mechanisms</td>
<td>Van Breda et al. [23,24]</td>
</tr>
</tbody>
</table>

* SPC — Soy Phytochemical Concentrate.
Synergistic effects of polyphenols and other phytochemicals

In two different studies, Zhou et al. investigated with use of mice models the potential synergistic effects of a combination of bioactive tea components and soy phytochemicals on androgen-sensitive human prostate tumours and estrogen-dependent human breast carcinoma [12,13]. A phytochemical soy concentrate (SPC) and green and black tea infusions were employed (Table 3.11.). In further investigations it was shown that bioactive compounds in tea (particularly EGCG [14]) and soy (the soy isoflavone genistein as well as SPC) inhibit growth of prostate cancer and tumour metastasis in vivo [15]. The synergistic inhibition of the progression and metastasis of prostate tumours by use of the combination of green tea and SPC was found to be associated with effective reduction in the serum levels of both testosterone and dihydrotestosterone, the latter a biologically more active metabolite of testosterone and a prerequisite for the development of benign prostatic hyperplasia and prostate cancer [12]. In an immune-deficient mouse model in which MCF-7 human breast cancer cells were implanted, SPC in combination with green tea showed a synergistic inhibition of tumour cell growth. This was accompanied by inhibition of tumour angiogenesis, reduced expression of estrogen-receptor (ER) alpha and reduced serum levels of the insulin-like growth factor (IGF)-I, all of these being factors in breast cancer development. The modulation of these two different mechanisms may be an explanation of the synergistic effects of the combined phytochemicals [13].

A tumour-specific growth protein possessing NADH oxidase activity (tNOX) has emerged as a potential target of the anticancer action of plant polyphenols and flavonoids [16]. NOX proteins are located at the cell surface and are responsible for the increase in cell size that occurs following cell division [17]. Cells in which NOX activity is blocked, such as by phytochemicals, are unable to enlarge. They cease to divide and eventually undergo apoptosis. Recently, Morré and Morré [16] showed there to be an exceptionally strong (10-fold) synergy between grape polyphenols and tea catechins in the inhibition of tNOX. The strongest synergistic activity was found for ethanol extracts of grape skins, whereas no activity was detected for extracts of grape seeds, indicating that the effects were not caused by resveratrol, which is found predominantly in the seeds. These results suggest that more effective cancer prevention and cancer therapy could be achieved by use of combinations of different phytochemicals.

Polyphenols and dietary antioxidant vitamins can also have synergistic inhibitory effects on lipid peroxidation and on the co-oxidation of dietary antioxidants. It was demonstrated with use of simulated stomach fluid that phytochemicals can prevent the build-up of oxidized lipid products (lipid hydroperoxides and malondialdehyde) as well as the destruction of vitamin E and β-carotene (and of vitamin C too, though to a lesser extent) [18]. In the gastric fluid, vitamin C can enhance the activity of polyphenols through a synergistic antioxidant effect. In line with this, the authors suggested that the antioxidant network in the stomach can decrease the level of hydroperoxides and other cytotoxic compounds, increasing at the same time the amounts of vitamin antioxidants reaching the blood system. The authors indicated that the resulting synergistic increase
in the systemic antioxidant effect might also explain the French paradox (the fact that people in France suffer from relatively low incidence of coronary heart disease, despite their unhealthy dietary habits and high consumption of alcohol in the form of red wine) and the beneficial effects of Mediterranean and Japanese diets that contain complex combinations of polyphenols and other antioxidants [18]. By studying the kinetics of the reaction of α-tocopheroyl radicals with green tea polyphenols by means of stopped-flow electron paramagnetic resonance, Zhou et al. [19] demonstrated clearly that several green tea polyphenols (EC, EGCG, EGC, ECG and gallic acid) can effectively reduce α-tocopheroxy radicals so as to allow α-tocopherol to be regenerated. These green tea polyphenols were also found to trap the initiating radicals (ROO•) as well as the propagating lipid peroxyl radicals (LOO•). It is particularly the elimination of the pro-oxidant effect of vitamin E (or the so-called tocopherol-mediated peroxidation) that can occur in the absence of other oxidants [20]. This, combined with an α-tocopherol regenerating reaction involving coexisting antioxidants, plays a major role in enhancing the antioxidant efficiency of vitamin E. These combined effects may also explain the synergistic antioxidant effects of the polyphenols in tea and of the α-tocopherol in both the micelles and human low-density lipoprotein that members of this same research group have reported [21,22].

