

SHORT COMMUNICATION

Inhibition of cellular transformation by berry extracts

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Recent studies have examined and demonstrated the potential cancer chemopreventive activity of freeze-dried berries including strawberries and black raspberries. Although ellagic acid, an abundant component in these berries, has been shown to inhibit carcinogenesis both *in vivo* and *in vitro*, several studies have reported that other compounds in the berries may also contribute to the observed inhibitory effect. In the present study, freeze-dried strawberries (*Fragara ananassa*, FA) or black raspberries (*Rubus ursinus*, RU) were extracted, partitioned and chromatographed into several fractions (FA-F001, FA-F003, FA-F004, FA-F005, FA-DM, FA-ME from strawberries and RU-F001, RU-F003, RU-F004, RU-F005, RU-DM, RU-ME from black raspberries). These extracts, along with ellagic acid, were analyzed for anti-transformation activity in the Syrian hamster embryo (SHE) cell transformation model. None of the extracts nor ellagic acid by themselves produced an increase in morphological transformation. For assessment of chemopreventive activity, SHE cells were treated with each agent and benzo[*a*]pyrene (B[*a*]P) for 7 days. Ellagic acid, FA-ME and RU-ME fractions produced a dose-dependent decrease in transformation compared with B[*a*]P treatment only, while other fractions failed to induce a significant decrease. Ellagic acid, FA-ME and RU-ME were further examined using a 24 h co-treatment with B[*a*]P or a 6 day treatment following 24 h with B[*a*]P. Ellagic acid showed inhibitory ability in both protocols. FA-ME and RU-ME significantly reduced B[*a*]P-induced transformation only when co-treated with B[*a*]P for 24 h. These results suggest that a methanol extract from strawberries and black raspberries may display chemopreventive activity. The possible mechanism by which these methanol fractions (FA-ME, RU-ME) inhibited cell transformation appear to involve interference of uptake, activation, detoxification of B[*a*]P and/or intervention of DNA binding and DNA repair.

Introduction

Chemoprevention has been acknowledged as an important and practical strategy for the management of cancer. Many naturally occurring substances present in the human diet have been

identified as potential chemopreventive agents (1–4). Animal investigations supported by epidemiological studies have suggested that consuming relatively large amounts of vegetables and fruit can prevent the development of cancers (5). Block *et al.* reviewed numerous epidemiological investigations and found an inverse association between the consumption of fruit and vegetables and the incidence of cancers in multiple organs including lung, larynx, oral pharynx, gastrointestinal tract and pancreas (6). Constituents and micronutrients in vegetables and fruit, including phytochemicals, vitamins, vitamin precursors and minerals, have been found to possess both complementary and overlapping mechanisms of chemopreventive activity in multistage carcinogenesis (5).

Recent studies have demonstrated potential cancer chemopreventive activity of berry derivatives including strawberries and black raspberries. Endogenous formation of *N*-nitrosoamino acids was reported to be significantly inhibited by the administration of strawberry juice in humans and this inhibitory activity was not solely related to the ascorbate content of the juice as was initially expected (7). Strawberries have also been shown to be high in antioxidant activity and thus the consumption of strawberries could increase the antioxidant capacity in humans (8,9).

Studies by Stoner *et al.* and Kresty *et al.* have shown that the supplementation of strawberries or black raspberries in the diet reduced the multiplicity and incidence of esophageal tumors in *N*-nitrosomethylbenzylamine-treated rats (10,11). Further, the decrease in the O⁶-methylguanine level found in esophageal DNA in rats fed strawberries or black raspberries suggested that a component(s) in these berries influenced the metabolism of *N*-nitrosomethylbenzylamine (10,11). The chemopreventive effects of these berries have primarily been attributed to ellagic acid, an abundant component in various fruits and nuts including strawberries and black raspberries. Ellagic acid by itself has been shown to inhibit cancers in rodents induced by several carcinogens (12). The inhibition of mutagenesis and carcinogenesis by ellagic acid appears to involve blockage of carcinogen metabolic activation, interference in the binding of reactive metabolites of carcinogens to DNA and the stimulation of detoxification enzymes (13–15).

