Effects of Black Raspberry Extract and Protocatechuic Acid on Carcinogen-DNA Adducts and Mutagenesis, and Oxidative Stress in Rat and Human Oral Cells

Joseph B. Guttenplan1,2, Kun-Ming Chen3, Yuan-Wan Sun3, Wieslawa Kosinska1, Ying Zhou1, Seungjin (Agatha) Kim1, Youngjae Sung1, Krishne Gowda4, Shantu Amin4, Gary D. Stoner5, and Karam El-Bayoumy3

Abstract

Effects of black raspberry (BRB) extract and protocatechuic acid (PCA) on DNA adduct formation and mutagenesis induced by metabolites of dibenzo[a,l]pyrene (DBP) were investigated in rat oral fibroblasts. The DBP metabolites, (±)-anti-11,12-dihydroxy-11,12-dihydrodibenzo[a]pyrene (DBP-diol) and 11,12-dihydroxy-13,14-epoxide-11,12,13,14-tetrahydrodibenzo[a]pyrene (DBPDE) induced dose-dependent DNA adducts and mutations. DBPDE was considerably more potent, whereas the parent compound had no significant effect. Treatment with BRB extract (BRBE) and PCA resulted in reduced DBP-derived DNA adduct levels and reduced mutagenesis induced by DBP-diol, but only BRBE was similarly effective against (DBPDE). BRBE did not directly inactivate DBPDE, but rather induced a cellular response—enhanced DNA repair. When BRBE was added to cells 1 day after the DBP-diol, the BRBE greatly enhanced removal of DBP-derived DNA adducts. As oxidative stress can contribute to several stages of carcinogenesis, BRBE and PCA were investigated for their abilities to reduce oxidative stress in a human leukoplasia cell line by monitoring the redox indicator, 2’,7’-dichlorodihydrofluorescein diacetate (H2DCF) in cellular and acellular systems. BRBE effectively inhibited the oxidation, but PCA was only minimally effective against H2DCF. These results taken together provide evidence that BRBE and PCA can inhibit initiation of carcinogenesis by polycyclic aromatic hydrocarbons; and in addition, BRBE reduces oxidative stress. Cancer Prev Res; 9(8): 704–12. ©2016 AACR.

Introduction

Cancers of the oral cavity and pharynx comprise the sixth most common malignancies worldwide, representing a major international health problem (1). The 5-year survival rate for patients with head and neck squamous cell carcinoma (HNSCC) is only about 50% (2). In the United States, over 45,000 cases and about 11,000 deaths from HNSCC occur annually (3). Worldwide, the annual incidence of new cases exceeds 300,000 (1). The number of annual deaths from oral cancer is similar to that from melanoma (3). Patients with resected oral cancers have an increased incidence of second primary tumors of the oral cavity (4, 5). Epidemiologic data provide strong support for exogenous factors such as tobacco, alcohol use, and human papillomavirus infection as being major causative agents (1, 3). In general, avoidance of risk factors has only been partially successful, as many individuals cannot change behavior—largely because smoking and alcohol have addictive effects.

As prevention is preferable to treatment, chemoprevention is a desirable approach. Numerous sources of phytochemicals have been proposed (6), and one that has shown promise in inhibiting head and neck cancers is freeze-dried black raspberry (BRB) (7). Many studies on black raspberries and their components have reported cancer preventive activity (8, 9). Topical treatment of human papillomavirus infection as being major causative agents (1, 3). In general, avoidance of risk factors has only been partially successful, as many individuals cannot change behavior—largely because smoking and alcohol have addictive effects. 

