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REVIEW

Laboratory and clinical studies of cancer chemoprevention by antioxidants in berries

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ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

AOM, azoxymethane; AP-1, activator protein-1; B(a)P, benz[a]pyrene; BE, Barrett’s esophagus; BPDE, benzo(a)pyrene diol-epoxide; COX-2, cyclooxygenase-2; DMBA, 7,12-dimethylbenz[a]anthracene; LBR, lycophilized black raspberry; NF-κB, nuclear factor kappa B; NMBA, N-nitrosomethylbenzylamine; 8-OHG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

Scavenging ROS and inhibition of oxidative DNA adduct formation

The ability of different berry juices (blackberry, strawberry, raspberry, cranberry and blueberry) to scavenge specific ROS including superoxide radicals (O2−), hydrogen peroxide (H2O2), hydroxyl radical (OH•) groups on aromatic rings. Phytochemicals are non-nutritive constituents produced by secondary metabolism in plants. They are known to defend plants against predators, microbial infections and ultraviolet light, to regulate metabolic pathways and provide color and flavor to the plant (20). The bioactive phytochemicals in berries fall into several structural and chemical classes including phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavonoids (anthocyanins, flavans, flavonols), condensed tannins (proanthocyanins), hydrolysable tannins (ellagitannins and gallotannins), stilbenoids, lignans, triterpenes and sterols (19). The structures of these berry bioactives are shown in Figure 1, and their chemical features, biological activities and content in different berry types have been described in detail by Seeram (19). Among the most prevalent compounds in berries are the anthocyanins and ellagitannins that, collectively, are responsible for much of their antioxidant activity (21–24). In addition to the bioactives listed in Figure 1, berries contain many other constituents known to exhibit cancer-preventive effects including vitamins, such as A, C, E and folic acid, and minerals, such as calcium, selenium and zinc (25). The inhibitory effects of most of these constituents are due, at least in part, to their antioxidant capacity.

Labaratory studies of the anticancer effects of berry bioactives

Berry bioactives have many roles in cancer prevention. These include protection against oxidative DNA damage by the scavenging of ROS, inhibition of the formation of carcinogen-induced DNA adducts, enhancement of DNA repair, inhibition of carcinogen-induced tumorigenesis in animals and modulation of signaling pathways involved with cellular proliferation, apoptosis, inflammation, angiogenesis and cell cycle arrest. The following sections will summarize laboratory studies, both in vitro and in vivo, to determine the mechanisms by which berries and berry components exert anticarcinogenic effects.

Abbreviations: AOM, azoxymethane; AP-1, activator protein-1; B(a)P, benzo(a)pyrene; BE, Barrett’s esophagus; BPDE, benzo(a)pyrene diol-epoxide; COX-2, cyclooxygenase-2; DMBA, 7,12-dimethylbenz[a]anthracene; LBR, lycophilized black raspberry; NF-κB, nuclear factor kappa B; NMBA, N-nitrosomethylbenzylamine; 8-OHG, 8-hydroxy-2′-deoxyguanosine; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

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and singlet oxygen \( (\cdot O_2) \) was investigated by Wang and Jiao (Table I) \(^{15}\). They found that the scavenging activity of berries varied as a function of berry type and cultivar, as well as the types of ROS that were scavenged. In general, blackberries had the highest antioxidant capacity for inhibition of \( O_2^- \), \( H_2O_2 \) and \( \cdot OH \). Strawberries were second best for these same three radicals; however, they were superior to blackberries in the scavenging of \( \cdot O_2 \). Cranberries had the lowest inhibition of \( H_2O_2 \) activity, whereas blueberries had the lowest antioxidant capacity against \( OH^- \) and \( \cdot O_2 \). Thus, there were significant differences among the different berry types in their abilities to scavenge different ROS.

In another study, the antioxidant capacity of Oregon grown blackberries, boysenberries, marionberries, black raspberries and red raspberries was compared using the oxygen radical absorbance capacity (ORAC) assay \(^{26}\). Antioxidant capacity, expressed as Trolox equivalents/gram of fresh berries, was greatest in black raspberries.
followed by boysenberries, red raspberries, blackberries and marionberries. The antioxidant capacity of these berries was related to their content of total phenolics and anthocyanins. It should be mentioned, however, that caution should be used in drawing conclusions regarding the antioxidant capacity of different berry types since, in addition to type and cultivar, the antioxidant capacity of berries can vary as a function of the climate and soil conditions in which they are grown, degree of ripeness, storage temperature, methods used for industrial processing and the specific assay used to measure antioxidant capacity (27–29).