The identification and development of cancer chemopreventive agents should proceed in a stepwise, mechanistic-based fashion. Several short-term *in vitro* and *in vivo* test systems have been used to assess the mutagenicity and carcinogenicity of chemicals. These tests can also be applied to identify and rank the potency of chemopreventive agents (3,16,17). *In vitro* cell transformation occurs by mechanisms similar to those involved in multistage *in vivo* carcinogenesis. Therefore, Syrian hamster embryo (SHE) cell transformation assay has been widely used both to predict the carcinogenicity of chemicals and to study the mechanisms by which chemicals induce cellular transformation (18–20). This assay can also be a potentially useful *in vitro* model for assessing the chemopreventive ability of chemicals and investigating their mechanisms of action (21,22).

Abbreviations: B[*a*]P, benzo[*a*]pyrene; EA, Ellagic acid; FA, *Fragara ananassa*; RU, *Rubus ursinus*; SHE, Syrian hamster embryo.

Table I. Effect of ellagic acid (EA) on B(a)P-induced morphological transformation in SHE cells following 7-day co-treatment

Treatment	Morphological transformation frequency (%)	Percentage from BP only group decrease of morphological transformation	Number of transformed colonies/ total number of colonies counted
DMSO 0.2%	0.19	–	3/1607
B[a]P 10 µg/ml	1.27 ^a	–	18/1414
EA 0.3 µg/ml	0.12	–	2/1685
1.5 µg/ml	0.06	–	1/1631
3.0 µg/ml	0.14	–	2/1459
4.5 µg/ml	0.08	–	1/1250
B[a]P + EA 10 µg/ml + 0.3 µg/ml	0.96 ^a	24	15/1562
10 µg/ml + 1.5 µg/ml	0.70 ^a	45	11/1567
10 µg/ml + 3.0 µg/ml	0.41 ^b	68	6/1465
10 µg/ml + 4.5 µg/ml	0.21 ^b	83	3/1437

^aSignificantly different from DMSO control (Fisher's exact test, $P < 0.05$).

^bSignificantly different from B[a]P group (Fisher's exact test, $P < 0.05$).

Table II. Effect of FA-ME on B(a)P-induced morphological transformation in SHE cells following 7-day co-treatment

Treatment	Morphological transformation frequency (%)	Percentage from BP only group decrease of morphological transformation	Number of transformed colonies/ total number of colonies counted
DMSO 0.2%	0.13	–	2/1556
B[a]P 10 µg/ml	1.18 ^a	–	17/1442
FA-ME 2 µg/ml	0.12	–	2/1634
5 µg/ml	0.12	–	2/1652
20 µg/ml	0.06	–	1/1611
50 µg/ml	0.14	–	2/1447
100 µg/ml	0.30	–	4/1352
B[a]P + FA-ME 10 µg/ml + 2 µg/ml	0.77 ^a	12/1561	
10 µg/ml + 5 µg/ml	0.47 ^b	7/1497	
10 µg/ml + 20 µg/ml	0.32 ^b	5/1568	
10 µg/ml + 50 µg/ml	0.20 ^b	3/1469	
10 µg/ml + 100 µg/ml	0.19 ^b	84	3/1559

^aSignificantly different from DMSO control (Fisher's exact test, $P < 0.05$).

^bSignificantly different from B[a]P group (Fisher's exact test, $P < 0.05$).

In the *N*-nitrosomethylbenzylamine-induced esophageal tumorigenesis model, Stoner *et al.* (23) found that the tumor inhibitory effects of the berries could not be solely attributed to their ellagic acid content. Other components in the berries appeared to contribute to their chemopreventive effects. In the present study, we utilized transformation in SHE cells by benzo[*a*]pyrene (B[a]P) to examine the potential chemopreventive activity of ellagic acid and selected strawberry and black raspberry extracts.

Ripe strawberries [*Fragara ananassa* (FA)] and ripe black raspberries [*Rubus ursinus* (RU)] were washed immediately after picking, frozen at -20°C , then freeze-dried as described by Stoner *et al.* (23). Freeze-dried strawberries were extracted with methanol. The extract was filtered and then dried under vacuum at 60°C (Fraction F001). The residue from Fraction F001 (F002) was not processed further. A portion of F001 was partitioned with water:dichloromethane (1:1). The aqueous

layer was concentrated under vacuum and dried (Fraction F003). The organic (dichloromethane) layer was vacuumed dried at 60°C resulting in a water insoluble fraction (F004). A small amount of insoluble fraction, F005, was obtained from the interface between the aqueous and organic layer. Additional F001 was dissolved in methanol and allowed to evaporate. The resulting precipitate was chromatographed on a silica gel column and eluted by dichloromethane:methanol (1:1). The resulting non-polar eluate (DM) and polar fraction (ME) were obtained. All extracts were stored at -20°C until examined in the transformation assay. Chemical analysis showed there was no ellagic acid in the ME fractions. All other fractions contained minor amounts of ellagic acid. For cell treatment each extract was dissolved in DMSO to give a final concentration of 200 mg of extract/ml, and was further used in cell transformation studies. The SHE cell transformation assay was conducted as described previously (24). Following