Although studies with whole BRB powder and with different BRB formulations are promising, there are multiple concerns regarding the “standardization” of whole berry powders. In view of this, Stoner’s laboratory has conducted bio-fractionation studies of BRB powder to identify the bioactive constituents and has shown that the chemopreventive activity in multiple assays can be attributed significantly to the anthocyanins (AC), the most abundant class of compounds in BRBs, but they are expensive for routine chemoprevention. In addition, they are difficult to synthesize. Protocatechuic acid (PCA), which is commercially available and relatively inexpensive, accounts for about 70% of the...
metabolites of BRB ACs in humans (10), and is an inhibitor of chemically induced cancers in rodents when administered sub-
sequent to the carcinogen (11). Previously, we reported that the tobacco smoke carcinogen, dibenz[a]pyrene (DBP; also known as Dibenzo[def,p]chry-
sene), and its ultimate carcinogenic metabolite, (±)-anti-11,12-
dihydroxy-13,14-epoxy-11,12,13,14-tetrahydrodibenzo[a,l]pyrene
(DBPDE), are mutagenic and carcinogenic in the mouse oral
cavity (refs. 12, 13; see Fig. 1). We also reported that DBP and
DBPDE form DNA adducts, preferentially with adenine (12, 13). Here, we report on the mutagenic effects and DNA adduct formation from the DBP metabolite, (±)-anti-11,12-dihydroxy-
11,12-dihydrodibenzo[a,l]pyrene (DBP-diol), and DBPDE in rat oral fibroblasts (OFB) containing a mutagenesis reporter
gene (14). We also determined the effects of a BRBE and PCA,
on mutagenesis and DNA adduct levels in OFB treated with
DBP-diol and DBPDE. In addition, we report the effects of BRBE
and PCA on oxidative stress, as assayed by their abilities to
inhibit the oxidation of 2’,7’-dichlorodihydrofluorescein diace-
tate (H2DCF) to its fluorescent product, DCF, in a precancerous
human oral epithelial cell line.

Materials and Methods

Chemicals
DBP-diol and (±)-anti-DB[a,l]PDE were prepared according to a
published method by our group (15). BRB powder and BRBE
were described as described and provided by Dr. Stoner (11). The
solvent used to obtain the BRBE from BRB powder was ethanol/
H2O (80:20). Using high-performance liquid chromatography
(HPLC), the extract was shown to be composed of approximately
70% ACs (16). The remaining compounds have not been iden-
tified. H2DCF was purchased from Biotium Inc. PCA was pur-
chased from Thermo Sci. Acros Organics.

Cell lines
OFB and rat epithelial cell lines were derived from a lacI
(BigBlue) Fisher 344 rat and were kindly provided by David
Josephy (University of Guelph, Guelph, Canada). The prepara-
tion, characterization, and maintenance of these lines have been
described (14). Cells were grown in DMEM/F12 medium (Lonza
–
cellgro) containing 5% FCS (Hyclone), Glutamine (0.29 mg/mL),
–
–
streptomycin (all from Media- 

–

Black Raspberry Extract, Protocatechuic Acid, and Mutagenesis

mutagenic effects and DNA adduct levels in OFB treated with
DBP-diol and DBPDE. In addition, we report the effects of BRBE
and PCA on oxidative stress, as assayed by their abilities to
inhibit the oxidation of 2’,7’-dichlorodihydrofluorescein diace-
tate (H2DCF) to its fluorescent product, DCF, in a precancerous
human oral epithelial cell line.

Materials and Methods

Chemicals
DBP-diol and (±)-anti-DB[a,l]PDE were prepared according to a
published method by our group (15). BRB powder and BRBE
were described as described and provided by Dr. Stoner (11). The
solvent used to obtain the BRBE from BRB powder was ethanol/
H2O (80:20). Using high-performance liquid chromatography
(HPLC), the extract was shown to be composed of approximately
70% ACs (16). The remaining compounds have not been iden-
tified. H2DCF was purchased from Biotium Inc. PCA was pur-
chased from Thermo Sci. Acros Organics.

Cell lines
OFB and rat epithelial cell lines were derived from a lacI
(BigBlue) Fisher 344 rat and were kindly provided by David
Josephy (University of Guelph, Guelph, Canada). The prepara-
tion, characterization, and maintenance of these lines have been
described (14). Cells were grown in DMEM/F12 medium (Lonza
–
cellgro) containing 5% FCS (Hyclone), Glutamine (0.29 mg/mL),
–
–
streptomycin (all from Media- 

–

Black Raspberry Extract, Protocatechuic Acid, and Mutagenesis

mutagenic effects and DNA adduct levels in OFB treated with
DBP-diol and DBPDE. In addition, we report the effects of BRBE
and PCA on oxidative stress, as assayed by their abilities to
inhibit the oxidation of 2’,7’-dichlorodihydrofluorescein diace-
tate (H2DCF) to its fluorescent product, DCF, in a precancerous
human oral epithelial cell line.