In addition to the direct scavenging of ROS, the antioxidant capacity of berries is also related to their content of antioxidant enzymes. For example, the juices of strawberries and blackberries have been shown to contain antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase and glutathione reductase, and the activities of these enzymes is positively correlated with antioxidant capacity of the berries (30). Thus, the ability of berry bioactives to scavenge ROS, coupled with the presence of antioxidant enzymes in berries, may result in attenuation of ROS concentration to maintain an intracellular oxidation and reduction (redox) balance in the berries themselves (19). It remains to be determined whether these berry enzymes exhibit antioxidant activity in mammals because it is not known whether they remain functional in the gastrointestinal tract.

The prevention of ROS-induced DNA damage is probably a first line of defense against the multistage process of carcinogenesis. Most studies with berry-based antioxidants have demonstrated their protective effects against oxidative DNA damage in human lymphocytes (31,32). For example, pretreatment of freshly isolated human lymphocytes from non-smokers with quercetin (1–100 μM) for 1 h was shown to protect against oxidative DNA damage induced by H2O2 as measured by the Comet assay (31). Similar findings were reported by Duthie et al. (32), who treated freshly isolated human lymphocytes with quercetin and myricetin. Inhibition of H2O2-induced DNA damage was prevented by quercetin at concentrations ranging from 10 to 50 μM and by myricetin at concentrations of ≥100 μM. In one animal study, the administration of 2.5, 5 and 10% lyophilized black raspberry (LBR) diets, which are abundant in anthocyanins and other antioxidants, given to rats treated with the colon carcinogen, azoxy-

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>% Inhibition</th>
<th>O2−</th>
<th>H2O2</th>
<th>OH</th>
<th>‘O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blackberry</td>
<td>64.3</td>
<td>66.3</td>
<td>72.0</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>Blueberry</td>
<td>60.1</td>
<td>61.2</td>
<td>58.7</td>
<td>7.71</td>
<td></td>
</tr>
<tr>
<td>Cranberry</td>
<td>59.0</td>
<td>59.8</td>
<td>64.2</td>
<td>8.64</td>
<td></td>
</tr>
<tr>
<td>Raspberry</td>
<td>57.3</td>
<td>60.9</td>
<td>66.9</td>
<td>8.88</td>
<td></td>
</tr>
<tr>
<td>Strawberry</td>
<td>64.2</td>
<td>65.3</td>
<td>68.6</td>
<td>15.41</td>
<td></td>
</tr>
<tr>
<td>LSD 0.05</td>
<td>3.02</td>
<td>2.33</td>
<td>1.79</td>
<td>1.16</td>
<td></td>
</tr>
</tbody>
</table>

Significant cultivars: ** **

Table I. Comparison of the mean values of the scavenging capacity of juice from different berry species (blackberry, blueberry, cranberry, raspberry and strawberry) on active oxygen species (O2−, H2O2, OH and ‘O2).

Inhibition of carcinogen-induced DNA adducts

Diets composed of berries and berry constituents that appear to produce reductions in the formation of oxidative DNA adducts have also been shown to inhibit the formation of DNA adducts from chemical carcinogens (Table II). Kresty et al. (25) reported that male rats fed diets containing either 5 or 10% LBRs or 0.04% ellagic acid for 14 days followed by a single injection of the esophageal carcinogen, N-nitrosomethylbenzylamine (N MBA), had reduced levels (73, 80 and 38%, respectively) of O6-methylguanine adducts in the DNA of their esophagi. In later experiments, the feeding of 5 and 10% LBRs to N MBA-treated rats of the same strain for 14–21 days was shown to result in enhanced mRNA expression levels of cytochromes P450 2a2 and 3a13 in the esophagus and of glutathione-S-transferase activity in the liver (37). It remains to be determined whether these effects are responsible for the reduced levels of O6-methylguanine adducts in the esophagus. Similar results were reported by Carlton et al. (38), who found that diets containing 5 or 10% lyophilized strawberries produced similar reductions in O6-methylguanine adducts in the DNA of esophagi from N MBA-treated rats. Casto et al. (39), in a study of oral cancer using Syrian golden hamsters, demonstrated a reduction in 7,12-dimethylbenz(a)anthracene (DMBA)-induced DNA adducts in the cheek pouch after feeding hamsters for 2 weeks with 5% LBRs. The adducts in berry-fed hamsters were reduced by 29 and 55% at 24 and 48 h, respectively, after topical application of DMBA to the surface of the cheek pouch.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pmol O6-mGua/mg</th>
<th>% Inhibition DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N MBA (0.25 mg/kg)</td>
<td>4.4 ± 0.9</td>
<td>—</td>
</tr>
<tr>
<td>N MBA + 5% STRWs</td>
<td>1.4 ± 0.1*</td>
<td>57</td>
</tr>
<tr>
<td>N MBA + 10% STRWs</td>
<td>1.9 ± 0.7*</td>
<td>73</td>
</tr>
<tr>
<td>N MBA + 5% BRS</td>
<td>1.2 ± 0.3*</td>
<td>73</td>
</tr>
<tr>
<td>N MBA + 10% BRS</td>
<td>0.9 ± 0.2*</td>
<td>80</td>
</tr>
</tbody>
</table>