Table III. Effect of RU-ME on B(a)P-induced morphological transformation in SHE cells following 7-day co-treatment

Treatment	Morphological transformation frequency (%)	Percentage from BP only group decrease of morphological transformation	Number of transformed colonies/ total number of colonies counted
DMSO 0.2%	0.13	–	2/1515
B[a]P 10 µg/ml	1.29 ^a	–	18/1392
RU-ME 2 µg/ml	0.00	–	0/1495
5 µg/ml	0.06	–	1/1549
20 µg/ml	0.07	–	1/1512
50 µg/ml	0.14	–	2/1424
100 µg/ml	0.08	–	1/1192
B[a]P + RU-ME 10 µg/ml + 2 µg/ml	1.06 ^a	18	15/1419
10 µg/ml + 5 µg/ml 0.88 ^a	32	12/1363	
10 µg/ml + 20 µg/ml 0.72 ^a	44	10/1391	
10 µg/ml + 50 µg/ml 0.51 ^b	60	7/1365	
10 µg/ml + 100 µg/ml 0.21 ^b	84	3/1417	

^aSignificantly different from DMSO control (Fisher's exact test, $P < 0.05$).

^bSignificantly different from B[a]P group (Fisher's exact test, $P < 0.05$).

Table IV. Effect of ellagic acid (EA) on B(a)P-induced morphological transformation in SHE cells following 1-day co-treatment and 1-day B(a)P + 6-day EA treatment

Treatment	Morphological transformation frequency (%)	Percentage from BP only group decrease of morphological transformation	Number of transformed colonies/ total number of colonies counted
DMSO 0.2%	0.17	–	3/1724
B[a]P 1-day 10 µg/ml	1.14 ^a	–	19/1667
(B[a]P + EA) (1 day) 10 µg/ml + 0.3 µg/ml	1.12 ^a	2	19/1704
10 µg/ml + 1.5 µg/ml	1.00 ^a	12	16/1603
10 µg/ml + 3.0 µg/ml 0.82 ^a	28	14/1716	
10 µg/ml + 4.5 µg/ml 0.52 ^b	54	8/1532	
B[a]P 1-day + EA 6-day 10 µg/ml + 0.3 µg/ml	0.85 ^a	25	15/1767
10 µg/ml + 1.5 µg/ml	0.83 ^a	27	15/1803
10 µg/ml + 3.0 µg/ml 0.52 ^b	54	9/1728	
10 µg/ml + 4.5 µg/ml 0.52 ^b	54	8/1547	

^aSignificantly different from DMSO control (Fisher's exact test, $P < 0.05$).

^bSignificantly different from B[a]P group (Fisher's exact test, $P < 0.05$).

7 days incubation, SHE cell colonies were fixed, stained and scored for morphological transformation (MT). DMSO (0.2%) was used as a solvent control. Two protocols were used to assess anti-transformation activity of the extracts and ellagic acid. B[a]P (10 µg/ml) treatment for 24 h or 7 days was included as a positive control. In the 7 day dosing protocol, cells were treated with non-toxic concentrations of either ellagic acid or each extract with and without B[a]P co-treatment for 7 days. For the agent(s) showing the anti-transformation activity in the 7 day treatment studies, a 24 h co-treatment with B[a]P and a 6-day treatment following a 24 h treatment of B[a]P were performed.

Initial studies determined that maximum subtoxic concentration of the various berry extracts on SHE cell colony formation was 100 µg/ml and for ellagic acid was 4.5 µg/ml, which was subsequently used as the highest concentration in subsequent assays (data not shown). The effects of ellagic acid and all

test berry extracts by themselves on SHE cell transformation following 7-day treatment was examined. Neither berry extracts nor ellagic acid produced an increase in morphological transformation. For chemopreventive activity assessment, SHE cells were treated with B[a]P and either ellagic acid or each extract continuously for 7 days. B[a]P (10 µg/ml) induced SHE cell transformation after 7 days of continuous treatment. Ellagic acid inhibited B[a]P-induced morphological transformation in a dose-dependent manner. Concentrations of ellagic acid from 0.3 to 4.5 µg/ml reduced B[a]P-induced transformation from 24 to 83% (Table I). Of the strawberry extracts, FA-001, FA-003, FA-004, FA-005 and FA-DM did not produce a statistically significant decrease in B[a]P-induced morphological transformation at any of the doses examined (data not shown). However, a concentration-dependent reduction in transformation was seen with these extracts, albeit not one that was statistically significant. In contrast, FA-ME, at