**Synergistic effects of whole foods and complex mixtures of compounds**

In addition to the synergistic effects of several individual compounds on biomarkers of cancer prevention, the synergistic effects of taking whole foods or complex mixtures of compounds have been reported. In two recent animal studies on the effects of vegetable consumption on the modulation of gene expression, it was found that most of the genes that were differentially expressed before and after vegetables were consumed, the changes in gene expression could be interpreted as a cancer preventive effect [23,24]. The effects of consuming one of four different vegetables on gene expression in the colon and the lungs of female C57B16 mice were found to differ from those of consuming a mixture of all four vegetables at once. Consumption of the mixture of the vegetables was able to modulate genes which were not significantly modulated by one of the specific vegetables present in the mixture. On the other hand, the individual vegetables were able to modulate genes which were not significantly modulated by the mixture, indicating that combinations of different foods containing different complex mixtures of phytochemicals can also have an antagonistic effect on gene expression.

Other examples of the assessment of synergistic effects in complex mixtures are to be found in studies aimed at unraveling the antioxidant capacity of red wines. In one study, the Trolox equivalent antioxidant capacity (TEAC) value and phenolic composition were determined for a large number of pinotage wines [25]. When the contributions of the separate phenolic compounds were calculated, it was found that only 11 to 24% of the TEAC values could be explained in terms of the sum of the values for the individual compounds. Taking account of the different mixtures found of the 12 phenolic compounds that were present, and assuming their concentrations to be those typical for red wines,
indicated 16 to 23% of the antioxidant activity to be synergistic. This implies that, in addition to the synergistic effects between the different phenolic compounds, synergistic effects between these and the other wine constituents may have contributed to the TEAC values. The author excludes a potential role of sulphur dioxide in the regeneration of phenolic compounds from their phenoxy radicals as it does not contribute to the total antioxidant potential at the concentrations normally present in red wines [26]. Jørgensen et al. [27] demonstrated, however, the regeneration of quercetin from its phenoxy radical by the presence of (+)-catechin. This suggests that the regeneration of phenolic compounds from phenoxy radicals is a mechanism that can contribute to the synergistic effects observed.

Other synergistic effects
In addition to investigating the synergistic effects of various phytochemicals, studying the combined effects of dietary factors and therapeutic compounds may be a promising approach toward optimising pharmacological strategies for the prevention and therapy of cancer [1,28]. Administering multiple agents can increase the efficacy and potency of the chemopreventive measures taken and reduce toxic side-effects. Various combinations of drugs have been proposed for further clinical development based on their synergistic activity as shown in vitro or in animal studies. Use of retinoids in combination with such SERMs (selective estrogen receptor modulators) as tamoxifen or raloxifene [29,30] are examples of this. Also, the effects reported by Suganuma et al. [7] of using EGCG to induce apoptosis in human lung cells in vitro have been synergistically enhanced by use of cancer preventive agents such as sulindac and tamoxifen. This effect has also been evaluated in an animal study in which treatment of rats with a combination of EGCG and sulindac was found to reduce aberrant crypt formation after their being treated with azoxymethane for colon carcinoma [31]. In that study, EGCG and sulindac were also found to synergistically enhance apoptosis. The results confirm earlier findings showing a combination of phytochemicals and therapeutic agents to provide more effective therapy for cancer than the therapeutic agents alone, its also reducing side effects, particularly those of sulindac, without a loss in treatment potency. A review of the effects of various combinations of chemotherapeutic and chemopreventive approaches in dealing with cancer has appeared recently [28].

Conclusions
An increasing number of in vitro and in vivo studies indicate that combinations of dietary chemopreventive agents can result in a significant level of activity being achieved at concentrations at which any single agent is virtually inactive. The fact that many of these phytochemicals act synergistically may why some food items or diets show cancer preventive effects that cannot be explained on the basis of their separate bioactive ingredients alone. Although our understanding of the molecular mechanism behind the observed combinatorial effects of these chemopreventive agents is still limited, it appears
that many different combinations of complementary modes of action may be involved. There is a clear need for further study of the synergistic effects of dietary phytochemicals. This may contribute to the improvement of cancer treatment and cancer prevention. Especially the development of new dietary supplement regiments, cancer therapies and nutraceuticals can benefit from improved insight into the mechanisms behind the synergistic effects that both natural and synthetic chemopreventive compounds show.