Materials and Methods

Chemicals
DBP-diol and (±)-anti-DB[a,l]PDE were prepared according to a
published method by our group (15). BRB powder and BRBE
were described as described and provided by Dr. Stoner (11). The
solvent used to obtain the BRBE from BRB powder was ethanol/
H2O (80:20). Using high-performance liquid chromatography
(HPLC), the extract was shown to be composed of approximately
70% ACs (16). The remaining compounds have not been iden-
tified. H2DCF was purchased from Biotium Inc. PCA was pur-
chased from Thermo Sci. Acros Organics.

Cell lines
OFB and rat epithelial cell lines were derived from a lacI
(BigBlue) Fisher 344 rat and were kindly provided by David
Josephy (University of Guelph, Guelph, Canada). The prepara-
tion, characterization, and maintenance of these lines have been
described (14). Cells were grown in DMEM/F12 medium (Lonza
–
cellgro) containing 5% FCS (Hyclone), Glutamine (0.29 mg/mL),
–
–
streptomycin (all from Media- 

–

Black Raspberry Extract, Protocatechuic Acid, and Mutagenesis

mutagenic effects and DNA adduct levels in OFB treated with
DBP-diol and DBPDE. In addition, we report the effects of BRBE
and PCA on oxidative stress, as assayed by their abilities to
inhibit the oxidation of 2’,7’-dichlorodihydrofluorescein diace-
tate (H2DCF) to its fluorescent product, DCF, in a precancerous
human oral epithelial cell line.
Concentrations mentioned above. Four hours later, H2DCF was added to the MSK-Leuk1 cells were plated in 96-well plates in keratinocyte growth medium, and the next day, BRBE or PCA was added at the concentrations mentioned above. Four hours later, H2DCF was added to a concentration of 5 μmol/L. Fluorescence was read in a Molecular Devices SpectraMax-M5 plate reader using 495 nm excitation and 525 nm emission wavelengths at 30-minute intervals up to 90 minutes. For the acellular assay, 0.1 mmol/L hydrogen peroxide, with and without BRBE or PCA in KGM, was incubated for 4 hours at 37°C. H2DCF was then added and fluorescence was read at 60 minutes. The background fluorescence of cells alone was about 3% of the final reading and was not subtracted. For the acellular assay without hydrogen peroxide, DCF with or without BRBE or PCA was incubated overnight with shaking at 37°C, and fluorescence was read 16 hours after the start of the incubation. H2DCF in PBS or KGM without cells was fluorescent at the concentrations of DBP-diol and DBPDE employed. Results of the mutagenesis assay were then used to determine concentrations of DBP-diol and DBPDE for subsequent experiments.

Analysis of DBP-tetrols

DBP tetrols were analyzed by an HPLC method previously described by us (18, 28). Elution was performed using a Shimadzu LC20AD system and a Waters C18 Symmetry column (2.1 × 150 mm, 3.5 micron particle size) at a flow rate of 0.17 mL/min in a pH 4.0, 10 mmol/L ammonium phosphate buffer containing 45% acetonitrile. A fluorescence detector (Shimadzu, RF10Axl) was set at 344 nm excitation and 400 nm emission wavelengths.

Results

Dose response for DNA adducts resulting from treatment with DBP, DBP-diol, and DBPDE

In order to determine an appropriate dose range for experiments using DBP, DBP-diol, and DBPDE, DNA adducts produced in OFB and epithelial cells by these compounds were measured. No measurable adducts were produced by DBP. The major DNA adducts produced by both DBP-dihydrodiol and DBPDE were the cis and trans anti-DBPDE-dA (Fig. 2A–D).