STRWs, strawberries; BRs, black raspberries; O6-mGua, O6-methylguanine.

LSD, least significant difference.

Data are taken from Wang and Jiao (15).

*Data expressed as percent inhibition of radical (O2−, H2O2, OH and ‘O2) production in the presence of 0.1 ml of fruit juice.

LSD, least significant difference.

1 Data are from Wang and Jiao (15).

2 Data expressed as percent inhibition of radical (O2−, H2O2, OH and ‘O2) production in the presence of 0.1 ml of fruit juice.

3 Data for the thornless blackberry were derived from the mean value calculated from six cultivars (Black Satin, Chester Thornless, Hull Thornless, Smoothstem, Thornfree, Triple Crown); blueberry data were derived from the mean value calculated from five cultivars (Bluecrop, Elliot, wild blueberry #1, wild blueberry #2, wild blueberry #3); cranberry data were derived from the mean value calculated from five cultivars (Ben Learn, Capper, Early Black, Franklin, Howes); raspberry data were derived from the mean value calculated from six cultivars (Allstar, Delmarvel, Earliglow, Lestar, Lester, Red Chief).

4 Significant difference from NMBA controls (P < 0.05).

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Wilms et al. (31) found that quercetin inhibits the formation of DNA adducts in human lymphocytes treated with benzo(a)pyrene [B(a)P]. Freshly isolated lymphocytes were treated with quercetin (0–100 μM), followed by treatment with 1 μM of B(a)P. Benzo(a)pyrene diol-epoxide (BPDE)-DNA adducts, evaluated by 32P-post-labeling, decreased in a dose-dependent manner and were undetectable in cells treated with 100 μM of quercetin. In contrast, when subjects were treated for 4 weeks with a quercetin-rich blueberry/apple juice, freshly isolated lymphocytes were not protected from BPDE-adduct formation following B(a)P treatment. Although the reasons for this discrepancy between in vitro and in vivo results are not known, the rather poor bioavailability of quercetin, and potentially other compounds in blueberry/apple juice, in vivo may be responsible for the differences.

Effects on DNA repair
A number of reports suggest that antioxidants in foods may have a dual action; i.e. reducing oxidative DNA damage by scavenging ROS and promoting DNA repair by enhancing the removal of oxidative adducts from DNA. Most research in support of the latter mechanism has revealed that cells or tissues treated with food constituents, after gamma irradiation, exhibit a faster rate of DNA repair than untreated cells or tissues. For example, Maurya et al. (40) examined the effect of ferulic acid (a phenolic acid in berries) on the induction of DNA strand breaks in peripheral blood leukocytes and bone marrow cells of mice exposed to whole-body γ-radiation. Intraperitoneal administration of ferulic acid (50–100 mg/kg body wt), 1 h prior to 4 Gy γ-radiation, resulted in a dose-dependent decrease in DNA strand breaks in both cell types. Administration of 50 mg/kg body wt of ferulic acid, after whole-body irradiation of mice, resulted in a disappearance of DNA strand breaks at a faster rate than was observed in irradiated controls, suggesting enhanced DNA repair in ferulic acid-treated animals. Similar results were obtained with troxerutin (a derivative of the flavonoid, rutin) by Maurya et al. (41). Thus, at least two compounds found in berries are effective in enhancing DNA repair, as evidenced by a more rapid rejoining of irradiation-induced DNA strand breaks in berry-treated cells. In contrast, another investigation found that resveratrol had only a modest effect. Ellagic acid had no effect, on stimulation of the DNA repair protein, O6-methylguanine-DNA-methyltransferase, in human lymphocytes and in glioblastoma and colon cancer cells (42). These results suggest that these berry compounds may have little or no effect on repair of O6-alkylguanine adducts in DNA.

