Suppression of Proinflammatory and Prosurvival Biomarkers in Oral Cancer Patients Consuming a Black Raspberry Phytochemical-Rich Troche

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Abstract

Black raspberries (BRB) demonstrate potent inhibition of aerodigestive tract carcinogenesis in animal models. However, translational clinical trials evaluating the ability of BRB phytochemicals to impact molecular biomarkers in the oral mucosa remain limited. The present phase 0 study addresses a fundamental question for oral cancer food–based prevention: Do BRB phytochemicals successfully reach the targeted oral tissues and reduce proinflammatory and antiapoptotic gene expression profiles? Patients with biopsy-confirmed oral squamous cell carcinomas (OSCC) administered oral troches containing freeze-dried BRB powder from the time of enrollment to the date of curative intent surgery (13.9 ± 1.27 days). Transcriptomic biomarkers were evaluated in patient-matched OSCCs and noninvolved high at-risk mucosa (HARM) for BRB-associated changes. Significant expression differences between baseline OSCC and HARM tissues were confirmed using a panel of genes commonly deregulated during oral carcinogenesis. Following BRB troche administration, the expression of prosurvival genes (AURKA, BIRC5, EGFR) and proinflammatory genes (NFKB1, PTGS2) were significantly reduced. There were no BRB-associated grade 3–4 toxicities or adverse events, and 79.2% (N = 30) of patients successfully completed the study with high levels of compliance (97.2%). The BRB phytochemicals cyanidin-3-rutinoside and cyanidin-3-xyllosylrutinoside were detected in all OSCC tissues analyzed, demonstrating that bioactive components were successfully reaching targeted OSCC tissues. We confirmed that hallmark antiapoptotic and proinflammatory molecular biomarkers were overexpressed in OSCCs and that their gene expression was significantly reduced following BRB troche administration. As these molecular biomarkers are fundamental to oral carcinogenesis and are modifiable, they may represent emerging biomarkers of molecular efficacy for BRB-mediated oral cancer chemoprevention. Cancer Prev Res; 9(2); 159–71. ©2015 AACR.

Introduction

Oral cancer is a growing global health problem due to the continued expansion of tobacco product use in the developing world contributing to significant morbidity, mortality, and economic burden (1, 2). Oral cancers, including oral cavity and oropharyngeal cancers, are some of the most common cancers worldwide with an expected 300,000 new cases and 145,000 deaths. Men are twice as likely as women to be diagnosed with oral cancers, with worldwide incidence and death rates highest in southern Asia, the Indian subcontinent, and many developing countries (3–5). Oral squamous cell carcinomas (OSCC) account for 90% of oral malignancies and are strongly associated with the use of tobacco products and alcohol, as well as poor nutrition, periodontal disease, exposure to high-risk human papillomaviruses, and genetic susceptibility (2, 6–13). More than 45,700 Americans will be newly diagnosed with oral cancer in 2015 and while the death rate has been dropping for the last several decades, an estimated 8,650 Americans will die from the disease this year (14).

Oral carcinogenesis is a multistep process marked by characteristic molecular changes in key regulators of cell growth (13, 15). The accumulation of molecular deficiencies results in unrepaired DNA damage, inappropriate transcriptional expression, epigenetic modifications, and the loss of orchestrated growth control (16). In OSCCs as well as other cancers, the role of the immune system and the inflammatory response appears to be biphasic. The acute and early responses directed by immune surveillance against the
developing tumor are considered beneficial. However, the chronic immune milieu created by an unresolved immune response in the tumor microenvironment promotes carcinogenic progression (17–21).

Cancer chemoprevention strategies are used to inhibit or delay the multistep carcinogenic process, and reduce the risk for recurrence or development of future cancer (22–27). Food-based models of cancer prevention leverage the complex interactions between bioactive phytochemicals to facilitate the reduction in procarcinogenic markers and negative outcomes concomitant with cancer risk. Black raspberries (BRB) feature many bioactive phytochemicals, including a rich complement of anthocyanins, flavonoids, and fruit phenolic acids (28). The antioxidant and anticancer activities of many of these individual agents are well documented; however, it is the potential for further synergistic contributions by multiple bioactive food components on the cancer microenvironment that makes a food-based intervention strategy most attractive (28–31).