References


3.6. Probiotics and prebiotics — potential anticarcinogenic food components

Joseph Rafter
Department of Medical Nutrition, Karolinska Institute, Huddinge, Sweden

Introduction

An example of a functional food, which has been the focus of intense research activity in recent years are probiotics — ‘living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition’ [1,2]. Probiotics usually refer to highly selected lactic acid bacteria, e.g. *Lactobacillus* spp. *Bifidobacterium* spp. and *Streptococcus* spp., with defined gut survival properties and associated biological activities and which can be ingested in fermented milk products or as a supplement. The list of healthful effects attributed to probiotic bacteria is extensive [3] and includes: alleviation of lactose intolerance symptoms; serum cholesterol reduction; anticancer effects; alleviating constipation; relieving vaginitis to name but a few. The vast majority of studies on the anticancer effects deal with colorectal cancer (CRC), although there are some on breast [4] and bladder cancer [5].

Mortality from CRC is second only to that of lung cancer in men and breast cancer in women and has shown little sign of decreasing in the last 20–30 years. Diet makes an important contribution to CRC risk [6] implying that risks of CRC are potentially reducible. Evidence from a wide range of sources supports the view that the colonic microflora is involved in the etiology of CRC. This has led to intense interest in factors, such as probiotics, prebiotics (‘a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve host health’) [3] and synbiotics (combinations of pro- and prebiotics), that can modulate gut microflora and its metabolism. Evidence for protective effects of pro- and prebiotics on cancer is derived from *in vitro* studies, animal models, epidemiology and human intervention studies.

Overall, the supportive evidence is stronger for probiotics than prebiotics (possibly because the latter have only recently come to prominence) and is recently suggestive that synbiotics are more effective than either pro- or prebiotics alone. The evidence from animal studies provides strongest support for anticancer effects and data from human studies (epidemiology and experimental) are limited.

Evaluation of anticarcinogenic dietary effects

Evidence from human studies

Consumption of lactobacilli by healthy volunteers has been shown to reduce the mutagenicity of urine and faeces associated with the ingestion of carcinogens in cooked meat. Administration of *L. acidophilus* to eleven volunteers on a fried meat diet known to increase faecal mutagenicity, resulted in a lower faecal mutagenic activity after 3 days.
compared to faecal mutagenic activity after 3 days consumption of ordinary fermented milk (although not significant) [7]. High levels of mutagenicity also appeared in urine on days 2 and 3 of the fried meat and ordinary fermented milk dietary regimen. During *L. acidophilus* administration, the urinary mutagenic activity on days 2 and 3 was significantly lower compared to the ordinary fermented milk period. In most cases, an increase in the number of faecal lactobacilli corresponded to a lower mutagen excretion, particularly in urine. Hayatsu and Hayatsu [8] also demonstrated a marked suppressing effect of orally administered *L. casei* on the urinary mutagenicity arising from ingestion of fried ground beef in the human.

As yet, there are few epidemiological studies addressing the association between fermented dairy products and colorectal cancer. Consumption of large quantities of dairy products such as yoghurt and fermented milk containing *Lactobacillus* or *Bifidobacterium* may be related to a lower incidence of colon cancer [9]. An epidemiological study performed in Finland demonstrated that, despite the high fat intake, colon cancer incidence was lower than in other countries because of the high consumption of milk, yoghurt, and other dairy products [10,11]. In two population-based case-control studies of colon cancer, an inverse association was observed for yoghurt [12] and cultured milk consumption [13], adjusted for potential confounding variables. In another case control study, an inverse relationship for yoghurt consumption with risk of large colon adenomas in men and women was reported [14]. It can also be mentioned that an inverse relationship has been demonstrated between the frequency of consumption of yoghurt and other fermented milk products and breast cancer in women [4,15]. On the other hand, two companion American prospective studies, the 1980–1988 follow-up of the Nurses’ Health Study and the 1986–1990 Health Professionals follow-up study, did not provide evidence that intake of dairy products is associated with a decreased risk of colon cancer [16]. In a cohort study in the Netherlands, it was shown that the intake of fermented dairy products was not significantly associated with colorectal cancer risk in an elderly population with a relatively wide variation in dairy product consumption, although a weak non-significant inverse association with colon cancer was observed [17]. In summary, it would appear that the case control studies indicate protective effects while the prospective studies do not.