Inhibition of carcinogen-induced tumors in animals
Berry bioactives have been shown to prevent chemically induced tumors in multiple animal model systems (43). Some of the earliest studies were done with ellagic acid [see summary by Stoner and Mukhtar (44)] that has been shown to inhibit carcinogen-induced cancer in the rodent lung, skin, esophagus, liver, colon and mammary gland. Mechanistic studies indicate that ellagic acid inhibits carcinogen-induced DNA adduct formation, scavenges the reactive metabolites of polycyclic hydrocarbons, stimulates the activity of various isofoms of glutathione-S-transferase and occupies sites in DNA that might otherwise react with carcinogens (45). Ellagic acid has also been shown to inhibit the activity of the enzyme, ornithine decarboxylase, resulting in reduced polyamine levels in mouse skin and an inhibition of skin tumorigenesis (46). Interest in ellagic acid as a chemopreventive agent was significantly reduced, however, by early studies that demonstrated its poor bioavailability in rodents (47,48).

While conducting studies with ellagic acid in the 1980s, our laboratory decided to identify foods in which it might be found (it was not known at that time to be widely present in berries). In examining a series of fruits, we found high concentrations (630–1500 μg/g dry wt) of ellagic acid in blackberries, red raspberries and strawberries and lesser amounts in cranberries (49). The ellagic acid was far more abundant in the pulp and seed of the berries; very little was found in the juice. Based upon these observations, we decided to take a ‘food-based’ approach to cancer prevention and determine if lyophilized berry powders would exhibit inhibitory effects on chemically induced cancer in animals. The rationale for this stems from the fact that berries are 85–90% water; thus, removal of water by the lyophilization process would increase the concentration of ellagic acid in berry powder at least 8- to 9-fold. At the time, we were unaware of the many other chemopreventive agents in berries. In initial tumor bioassays, lyophilized berry powders were evaluated for their ability to inhibit NMBA-induced esophageal carcinogenesis in rats when fed to animals either before, during and after NMBA treatment (complete prevention protocol) or only after treatment with NMBA (postinitiation protocol). The berry powders were fed at 5 and 10% of synthetic AIN-76A diet, which, in humans, would be equivalent to the consumption of 2–4 cups of fresh berries per day.

The chemopreventive effects of lyophilized strawberries, black raspberries and blackberries on NMBA-induced tumors (papillomas), in the rat esophagus, when administered in the complete prevention protocol are given in Table III. Administration of 5 and 10% LBRs and strawberries had no effect on tumor incidence but significantly reduced tumor multiplicity when compared with NMBA controls (P < 0.05) (25,38). Surprisingly, 5% dietary blackberries were effective in reducing tumor multiplicity, whereas 10% blackberries were ineffective (43). There were no significant differences in tumor size among any of the groups in these studies. One mechanism for the chemopreventive effects of strawberries and black raspberries in the complete prevention protocol was that of inhibiting the formation of mutagenic O6-methylguanine adducts in the DNA of esophageal epithelium (Table II).

The chemopreventive effects of lyophilized strawberries and LBRs when administered to rats after treatment with NMBA (postinitiation protocol) are shown in Table IV. When administered postinitiation, LBRs reduced tumor incidence whereas strawberries had no effect on tumor incidence (25,38). Both strawberries and LBRs produced significant reductions in tumor multiplicity; however, the 5% dietary concentrations were more effective than the 10%. The reason(s) for this result is unknown, but did not appear to be due to toxic effects of 10% berries for esophageal epithelium. In the postinitiation protocol, LBRs were found to inhibit the conversion of preneoplastic (dysplastic) lesions to papillomas and to reduce cellular proliferation, as determined by measuring the expression of proliferating cell nuclear antigen in esophageal epithelium, at different time points during the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rats (n)</th>
<th>Tumor incidence (% inhibition)</th>
<th>Tumor multiplicity (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>15</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>10% STRWs</td>
<td>15</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>NMBA control</td>
<td>15</td>
<td>100</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>NMBA + 5% STRWs</td>
<td>15</td>
<td>100</td>
<td>3.1 ± 1.0 (24)*</td>
</tr>
<tr>
<td>NMBA + 10% STRWs</td>
<td>15</td>
<td>80 (20)</td>
<td>1.8 ± 1.4 (56) a</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>15</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>10% BRs</td>
<td>14</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>NMBA control</td>
<td>13</td>
<td>100</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>NMBA + 5% BRs</td>
<td>14</td>
<td>79 (21)</td>
<td>1.9 ± 0.4 (41)*</td>
</tr>
<tr>
<td>NMBA + 10% BRs</td>
<td>13</td>
<td>92 (8)</td>
<td>1.6 ± 0.3 (50)*</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>10</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>10% BBs</td>
<td>10</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>NMBA control</td>
<td>17</td>
<td>82</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>NMBA + 5% BBs</td>
<td>20</td>
<td>75 (8)</td>
<td>1.5 ± 0.4 (46)*</td>
</tr>
<tr>
<td>NMBA + 10% BBs</td>
<td>18</td>
<td>70 (15)</td>
<td>2.3 ± 0.5 (18)</td>
</tr>
</tbody>
</table>