The current phase 0 study design was chosen to answer two fundamental questions. First, can newly diagnosed oral cancer patients complete a short-term BRB protocol using oral lozenges (troches) with high compliance? Second, could regional targeting using BRB troches significantly inhibit the transcriptional profile of key procarcinogenic genes in oral squamous cell carcinoma (OSCC) tissues? We hypothesized that oral cancer patients would self-administer BRB troches with strong compliance, and that this exposure to BRB phytochemicals would reduce procarcinogenic gene expression in contacted OSCC tissues. We employed a presurgical model that focused on obtaining oral tissues during the interval between OSCC diagnosis and scheduled surgical resection as part of curative therapeutics. This model provides the opportunity to assess compliance, safety, and toxicity while providing oral biopsy tissues to characterize transcriptional biomarkers without impeding normal standard-of-care clinical practices.

Fundamental to the success of this early biomarker study was the presence of BRB phytochemicals (i.e. anthocyanins) in the targeted tumor tissues and a testable/predictable biologic outcome in response to those phytochemicals. Detection of BRB components in OSCC tissues acts as an analytic assessment of BRB exposure, confirms the utility of the oral troches as a delivery vehicle, and corroborates patient self-reported compliance. In conjunction with BRB bioactive components present in the oral tumor tissues, the reduced expression of genes fundamental to oral cancer progression following BRB troche administration further supports the role of these genes as reversible biomarkers of molecular efficacy for this BRB troche intervention.

Materials and Methods

BRB source, troche compounding, and topical delivery

Black raspberries (Rubus occidentalis, Jewel variety) were obtained from Dale Stokes Raspberry Farm, LLC. A dedicated single lot of BRBs was mechanically harvested, washed, and frozen at −20°C. BRBs were shipped frozen to Van Drunen Farms, lyophilized in a VirTis Sublimator Freeze Dryer (SP Scientific), and ground into a freeze-dried powder. Dissolvable BRB troches were compounded to prolong oral mucosa contact time and facilitate bioactive component delivery using established industry standards (Central Ohio Compounding Pharmacy, Columbus, OH). BRB troches were packaged in protective plastic containers, containing 30 prescored troches (Fig. 1A). Each 25 mm × 25 mm square troche contained 360 mg BRB freeze-dried powder plus inert ingredients and binders, including mannitol USP, citric acid monohydrate USP, polyethylene glycol 1450 NF, and sodium benzoate. The daily administered BRB dosage for the current study was markedly less than the BRB amounts previously administered to either healthy participants (45 g/day; ref. 32) or Barrett esophagus patients (32–45 g/day; ref. 33) without toxicity. Importantly, in the current study, the effective BRB dose was based on accessible oral cavity surface area rather than total body weight, and focused on a localized delivery instead of systemic dissemination of BRB bioactive components. Patients with oral cancer were instructed to actively “tumble” the BRB troches during administration to facilitate oral cavity coverage and dissolution.

BRB dosing rationale and physicochemical properties

BRB dose was extrapolated from accessible oral cavity surface area data from previous preclinical animal studies that demonstrated a significant reduction (44%, P < 0.05) in the number of oral cavity tumors (34). Consequently, the equivalent topical daily dose of orally administered BRBs for humans was estimated at 4.3 g of freeze-dried BRB powder. BRB troches were characterized for phytochemical release using dissolution kinetic analysis. Total phenolic release measurements (tmax 765 nm, N = 9) in a pH 6.5 phosphate buffer system were obtained for BRB troches over 90 minutes.

Phase 0 human clinical trial

Eligibility and inclusion

Male and female OSCC cancer patients (N = 38) ≥ 21 years of age of any race or ethnicity with newly diagnosed, untreated, biopsy confirmed OSCC of any stage were consented and enrolled onto the study protocol in accordance with Internal Review Board directives for The Ohio State University Wexner Medical Center/The Arthur G. James and Richard J. Solove Research Institute. Participants were instructed to follow a low-phenolic diet, document (self-report) alcohol and tobacco use, and record adherence/compliance with daily BRB troche administration using provided log books. Patients were excluded for any of the following criteria: (i) inability to provide informed consent, (ii) requirement of chemotherapy and/or radiotherapy prior to scheduled standard-of-care surgery, (iii) pregnancy, (iv) use of COX inhibitors that could not be discontinued, (v) inability to take nutrition orally, (vi) intolerance or hypersensitivity to BRB products, (vii) exclusive vegetarian or vegan diet. In this short-term presurgical protocol, participants consumed three dissolvable slow-release BRB troches four times a day for a cumulative daily dose of 4.3 g freeze-dried BRBs. Each patient provided oral tissue biopsies from oral tumor (OSCC) and distal, histologically “normal,” high at-risk mucosa (HARM) before (OSCC1, HARM1) and following (OSCC2, HARM2) BRB troche administration. The duration of BRB administration corresponded to existing standard-of-care scheduled surgical resection for curative therapeutic appointments (Fig. 1B).