In conclusion, data from human intervention studies are of paramount importance in providing evidence that probiotics, prebiotics or fermented milk consumption are causally related to reduction in cancer risk. Thus, this is an area of high priority for future studies. Presently however the lack of well validated biomarkers for colon cancer limits the relevance of such studies although a wide range of potential biomarkers of risk are under development. Once such markers are available, it will become possible to perform studies in healthy volunteers, at-risk groups and patients. It will be important to define dose and time relationships and it would appear at present, from animal studies, the most profitable approach would be to use combinations of pro- and prebiotics. There will also be, in the near future, the opportunity to exploit genomics and proteomics in investigations of effects of pro/prebiotics on gene expression and post-transcription
events in colonic biopsies and to identify human groups responsive to pro/prebiotic intervention. It will also be particularly important to use data on mechanisms of action to develop hypothesis based intervention studies in humans.

Of relevance here is a clinical trial which is presently ongoing i.e. to examine the effect of a synbiotic preparation on colon cancer risk biomarkers in humans (SYNCAN project, funded by EU, and involving 8 research centres in Europe; http://www.syncan.be). It involves a twelve-week randomised, double blind, placebo controlled trial of a food supplement containing *Lactobacillus GG*, *Bifidobacterium* Bb-12 and Raftilose Synergyl in adenoma patients. In this study, all of the “state of the art” colon cancer risk biomarkers, including colonic mucosal markers, faecal water markers and immunological markers, are being measured. It is hoped that the results of this study will provide much needed information on the cancer protective effects of synbiotics in humans and supply us with additional valuable information on the underlying mechanisms.

**Evidence from laboratory animal studies**

There are several good animal models for colon cancer which have proved useful for identifying dietary factors which may protect us against the development of this tumour. End points used are the tumours themselves or early lesions, such as aberrant crypt foci (ACF). ACF are putative preneoplastic lesions from which adenomas and carcinomas may develop. In recent years, there have been many studies, using these models, which clearly demonstrate a protective effect of dietary supplements of lactic acid bacteria against colon tumour development.

Oral administration of lactic acid bacteria has been shown to effectively reduce DNA damage, induced by chemical carcinogens, in gastric and colonic mucosa in rats. Pool-Zobel et al. [18] reported, using the comet assay, that *Lactobacillus acidophilus*, *L. gasseri*, *L. confusus*, *Streptococcus thermophilus*, *Bifidobacterium breve* and *B. longum* were antigenotoxic toward *N’*-nitro-*N*-nitrosoguanidine (MNNG). These bacteria were also protective toward 1,2-dimethylhydrazine (DMH)-induced genotoxicity. Metabolically active *L. acidophilus* cells, as well as an acetone extract of the culture, prevented MNNG-induced DNA damage, while heat-treated *L. acidophilus* was not antigenotoxic. Among different cell fractions from *L. acidophilus*, the peptidoglycan fraction and whole freeze-dried cells were antigenotoxic.

Certain strains of lactic acid bacteria have also been found to prevent putative preneoplastic lesions or tumours induced by carcinogens. Goldin et al. [19] showed that a specific strain of *L. casei* subsp. *rhamnosus* designated GG can interfere with the initiation or early promotional stages of DMH-induced intestinal tumourigenesis and that this effect is most pronounced for animals fed a high-fat diet. Overnight cultures of *L. acidophilus* also inhibited the formation of ACF, induced by azoxymethane (AOM) [20]. Although *B. adolescentis* culture and its supernatant did not show an inhibitory effect in this study [20], feeding of bifidobacteria suppressed the ACF formation induced by AOM [21,22] or DMH [23,24]. Consumption of *B. longum* or inulin was associated with a decrease in AOM-induced colonic small ACF in rats and combined administration
significantly decreased the incidence of large ACF [25]. In addition, it has been reported that colonisation of bacteria with an ability to produce genotoxic compounds and high β-glucuronidase activity enhanced progression of ACF induced by DMH in rats, and that the additional colonisation of B. breve reduced the number of ACF with four or more crypts/focus and crypt multiplicity which are reliable predictors of malignancy [26].

Reddy and Rivenson [27] reported that lyophilised cultures of B. longum administered in the diet to rats inhibited liver, colon and mammary tumours, induced by the food mutagen 2-amino-3-methyl-3H-imidazo(4,5-f)quinoline (IQ). Goldin and Gorbach [28] showed that dietary supplements of L. acidophilus not only suppressed the incidence of DMH-induced colon carcinogenesis but also increased the latency period in rats. Feeding of fermented milk increased the survival rate of rats with chemically induced colon cancer [29]. Dietary administration of a lyophilised culture of B. longum resulted in a significant suppression of colon tumour incidence and tumour multiplicity and also reduced tumour volume induced by AOM in rats [30]. Ingestion of B. longum also significantly inhibited AOM-induced cell proliferation, ornithine decarboxylase activity and expression of the ras-p21 oncoprotein. Recently, there was a report on the anti-tumourigenic activity of the prebiotic inulin, enriched with oligofructose, in combination with the probiotics Lactobacillus rhamnosus and Bifidobacterium lactis in the AOM-induced colon carcinogenesis rat model [31]. The authors concluded that, while a possible protective effect of probiotics was observed, the results indicated that the prebiotic decreased AOM-induced carcinogenesis. The mechanisms by which they act are less clear, but the data presented suggested that they may act through a combination of mechanisms involving an increase in short-chain fatty acid (SCFA) production, lower proliferative activity and a variation in the expression of some enzymes involved in the pathogenesis of colon cancer.