STRWs, strawberries; BRs, black raspberries; BBs, blackberries.
*Statistically significant relative to NMBA controls (P < 0.05). STRW data are taken from Carlton et al. (38), BR data are from Kresty et al. (25) and BB data are taken from Stoner et al. (43).
bioassay (25). In these studies, the inhibitory effects of all lyophilized berry types on tumor multiplicity were not dose related; i.e. 10% berry diets were not twice as effective in reducing the number of NMBA-induced tumors as 5% diets. Similarly, the inhibitory effects of ellagic acid on NMBA-induced tumors in the rat esophagus were not dose related (50). These results suggest that there may be a maximum threshold level in the uptake of dietary berry phenolics into the esophagus, beyond which additional amounts of dietary phenolics have no added benefit.

LBRs were also found to inhibit AOM-induced colon carcinogenesis in rats, when given in a postinitiation protocol (Table V) (33). When fed at 2.5, 5.0 and 10% of the diet, LBRs did not reduce tumor incidence; however, they reduced the total tumor (adenoma + adenocarcinoma) multiplicity by 42, 45 and 71% (P < 0.05 in all groups), respectively, relative to AOM controls. Inhibition of oral cancer by the dietary administration of LBRs was demonstrated in the hamster cheek pouch when administered in the diet before, during and after DMBA treatment (complete prevention protocol) (39) (Table VI). Tumors were induced by painting both cheek pouches of hamsters with DMBA. Hamsters were fed either 5 or 10% LBRs in AIN-76A diet, prior to, during and after treatment with DMBA. Treatment with 5% LBRs caused a significant reduction in tumor multiplicity (P < 0.05), but the reduction with 10% berries was not significant. The reasons for the lack of inhibitory effect in animals administered 10% LBRs were not determined.

A recent study from our laboratory showed that an anthocyanin-rich fraction, derived from LBRs and an alcohol:water (80:20) extract of LBRs, were equally as effective as a 5% LBR diet in reducing NMBA-induced tumors in the rat esophagus (51). The content of anthocyanins in the anthocyanin-rich diet and in the alcohol:water extract diet was essentially identical to that in the 5% LBR diet (~3.5 μmol/g diet). Since all three diets contained the same amounts of anthocyanins and were equally effective in preventing esophageal cancer, these data suggest the importance of the anthocyanins as chemopreventive agents in black raspberries. However, in the same study, another diet containing an alcohol-insoluble (residue) fraction of LBRs, that contained only trace amounts of anthocyanins, was nearly as effective as the anthocyanin-rich and alcohol:water extract diets in preventing NMBA-induced esophageal tumorigenesis. Recent results suggest that the residue fraction contains high levels of ellagitannins (S.S. Hecht, S. Carmella, L-S Wang and G.D. Stoner, unpublished data). Thus, both the anthocyanins and ellagitannins appear to be important for the chemopreventive effects of berries.

In contrast to the above observations, Carlton et al. (52), found that a diet containing 10% lyophilized strawberries was ineffective in reducing lung tumors in mice that were induced by 4-(methylnitrosa-
In vitro studies. Most in vitro studies of the antiproliferative effects of berry extracts and components have been conducted with human tumor cell lines. For example, extracts from strawberry cultivars have been shown to reduce the proliferation of human HepG2 liver tumor cells and of human colon (HT29) and breast (MCF-7) cancer cells in a dose-dependent manner (55). However, there was no correlation between the antioxidant capacity of the cultivars or the levels of anthocyanins and flavonols in the extracts and their antiproliferative effects for the three cell lines. Jurinac et al. (56) compared the effects of a red raspberry extract with ellagic acid alone on the growth of human colon cancer LS174 cells and normal immune competent cells. Results from this comparison indicated that the extract produced a selective inhibitory effect on the growth of the colon cancer cells, which correlated positively with its content of ellagic acid.