Table V. Effect of FA-ME on B(a)P-induced morphological transformation in SHE cells following 1-day co-treatment and 1-day B(a)P + 6-day FA-ME treatment

Treatment	Morphological transformation frequency (%)	Percentage from BP only group decrease of morphological transformation	Number of transformed colonies/ total number of colonies counted
DMSO 0.2%	0.15	–	3/1977
B[a]P 1-day 10 µg/ml	0.96 ^a	–	17/1771
(B[a]P + FA-ME) 1-day			
10 µg/ml + 2 µg/ml	0.76 ^a	14/1842	
10 µg/ml + 5 µg/ml	0.54 ^a	11/2031	
10 µg/ml + 20 µg/ml	0.37 ^b	7/1867	
10 µg/ml + 50 µg/ml	0.21 ^b	4/1878	
10 µg/ml + 100 µg/ml	0.22 ^b	4/1854	
B[a]P 1-day + FA-ME 6-day			
10 µg/ml + 2 µg/ml	0.75 ^a	13/1734	
10 µg/ml + 5 µg/ml	0.82 ^a	14/1708	
10 µg/ml + 20 µg/ml	0.69 ^a	14/2038	
10 µg/ml + 50 µg/ml	0.61 ^a	36	11/1790
10 µg/ml + 100 µg/ml	0.48	50	9/1889

^aSignificantly different from DMSO control (Fisher's exact test, $P < 0.05$).

^bSignificantly different from B[a]P group (Fisher's exact test, $P < 0.05$).

Table VI. Effect of RU-ME on B(a)P-induced morphological transformation in SHE cells following 1-day co-treatment and 1-day B(a)P + 6-day RU-ME treatment

Treatment	Morphological transformation frequency (%)	Percentage from BP only group decrease of morphological transformation	Number of transformed colonies/ total number of colonies counted
DMSO 0.2%	0.18	–	3/1681
B[a]P 1-day 10 µg/ml	1.31 ^a	1.31 ^a	19/1449
(B[a]P + RU-ME) 1-day			
10 µg/ml + 2 µg/ml	1.13 ^a	18/1593	
10 µg/ml + 5 µg/ml	0.82 ^a	13/1579	
10 µg/ml + 20 µg/ml	0.79 ^a	12/1528	
10 µg/ml + 50 µg/ml	0.79 ^a	11/1401	
10 µg/ml + 100 µg/ml	0.57 ^b	7/1234	
B[a]P 1-day + RU-ME 6-day			
10 µg/ml + 2 µg/ml	1.22 ^a	19/1563	
10 µg/ml + 5 µg/ml	1.41 ^a	21/1487	
10 µg/ml + 20 µg/ml	1.29 ^a	19/1477	
10 µg/ml + 50 µg/ml	1.03 ^a	15/1356	
10 µg/ml + 100 µg/ml	0.93 ^a	12/1296	

^aSignificantly different from DMSO control (Fisher's exact test, $P < 0.05$).

^bSignificantly different from B[a]P group (Fisher's exact test, $P < 0.05$).

5 µg/ml and higher concentrations, produced a significant decrease (60–84%) in B[a]P-induced transformation frequency (Table II). Similar effects were observed for black raspberry extracts. RU-F001, RU-F003, RU-F004, RU-F005 and RU-DM did not exhibit significant inhibitory effects on the transformation frequency (data not shown). As observed with strawberry extracts, black raspberry extracts produced a concentration-related reduction in transformation. RU-ME (2–100 µg/ml) decreased B[a]P-induced morphological transformation 18–84% in a dose-dependent manner (Table III).