There is additional direct evidence for anti-tumour activities of lactic acid bacteria obtained in studies using pre-implanted tumour cells in animal models. It has been demonstrated that feeding of fermented milk or cultures containing lactic acid bacteria inhibited the growth of tumour cells injected into mice [32,33]. Sekine et al. [34] using whole peptidoglycan isolated from B. infantis strain ATCC15697, reported that a single subcutaneous injection significantly suppressed tumour growth. In addition, five intralesional injections resulted in 70% tumour regression in the mice.

More recently, mindful of the fact that the composition and metabolic activities of the intestinal flora of experimental animals are significantly different from those of humans [35], we exploited human flora associated (HFA) mice to test the effects of a probiotic mixture on a parameter of relevance for colon carcinogenesis i.e. DNA adduct formation [36]. Indeed, the results from a previous report from our laboratory, demonstrated that human intestinal microflora had different effects than mouse microflora concerning DNA adduct formation after exposure to mutagens [37]. The probiotic mixture, Biothree®, used in this study contained Streptococcus faecalis T-110, Clostridium butyricum TO-A and Bacillus mesentericus TO-A, which are acid resistant in contrast to most bacteria, which do not survive contact with gastric acid. It has been
reported that *S. faecalis* T-110 and *C. butyricum* TO-A showed strong symbiosis with each other and the growth of enteropathogens (*enterotoxigenic Escherichia coli*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*, *C. difficile* and *C. botulinum*) was inhibited in mixed cultures of *S. faecalis* T-110 and *C. butyricum* TO-A [38]. It has also been reported that *B. mesentericus* TO-A stimulated the growth of *Bifidobacterium* by producing 3,3-dihydroxyazetidine [39,40]. Biothree® is used as a clinical therapy in Japan. It is effective for the improvement of symptoms caused by abnormal intestinal flora, i.e. diarrhoea and constipation. Interestingly, the results of this study demonstrated that the above probiotic mixture had an effect to significantly decrease the DNA adduct formation in the colonic epithelium induced by the food mutagen 2-amino-9H-pyrido[2,3-b]indole (2-amino-alpha-carboline; AAC), given by gavage. Two possible mechanisms may be involved: reduction of direct exposure to AAC and/or induction of DNA repair of the DNA adducts in the colonic epithelium.

**Mechanisms by which probiotic bacteria may be inhibiting colon cancer**

The precise mechanisms by which lactic acid bacteria may inhibit colon cancer are presently unknown. However, such mechanisms might include: alteration of the metabolic activities of intestinal microflora; alteration of physicochemical conditions in the colon; binding and degrading potential carcinogens; quantitative and/or qualitative alterations in the intestinal microflora incriminated in producing putative carcinogen(s) and promoters (e.g. bile acid-metabolizing bacteria); production of antitumourigenic or antimutagenic compounds; enhancing the host's immune response; effects on physiology of the host.

**Conclusion**

Many healthful effects are attributed to the probiotic bacteria and some of these effects have more scientific support than the anticancer effect. The strongest evidence for anticancer effects of probiotics comes from animal studies and evidence from human studies (epidemiology and experimental) is still limited. An important goal for the future should be carefully designed human clinical trials to corroborate the wealth of experimental studies. Also, as mentioned above, there are several possible mechanisms which might explain how lactic acid bacteria might protect against tumour development in the colon. It is possible that different strains target different mechanisms. All of the mechanisms have various degrees of support, mainly originating from *in vitro* and animal experiments and some of them even have some support from human clinical studies. Thus, more work needs to be done to identify the specific strains and strain characteristics responsible for specific antitumour effects and the mechanisms by which these effects are mediated. However, even with the above reservations in mind and mindful of the limited number of human studies available, the use of lactic cultures for human cancer suppression is interesting, holds promise and certainly deserves more scrutiny.
References