Oxidative stress

The oxidative stress assays are dependent on the oxidation of H2DCF to DCF—the latter compound being much more fluorescent than the reduced form. In the cellular assays, 5,000 to 10,000 MSK-Leuk1 cells were plated in 96-well plates in keratinocyte growth medium, and the next day, BRBE or PCA was added at the concentrations mentioned above. Four hours later, H2DCF was added to a concentration of 5 μmol/L. Fluorescence was read in a Molecular Devices SpectraMax-M5 plate reader using 495 nm excitation and 525 nm emission wavelengths at 30-minute intervals up to 90 minutes. For the acellular assay, 0.1 mmol/L hydrogen peroxide, with and without BRBE or PCA in KGM, was incubated for 4 hours at 37°C. H2DCF was then added and fluorescence was read at 60 minutes. The background fluorescence of cells alone was about 3% of the final reading and was not subtracted. For the acellular assay without hydrogen peroxide, DCF with or without BRBE or PCA was incubated overnight with shaking at 37°C, and fluorescence was read 16 hours after the start of the incubation. H2DCF in PBS or KGM without cells was essentially nonfluorescent after 90 minutes. BRBE and PCA at the concentrations employed did not affect the fluorescence of DCF, and were not significantly fluorescent at the concentrations employed.

Analysis of DBP-tetrols

In Fig. 2D, peaks with retention time (RT) 26.5 and 29 minutes are DBPDE-dG adducts; peaks with RT 30.5 and 36.4 are (+)-DBPDE-dA adducts. They are all correlated with the doses of DBPDE, but we only quantified (-)-DBPDE-dA adducts because they are more important to DBP-induced carcinogenesis as reported in our previous publications (21, 29). DBPDE was much more effective at producing adducts than DBP-diol. The dose–response results were then used to determine concentrations of DBP-diol and DBPDE for subsequent experiments.

Mutagenesis induced by DBP, DBP-diol, and DBPDE

Mutagenesis induced by DBP, DBP-diol, and DBPDE was monitored in the lacI rat oral epithelial and fibroblast cells. DBP was at best very weakly mutagenic (results not shown) and was not studied further. Preliminary studies revealed that DBP-diol and DBPDE were similarly mutagenic in both cell lines, but background mutation levels were greater in the epithelial cell line, resulting in the fibroblast line being more sensitive to mutagenesis. Therefore, the fibroblast cell line was used for further experiments. Results of the mutagenesis assay are shown in Fig. 3A and B. DBPDE was much more potent than DBP-diol. The relative potencies were DBPDE, 9,600; DBP-diol, 14; DBP, 1.

Effects of BRBE and PCA on DNA adduct formation by DBP-diol and DBPDE

BRBE produced a significant inhibition (about 50%) of DBP-diol–induced DNA adduct formation at 66 μg/mL medium and greater inhibition at higher doses (Fig. 4A). It also inhibited DNA adduct formation by DBPDE; similar to the effects on adduct formation by DBP-diol, it was very effective at concentrations of 75 μg/mL and above (Fig. 4B). PCA also inhibited DNA adduct formation by DBP-diol (Fig. 4A), but in contrast to BRBE, it had no effect on adduct formation by DBPDE at the concentrations employed (Fig. 4C).

Effect of BRBE on removal of DBP-diol–induced DNA adducts

When BRBE was added to cells 1 day after the DBP-diol, it greatly enhanced removal of DBP-derived DNA adducts over the next 24 hours (Fig. 4D). One day after treatment with 1.5 μmol/L DBP-diol, levels of the DBP-dA adduct were about 16 × 10^-6 adducts/da. One day later, they declined to
8.1 adducts x 10^{-6} adducts/dA. However, when the OFB were treated with 75 μg/mL BRBE 1 day after DBP-diol treatment, the adduct level declined to 2.9 adducts x 10^{-6} adducts/dA, a major enhancement in removal.

Effects of BRBE and PCA on mutagenesis by DBP-diol and DBPDE

BRBE was an effective inhibitor of mutagenesis induced by DBP-diol at the concentrations employed (Fig. 5A). The inhibition was dose-responsive with slightly above 50% inhibition at 25 μg/mL. BRBE was also effective at reducing mutagenesis induced by DBPDE (Fig. 5C). PCA was moderately effective at inhibiting mutagenesis induced by DBP-diol, showing a dose-response, although the inhibition only reached significance at 1 μmol/L and above (Fig. 5B). PCA had no noticeable effect on mutagenesis induced by DBPDE at the concentrations employed (Fig. 5D).