Seeram et al. (57) evaluated the antiproliferative effects of methanol extracts of blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry for human oral (KB and CAL27), breast (MCF-7), colon (HT29, HCT116) and prostate (LNCaP) cancer cell lines. The extent of inhibition of cell proliferation was found to vary markedly with both the type of berry extract and the specific cell line studied. The effects of the different berry extracts on cell apoptosis were assessed using HT29 cells that were treated with 200 μg/ml of each berry extract (a significantly higher level than can be achieved in vivo). Only black raspberry and strawberry extracts produced a significant induction of apoptosis. The authors concluded that there are several factors that must be considered when using cell culture studies to rank the chemopreventive activities of berry extracts. These include the cell line being used, the artificially high concentrations of extracts, the stability of extract components in different media, length of treatment time, differential uptake of phenolics and generation of artifacts such as H2O2 that is known to induce apoptosis.

Bioactivity-guided fractionation has been used to identify the active constituents in different berry types. Using bioactivity-guided fractionation, Murphy et al. (58) identified triterpenoid esters in cranberries as important for the inhibition of the growth of human MCF-7 breast, ME180 cervical and PC3 prostate tumor cells. Similarly, Han et al. (59) used bioactivity-guided fractionation to identify chemopreventive phytochemicals in black raspberries. They compared the effects of several extract fractions of black raspberries with those of the berry constituents: ellagic acid, ferulic acid and β-sitosterol on the growth and cell cycle of normal, premalignant and malignant human oral cancer cell lines. An ethanol extract was found to inhibit growth of the premalignant and malignant but not the normal oral cell lines. Ellagic acid inhibited the growth of normal, premalignant and malignant cell lines, whereas ferulic acid and β-sitosterol exhibited selectivity for the premalignant and malignant lines. Thus, these workers concluded that ferulic acid and β-sitosterol were effective bioactives in black raspberries. Using western blot analysis, all treatments were found to affect different cyclins and cyclin-dependent kinases in the premalignant and malignant lines. This led to the conclusion that the growth-inhibitory effects of black raspberries on premalignant and malignant oral epithelial cells reside in the specific effects of different berry components on signaling pathways regulating cell cycle progression. Rodrigo et al. (60) also evaluated the preventative effects of an ethanol fraction of black raspberries on human oral cancer cell lines and found that the extract inhibited cell proliferation, vascular endothelial growth factor (VEGF) production and nitric oxide synthase activity, and stimulated apoptosis and terminal cell differentiation. In an extension of these studies, another ethanol fraction of black raspberries was extensively subfractionated by high-performance liquid chromatography and the individual subfractions compared for their ability to downregulate the expression levels of nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1) in mouse epidermal JB-6 Cl 41 cells (61). Interestingly, the major constituents of the most active subfractions were the three most prevalent anthocyanins in black raspberries: cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside and cyanidin-3-O-(2'-xylosylrutinoside). These results further suggest that the cyanidin glucosides (anthocyanins) account for at least some of the chemopreventive activity of black raspberries.

The effects of berry extracts on signal transduction pathways have also been discussed (62). In initial studies with the JB-6 Cl 41 mouse epidermal cell model, Huang et al. (63) demonstrated the inhibitory effects of a methanol fraction of black raspberries and of other purified fractions on transactivation of AP-1 and NF-kB induced by BPDE in JB-6 Cl 41 cells. The inhibitory effects appeared to be mediated via inhibition of mitogen-activated protein kinase activation and inhibitory subunit κB phosphorylation, respectively. Pretreatment of cells with the methanol fraction did not result in an inhibition of BPDE binding to DNA; thus, this was not a mechanism of reduced AP-1 and NF-kB activity. None of the tested fractions affected p53-dependent transcription activity. The ability of black raspberries to inhibit tumor development may be mediated by impairing signal transduction pathways leading to activation of AP-1 and NF-kB. This concept is supported by the observation that the methanol fraction markedly inhibited activation of PI-3K, Akt and p70 S6 kinase, suggesting that another mechanism for the chemopreventive activity may be through inhibition of the PI-3K/Akt/VEGF pathway (64).