Previously, the multistage nature of SHE cell morphological transformation has been associated with both the initiation and promotion stages of *in vivo* carcinogenesis. Thus, different exposure duration could be applied to assess the effects of

agents on different stages of cell transformation process. In one protocol, SHE cells were co-treated for only 24 h with B[a]P (10 µg/ml) and either ellagic acid, FA-ME or RU-ME, medium was changed and cells were grown in fresh medium for an additional 6 days. In a second protocol, SHE cells were treated with B[a]P for the first 24 h, the medium was changed and cells were incubated with different concentrations of ellagic acid, FA-ME or RU-ME for the remaining 6 days. Using these two protocols, ellagic acid inhibited B[a]P-induced transformation (Table IV). When co-incubated with B[a]P for the first 24 h, 4.5 µg/ml of ellagic acid decreased the transformation frequency by 54%, whereas 3.0 and 4.5 µg/ml of ellagic acid treated for 6 days following 24 h of B[a]P produced similar decreases. FA-ME reduced the B[a]P-induced

transformation in a dose-dependent manner when co-incubated with B[a]P for 24 h. In contrast, a decrease of 50% in transformation frequency by the highest dose of FA-ME (100 µg/ml) was seen, but was not significant, when the cells were incubated with FA-ME for 6 days following B[a]P treatment (Table V). RU-ME only showed a significant inhibitory effect on B[a]P-induced transformation at a dose of 100 µg/ml when co-treated with B[a]P in the first 24 h (Table VI).

Previous studies have shown that intake of fruit and vegetables is associated with a decreased risk of chemically induced cancer. Strawberries and black raspberries were found to inhibit *N*-nitrosomethylbenzylamine–esophageal tumorigenesis in rats in a dose-dependent manner, when provided in the diet (10,11). Ellagic acid, a naturally occurring plant polyphenol found in various fruits and nuts, has exhibited anticarcinogenic activity in both *in vitro* and *in vivo* systems (25,26). However, the poor solubility of ellagic acid in water and organic solvents and its low bioavailability potentially restrict its use as a chemopreventive agent (27). In the present study, the chemopreventive activity of ellagic acid was confirmed because B[a]P-induced transformation in SHE cells was inhibited in a dose-dependent manner.

Stoner *et al.* (11,23) previously found that esophageal tumor formation was inhibited more in rats fed dietary strawberries or black raspberries than with ellagic acid only. Thus, there appear to be compounds other than ellagic acid in the berries that contribute to the observed chemopreventive effects. This was confirmed in the present study. Among the tested extracts, FA-ME from strawberries and RU-ME from black raspberries showed a significant inhibitory effect on B[a]P-induced morphological transformation, suggesting their potential chemopreventive activity. For all other fractions examined (FA-F001, FA-F003, FA-F004, FA-F005, FA-DM, RU-F001, RU-F003, RU-F004, RU-F005 and RU-DM), decreases of 30–50% in transformation frequency were also found, but not statistically significant. These fractions contained low but detectable levels of ellagic acid that may be contributory to the anti-transforming activity seen. Further evaluation of this issue needs to be performed. In contrast, both FA-ME and RU-ME fractions were determined to be devoid of ellagic acid and thus, the chemopreventive activity seen is related to other chemical components.

The mechanism by which ellagic acid, FA-ME or RU-ME inhibited the B[a]P-induced transformation in SHE cells were further studied through the two treatment protocols. (Co-cultured with B[a]P for 24 h or B[a]P treatment for 24 h followed by treatment with extracts for 6 days.) When SHE cells were treated with B[a]P and either ellagic acid, FA-ME or RU-ME in different stages, only ellagic acid decreased the cell transformation frequency in both treatment procedures. This is consistent with previous studies which have revealed that ellagic acid appears to function as an anticarcinogen at both the initiation and post-initiation stages (28). FA-ME clearly showed an inhibitory effect in the early stage and to a lesser extent when administered following treatment with B[a]P, suggesting an effect predominantly on the ‘initiation’ stage of B[a]P-induced transformation. FA-ME fraction might therefore be functioning to inhibit uptake or activation of B[a]P, to interfere with B[a]P-DNA binding, to enhance B[a]P detoxification and/or to increase DNA repair. A similar mechanism was also suggested for RU-ME, as this fraction also exhibited its main effect in the early stage of the transformation process. Compared with ellagic acid, which

functioned at both ‘initiation’ and ‘promotion’ stages and the inhibition in the latter was higher, FA-ME and RU-ME showed their most inhibitory effects in the ‘initiation’ step. This supports the theory that other active component(s) in berries besides ellagic acid contribute to their chemopreventive effects.

Further studies are needed to identify specific compound(s) in the FA-ME and RU-ME fractions that are responsible for their chemopreventive activity and to further clarify the possible mechanisms involved in this process. The identification of compounds in these fractions is currently in progress.

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