Investigation of possible direct effects of BRBE on DBPDE

In contrast to DBP-diol, which must be further metabolized to exert its mutagenic and carcinogenic effects, fjord-region diol-epoxides like DBPDE are short-lived highly reactive electrophiles that bind to DNA without further metabolism (30). Because BRBE inhibited DNA adduct formation by DBPDE, we considered the possibility that there was a direct reaction between the extract and DBPDE. If some component(s) of BRBE reacted with DBPDE before the DBPDE could permeate the cells or when DBPDE was within the cells, this could explain the mechanism of the observed inhibitory effects. As DBP-diol is metabolized to DBPDE, this mechanism could also apply to inhibition of DNA adduct formation and mutagenesis by DBP-diol. To test this possibility, we investigated whether the products from the spontaneous decomposition of the DBPDE were affected by the presence of BRBE. Upon spontaneous hydrolysis of DBPDE in aqueous medium, two products are formed—the cis and trans (-)-anti-11,12,13,14-tetrahydro-11,12,13,14-tetrahydroxy-DBP (DBP-tetrols). DBPDE was incubated in keratinocyte growth medium for 30 minutes, 1 hour, 2 hours, and overnight at 25°C. After incubation, the solutions were applied to an HPLC, and the two expected peaks were observed (Fig. 1; Supplementary Data). The concentrations of the products increased from 30 to 60 minutes, but did not change after that—even after an overnight incubation. A second solution containing the same concentration of DBPDE plus 50 μg/mL BRBE, and incubated in parallel, resulted in the same two products at the same final concentrations, indicating that BRB components do not react with DBPDE and prevent it from reacting with DNA and other molecules.
Effects of BRBE and PCA on oxidative stress

As oxidative stress can play a role in initiation of carcinogenesis, as well as during the promotion and progression stages (31, 32), we investigated whether BRBE and PCA could inhibit oxidative stress. To determine whether BRBE affects intracellular oxidative stress, we monitored the ability of the precancerous human oral epithelial cell line, MSK Leuk1, to oxidize H$_2$DCF to DCF in the presence and absence of BRBE. The oxidized form (DCF) is highly fluorescent, in contrast to the reduced form (H$_2$DCF). BRBE was very effective at reducing the rate of oxidation. Even at a relatively low dose (2.5 µg/mL), it reduced the rate of oxidation by over 50%, and the effect was even more pronounced at higher concentrations (Fig. 6A). In contrast, PCA exhibited only a minor effect on oxidative stress, with a statistically significant inhibition reached at the relatively high dose of 4 µmol/L (Fig. 6B).

We also tested whether BRBE could inhibit the oxidation of H$_2$DCF extracellularly. We preincubated H$_2$DCF with 1 mmol/L hydrogen peroxide in the presence and absence of BRBE for 3 hours in PBS before addition of DCF. BRBE reduced the rate of oxidation. Even at a relatively low dose (2.5 µg/mL), it reduced the rate of oxidation by over 50%, and the effect was even more pronounced at higher concentrations (Fig. 6A). In contrast, PCA exhibited only a minor effect on oxidative stress, with a statistically significant inhibition reached at the relatively high dose of 4 µmol/L (Fig. 6B).

Figure 3.
A, dependence of mutagenesis on concentration of DBP-diol in OFB. B, dependence of mutagenesis on concentration of DBPDE in OFB. *, P < 0.05; **, P < 0.01 in a one-tailed t test versus control (no DBP metabolite).