The effects of individual berry bioactives on growth and apoptosis of human cancer cell lines have also been investigated. Quercetin, a flavonol found in several varieties of berries and black currents, at concentrations up to 70–80 mg/kg dry wt, was found to inhibit growth and stimulate apoptosis of lung cancer cells (65). Survival of a lung cancer cell line (NCI-H209) was decreased in a concentration-dependent manner after treatment with quercetin glucuronide. Importantly, quercetin was effective in vitro at doses (1–10 μM) that are pharmacologically relevant in vivo. Quercetin treatment resulted in an increase in cell cycle arrest with the cells accumulating in the S and G2/M phases of the cell cycle and in subG0, while undergoing apoptosis. Apoptosis was associated with decreases in mitochondrial membrane potential and in the antiapoptotic protein, Bcl-2, and increases in the proapoptotic proteins, p21CIP/WAF1, Bak, Bax, cytochrome c and caspase 3. Nguyen et al. (66) treated A549 human lung epithelial cells with quercetin and found a similar dose-dependent reduction in cell viability and induction of apoptosis. Most of the reduction in cell viability at the two highest doses (43.5 and 58 μM) was attributed to apoptosis. In addition, quercetin treatment led to phosphorylation of the extracellular signal-related kinase, ERK 1/2, and ERK 1/2 activation was accompanied by phosphorylation of the mitogen-activated kinase, MEK.

Individual anthocyanins in berries have also been shown to exhibit chemopreventive effects in vitro. Ding et al. (67) investigated the effects of cyanidin-3-O-glucoside, isolated from blackberries, on gene expression in JB-6 cells. Cyanidin-3-O-glucoside pretreatment led to a dose-dependent decrease in the expression of cyclooxygenase-2 (COX-2) and activities of AP-1, NF-kB and tumor necrosis factor (TNF)ζ, when the cells were treated with 12-O-tetradecanoylphorbol-13-acetate or ultraviolet. Finally, an anthocyanin-rich preparation, from berries (wild blueberry, bilberry, cranberry, elderberry, raspberry and strawberry), was found to reduce VEGF expression in a spontaneously immortalized human keratinocyte cell line (HaCaT) and inhibit endothelial tube formation in a Matrigel assay (68).

It should be mentioned that there is growing concern that observations of the effects of phenolic antioxidants in vitro as a measure of their chemopreventive activity may be deceptive and should not be extrapolated to activity in vivo (69–72). Long et al. (70) suggested that the effects of several antioxidants in vitro are artifacts due to the production of hydrogen peroxide generated from these antioxidants in the culture medium. They pointed out that H2O2 can produce many of the effects observed in vitro with phenolics. These effects include elevation of intracellular Ca++ levels, activation of transcription factors, inhibition of cell proliferation, cytotoxicity, alteration of signal transduction pathways, stimulation of apoptosis, expression of adhesion molecules such as intercellular adhesion molecule 1, promotion of differentiation or cell senescence, downregulation of activation of AP-1 and suppression of protein kinase activation. These findings are supported by the work of other investigators (69,71,72), who also suggest caution in interpreting the results of studies of the effects of antioxidants on normal and tumor cells in vitro.
An additional concern of considerable importance in the extrapolation of in vitro data to the in vivo situation is that of dose extrapolation. Most, if not all, berry bioactives produce chemopreventive effects in cell cultures when added at micromolar concentrations (~10 to 150 μM). In contrast, pharmacokinetic studies indicate that berry bioactives, such as the anthocyanins and ellagitannins, reach only nanomolar concentrations (~1 to 20 nM) in blood and tissues when administered in the diet (13,77). Thus, caution must be used when extrapolating in vitro results to the in vivo situation; perhaps, the most appropriate use of the in vitro data is that of identifying possible mechanistic biomarkers for clinical investigations.