Figure 4.
A, effects of BRBE and PCA on DNA adducts produced by DBP-diol in OFB. DBP-diol, 0.5 µmol/L. B, effect of BRBE on DNA adducts produced by DBPDE in OFB. DBPDE, 70 nmol/L. For the highest dose of DBPDE, there was only one sample that yielded sufficient DNA for adduct analysis; the single result is plotted in the figure. C, effect of PCA on DNA adducts produced by DBPDE in OFB. DBPDE, 140 nmol/L. *, P < 0.05; **, P < 0.01 in a one-tailed t test versus control (no DBP metabolite). Experiments using BRBE and PCA in the presence of DBP-diol were performed on different days and are expressed as a percentage of the control (DBP-diol-alone). D, effect of BRBE on removal of DBPDE-da adducts. OFB were treated with 1.5 µmol/L DBP-diol. One day later, DNA adducts were analyzed in one group, and 75 µg/mL BRBE was added to a second group of cells, whereas a third group of DBP-diol-treated cells was incubated without further additions. One day later (48 hours after addition of DBP-diol), adducts were analyzed in DBP-diol-treated OFB, with and without BRBE. *, P ≤ 0.05 in a Mann-Whitney U test, and 0.055 in a t test with unequal variances versus 48-hour group with no BRBE.
of oxidation by about 50% at 5 μg/mL, indicating it need not generate an intracellular response to act as an antioxidant (Fig. 6C). This experiment demonstrated that a component of BRBE reacted with preformed hydrogen peroxide. We also investigated whether BRBE could inhibit the ambient atmospheric oxidation of H$_2$DCF. H$_2$DCF is oxidized to DCF in aqueous solution, but less rapidly than in the presence of hydrogen peroxide or when taken up by the MSK cells. We incubated H$_2$DCF with and without BRBE, in the absence of any added oxidant for 16 hours at 37°C and then measured fluorescence. Similar to the results with hydrogen peroxide, BRBE inhibited the oxidation of H$_2$DCF by about 50% at a concentration of 5 μg/mL (Fig. 6D).

**Discussion**

BRB powder and the ethanol/H$_2$O-soluble (80:20) extract have been effective at inhibiting carcinogenesis in a number of *in vitro* and *in vivo* experimental systems (7, 9, 11). Exposure to BRB results in a reduced rate of growth of precancerous cells, reduced inflammation, oxidative stress and angiogenesis, increased apoptosis, and a reduced tumor multiplicity in initiated animals. In those studies, BRB is functioning to inhibit the promotion and progression stages of carcinogenesis. Here, we investigated the effects of an ethanol/H$_2$O soluble (80:20) extract of BRB and a major *in vivo* metabolite, PCA, on processes important for initiation of carcinogenesis—DNA damage, mutagenesis, and potentially, oxidative stress. In experimental animals, DBP is metabolized to (among other metabolites) DBP-epoxide, then to DBPDE, and finally to DBPDE (20). The diol is thought to represent the proximate mutagenic and carcinogenic metabolite of DBP, and DBPDE is the ultimate mutagen and carcinogen (20). We have previously reported that in mice, DBP and DBPDE form DNA adducts in oral tissue—predominantly with adenine (21) and a much higher percentage of the mutations were found at A:T base pairs than in untreated mice (12, 13). Significant fractions of mutations at G:C pairs were also observed.

Consistent with the above metabolic pathway for DBP, DBPDE was much more potent than DBP-diol, and DBP-diol was more potent than DBP in the induction of DNA adducts and mutations. Because the OFB were unable to metabolize sufficient DBP to produce detectable levels of DNA adducts or lead to mutagenesis, further experiments on inhibition of DNA adduct formation and mutagenesis were carried out using DBP-diol and DBPDE. We then investigated inhibition of these processes by the BRBE and PCA. BRB contains about 4% to 5% by dry weight of ACs, and they are thought to be responsible for many of the reported effects of BRB on carcinogenesis and inflammation (11, 16). PCA is a major metabolite of ACs and is produced by microbial metabolism of ACs in the gut (11, 33). There is evidence that some inhibitory effects of BRB and ACs on carcinogenesis can be attributed to PCA (11).

BRBs may act on different stages of carcinogenesis, and in a number of experimental models of carcinogenesis, BRB and ACs were shown to inhibit growth of implanted tumor cells or retard growth of tumors in animals predisposed for cancer (34). In some cases, they were administered in the diet after treatment with a carcinogen (11). In such models, their effects were on promotion and progression of carcinogenesis. There have been studies where they were administered before, during, and after treatment with a carcinogen, but in those cases, it was not possible to determine at which stage of carcinogenesis inhibition was occurring (34). Here, we investigated effects of BRB on processes involved in tumor initiation, using an *in vitro* system, in which formation of DNA adducts by a proximate and ultimate carcinogen was measured, and mutagenesis (a consequence of DNA damage) was also monitored. In addition, effects of BRBE on oxidative stress were examined, as oxidative stress contributes to several stages of carcinogenesis. Pretreatment of cells with BRBE followed by treatment with DBP-diol led to a reduction in DBPDE-induced DNA adducts and mutagenesis, compared with cells treated with DBP-diol alone. Presumably, the reduction in adducts was responsible for the reduction in mutagenesis. Potential mechanisms of inhibition of initiating effects of DBP-diol are (i) direct inhibition of its metabolism to DBPDE or altering its metabolism so that less DBPDE is produced, and/or (ii) trapping or detoxifying DBPDE before it reacts with DNA. BRBE also inhibited mutagenesis and DNA adduct formation by DBPDE. In contrast to DBP-diol, DBPDE is the ultimate mutagenic metabolite of DBP-diol, and inhibition of the reaction of DBPDE with DNA cannot be attributed to inhibition of its metabolism to a more
potent mutagen. The inhibitory effects of BRBE on adduct formation by DBPDE likely result from trapping or enzymatic detoxification of DBPDE.

To investigate the possibility that BRBE can trap DBPDE and prevent it from reacting with nucleophiles (including DNA), we incubated DBPDE in culture medium in the presence and absence of 50 µg/mL BRBE. DBPDE spontaneously decomposed to form two DBP-tetros. We allowed the incubation proceed to completion and then measured the concentrations of the two DBP-tetros. The concentration of tetros produced was unchanged by the presence of BRBE in the incubation solution. If a component of the BRBE reacted with DBPDE before it could hydrolyze, then the concentration of the tetros would have been reduced. This was not the case, and we conclude that the inhibitory effects of BRB on DNA adduct formation and mutagenesis likely result from a cellular response to DBPDE. One possibility is that ACs induce the expression of NAD(P)H dehydrogenase (quinone) (NQO1) which is a sentinel for induction of phase II enzymes (35). Several of these enzymes, such as the glutathione transferases, may conjugate DBPDE and thus reduce its concentration. It has already been reported that several glutathione transferases are capable of catalyzing the conjugation of DBPDE with glutathione (36, 37). We also show here that BRBE enhances the removal of DBPDE-DNA adducts. The mechanism for inhibition by BRB of DBP-diol-induced adduct formation and mutagenesis may be the same as that for its inhibition of DBPDE, as DBP-diol is metabolized to DBPDE. However, the conversion of DBP-diol to DBPDE may also be subject to enzyme inhibition.

We also investigated the effects of PCA, a major metabolite of ACs, on DNA damage and mutagenesis by DBP-diol and DBPDE. Pretreatment with PCA also led to inhibition of DNA adduct formation and mutagenesis by DBP-diol, but not by DBPDE. Hence, the reason for its inhibitory effects likely involves its inhibition of the formation of DBPDE, either by enzyme inhibition or induction of phase II enzymes. PCA is readily available and would represent a convenient substitute for BRBE; the latter must be produced by more involved processing.

An important consideration is whether the levels of inhibitors employed here are relevant to in vivo levels. Plasma levels of PCA in volunteers consuming berry powder were 1 to 2 orders of magnitude below those used here (38). However in mice fed PCA in food, higher plasma levels (38) were achieved. In principle then, blood levels of PCA similar to those used here in cell culture could be achievable by use of supplemental dietary PCA.

For ACs, it has been reported that intraoral delivery of a 10% (w/v) bioadhesive freeze-dried BRB gel to human subjects led to a mean oral tissue concentration of about 0.5 µg AC/mg protein 5 minutes after administration of the gel (39), although there were wide interindividual variations. As typical tissue concentrations of proteins are about 15%, an average tissue concentration would be 75 µg/g tissue (150 mg/g tissue x 0.5 µg AC/mg tissue). This is only for a single time point, but suggests the concentrations employed here are consistent with levels delivered to humans. Also, levels in saliva are in the same range as employed here, but again with very wide interindividual variations (39). The results reported here, taken together with the previous report, suggest that topical application of an BRBE or AC-enriched gel could inhibit DNA damage and mutagenesis induced by polycyclic aromatic hydrocarbon (PAH) in the human oral cavity.