**In vivo studies.** The effects of diets containing lyophilized berry powders on the expression of genes involved in signal transduction have been investigated. As indicated in Table IV, at dietary concentrations of 5 and 10%, lyophilized strawberry and black raspberry powders significantly reduced tumor multiplicity by 30–60%, when administered in a postinitiation scheme, indicating their ability to inhibit tumor progression in the rat esophagus (25,38). The molecular mechanisms of this inhibitory effect on tumor progression were investigated by Chen et al. (73), who showed that the 5% black raspberry diet downregulated the mRNA and protein expression levels of COX-2 and -c-Jun (a component of AP-1), in preneoplastic tissues of NMBA-treated esophagus. This reduction in COX-2 expression correlated with lowered levels of prostaglandin E2 in esophageal epithelium that might be expected to reduce cell proliferation. The inhibition in c-Jun expression might also be expected to reduce the rate of cell proliferation since this gene is a component of the transcription activator, AP-1, which is induced by activated Ras oncoproteinic proteins (74). Nearly all NMBA-induced tumors in the rat esophagus have an activated H-ras oncogene (75). The reduction in inducible nitric oxide synthase (iNOS) expression correlated with lowered levels of nitrate/nitrite in the esophagus and, along with reduced COX-2, might be expected to inhibit inflammatory events in the tissue. The lowered levels of VEGF expression were accompanied by a significant reduction in microvessel density in the esophagus, indicating that the berries inhibited angiogenesis (76). Thus, these studies demonstrated that lyophilized berries reduce molecular markers of cell proliferation, inflammation and angiogenesis in carcinogen-treated rat esophagus. A summary of the effects of berry bioactives on cellular functions and signal transduction pathways is shown in Figure 2.

**Clinical studies of the anticancer effects of berry bioactives**

There are relatively few clinical studies of the anticancer effects of berries. Most of the available data on berry bioactives, in humans, derive from pharmacokinetic studies of the absorption, distribution, metabolism and excretion of berry compounds, obtained either from foodstuffs or as purified compounds. These studies have been summarized extensively by Seeram (19). Recently, our laboratory conducted a phase I trial in 11 subjects to determine the safety/tolerability of LBRs and to measure anthocyanins and ellagic acid in plasma and urine (77). Subjects were fed 45 g (equivalent to a 5% berry diet in animals) of black raspberry powder as a slurry in water daily for 7 days. Blood and urine samples were collected prior to and after berry treatment. The berries were found to be well tolerated; the only clinical observation was a low incidence of mild or moderate constipation (77). Subjects had reduced levels (77). The authors concluded that the daily consumption of lyophilized black raspberries promotes reductions in the urinary excretion of two biomarkers of oxidative stress, 8-Iso-PGF2, and to a lesser more-variable extent, 8-OhdG, among patients with BE. One concern from this study is that the transit time of the black raspberry slurry across the esophagus, when consumed orally, is very rapid and may not permit sufficient localized absorption of berry compounds into Barrett’s lesions to be effective.

**Colon polyps and cancer**

Based upon positive results of the inhibitory effects of LBRs on colon tumor development in rodents (33), we initiated a study in 50 subjects with colorectal cancer and/or polyps to determine if the oral administration of LBRs would modulate biomarkers of colon cancer development. Biopsies of normal and tumor/polyp tissues are collected at baseline. Subjects consume 20 g of freeze-dried LBR powder, as a slurry in water, three times per day (60 g total), until their scheduled surgery date, usually within 2–4 weeks. Posttreatment biopsy specimens are collected during the surgery. Pre- and posttreatment specimens from 23 patients have been analyzed to date for the effects of LBRs on cell proliferation using Ki67, apoptosis by TUNEL and angiogenesis by staining for CD105. Proliferation and
that this is the sole mechanism for their inhibitory effects. \textit{In vitro} studies have clearly demonstrated the ability of berry compounds to enter cells and to bind covalently to cellular proteins (84); this could be responsible for at least some of their effects on cell signaling pathways and other cellular functions.

Investigations in animals indicate that dietary berry bioactives are effective in preventing cancer in the oral cavity, esophagus and colon. Preliminary results suggest that they might also exhibit preventative effects in the human oral cavity, esophagus and colon. In these tissues, localized absorption of berry compounds appears to be important for their chemopreventive effects. Berries were not effective in preventing lung cancer in mice when administered in the diet (52), presumably because they do not reach the lung in sufficient concentrations for chemoprevention. This is consistent with pharmacokinetic studies in animals and humans in which it has been shown that the uptake of berry bioactives, such as the anthocyanins, ellagitannins and quercetin, into the bloodstream is low; i.e. in the nanomolar range. Thus, the future development of whole berry formulations that augment the absorption of berry bioactives is worthy of pursuit and could result in more effective prevention in the oral cavity, esophagus and colon, as well as in other organ sites in which berries have not been shown to exhibit chemopreventive efficacy.

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References


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