BRBE was an effective inhibitor of cellular oxidative stress as measured by its ability to reduce the rate of oxidation of H$_2$DCF to DCF. Significant inhibition was observed at concentrations well below those necessary to inhibit DNA adduct formation. It has been reported that BRB can induce the antioxidant response element in liver cells, resulting in increased levels of antioxidant enzymes and glutathione (40). Also, grape seed ACs induce phase II enzymes in MCF10A breast cells and inhibit H$_2$DCF oxidation (41). These reports suggest that cellular responses to AC treatment can lead to the antioxidant effect of BRB. However, we observed that BRB inhibited the oxidation of DCF by hydrogen peroxide or molecular oxygen in the absence of cells. Our results are consistent with either inhibition resulting from a cellular response to BRBE.

**Figure 6.** A, effect of BRBE on fluorescence intensity of DCF in human oral epithelial cells. B, effect of PCA acid on fluorescence intensity of DCF in human oral epithelial cells. C, effect of BRBE on fluorescence intensity of DCF in the presence of hydrogen peroxide in the absence of cells. D, effect of BRBE on fluorescence intensity of DCF in the absence of cells when incubated overnight at 37°C. Results are expressed as relative fluorescence intensity, where the control (no BRBE) is 100%. The trend lines are logarithmic. For all points with BRBE, $P<0.01$ in a one-tailed t test versus control (no BRBE) except the 2 µg/mL point in D, which was not significantly different than control. There was no statistically significant effect of PCA on fluorescence intensity at any of the concentrations tested.
or direct reduction of intracellular oxidants by BRB. The results thus far do not allow us to choose between the two possibilities, or a combination of the two.

In conclusion, BRB at concentrations leading to AC levels achievable by topical application of BRB led to inhibition of DNA adduct formation resulting from the DBP metabolites, DBP-diol, and DBPDE in a rat oral cell model. Similar inhibition of mutagenesis induced by DBP-diol and DBPDE was also observed. PCA was an inhibitor of DNA adduct formation and mutagenesis induced by DBP-diol, but not DBPDE. BRB was not an inhibitor of carcinogenesis initiation by tobacco smoke PAH, and its antioxidant ability may play a role in inhibiting several steps in multistage carcinogenesis.

**Disclosure of Potential Conflicts of Interest**

G.D. Stoner has ownership interest (including patents) in BerriProducts, LLC. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: J.B. Guttenplan, S. Amin, K. El-Bayoumy


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.B. Guttenplan, K.-M. Chen, Y.-W. Sun, W. Kosinska, Y. Zhou, S. (Agatha) Kim, Y. Sung, K. Gowda, K. El-Bayoumy

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.B. Guttenplan, K.-M. Chen, W. Kosinska, K. El-Bayoumy

Writing, review, and/or revision of the manuscript: J.B. Guttenplan, K.-M. Chen, G.D. Stoner, K. El-Bayoumy

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.B. Guttenplan, W. Kosinska, G.D. Stoner, K. El-Bayoumy

Study supervision: J.B. Guttenplan, S. Amin, K. El-Bayoumy

**Acknowledgments**

J.B. Guttenplan thanks Vera Golgotou for assistance in the performing the oxidative stress assays and Tia Han for cell maintenance.

**Grant Support**

This study was supported by NIH grant # R01-CA173465.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 6, 2016; revised May 3, 2016; accepted May 18, 2016; published OnlineFirst June 7, 2016.

**References**


Cancer Prevention Research

Effects of Black Raspberry Extract and Protocatechuic Acid on Carcinogen-DNA Adducts and Mutagenesis, and Oxidative Stress in Rat and Human Oral Cells


Updated version
Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-16-0003

Supplementary Material
Access the most recent supplemental material at:
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2016/06/07/1940-6207.CAPR-16-0003.DC1
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2016/06/21/1940-6207.CAPR-16-0003.DC2

Cited articles
This article cites 41 articles, 7 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/9/8/704.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://cancerpreventionresearch.aacrjournals.org/content/9/8/704.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/9/8/704. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.