Compliance. Adherence to the study protocol was assessed by comparing the number of BRB troches assigned for presurgical administration, the number of unused BRB troches returned at the
date of surgery, and the number of BRB doses documented in their daily log books.

Analytic measurements of BRB components in OSCC tissues

Liquid chromatography/mass spectrometry (LC/MS) assays were performed on the basis of previously described methodologies (refs. 33, 36; Supplementary Data). Briefly, representative oral tumor tissues obtained during surgical resection (OSCC2) were suspended in 5% (v/v) formic acid, sonicated, precipitated, dried under nitrogen gas, and resuspended in 5% (v/v) formic acid/methanol. Extracted samples were applied to an ACQUITY UPLC System (Waters Corporation) operated with BEH C18 column and eluent introduced to a Micromass Quattro Ultima triple quadrupole mass spectrometer with an electrospray probe in positive ion mode. Anthocyanins were detected by selected reaction monitoring (SRM) with the following m/z transitions: cyanidin-3-rutinoside 595>287, cyanidin-3-xylosylrutinoside 727>287, cyanidin-3-glucoside 449>287, cyanidin-3-sambubioside 581>287 using collision energies between 15 and 25 eV.

Total RNA isolation from HARM and OSCC tissues

Oral tissue incisional biopsies were collected into Ambion RNAlater reagent and batch processed for RNA isolation using the Qiagen RNeasy Fibrous Tissue Kit. Total RNA was treated with DNase I to remove contaminating coisolated genomic DNA. The DNA-free RNA was assessed for yield using a NanoDrop ND-1000 microvolume spectrophotometer and integrity using an Agilent Technologies Bioanalyzer 2100. RNA samples with RNA Integrity Number (RIN) values between 6 and 9 were used as templates for cDNA syntheses and reverse transcription quantitative PCR (RT-qPCR) analysis.

Prognostic biomarkers of OSCC and biomarkers of BRB exposure/molecular efficacy

Prognostic cancer biomarkers define the likely course of carcinogenic progression in the absence of treatment. Pathways associated with "hallmarks of cancer," (16) including apoptosis and inflammation, were used to focus on known prognostic biomarkers that could further represent relevant biomarkers of BRB molecular efficacy in some OSCC patients. Potential gene targets were identified that demonstrated deregulated patterns of expression in tumor tissues with an emphasis on OSCC and HNSCC cancers. Clinical tissue samples from current oral cancer patients were obtained from tumor (OSCC) and distant, noninvolved, phenotypically "normal" HARM at the time of surgical resection. HARM tissues, while distant from the tumor, also represent potential regions high at-risk for oral cancer development as a consequence of field cancerization defects. First, genes were characterized for differential expression between baseline tumor and high at-risk mucosal tissues (OSCC1/C0 HARM1), thereby supporting a biologic role in oral carcinogenesis. Second, genes demonstrating a differential expression between baseline tumor tissue and post-BRB administered tumor tissue (OSCC2/OSCC1) were identified as biomarkers of BRB-associated exposure and molecular efficacy in OSCC tissues.
Reverse transcription quantitative PCR
An 11-gene panel of oral cancer prognostic biomarkers (AURKA, BAX, BCL2, BIRC5, CASP3, CASP14, EGFR, NFKB1, PTGS1, PTGS2, TXA2R) was validated using RT-qPCR and 384-well microfluidic cards in triplicate. Prevalidated Applied Biosystems human TaqMan Assays and qPCR chemistry were used to interrogate molecular biomarker expression patterns in OSCC tumor tissues prior to and following short-term, low-dose administration of BRB troches.

Statistical analysis of gene expression profiles
Gene expression values were normalized to an evidence-based active reference gene (DUSP1) and examined for significant changes in OSCC–HARM1 (oral carcinogenesis prognostic biomarkers) and OSCC2–OSCC1 (OSCC BRB predictive biomarkers) gene expression. Differences in ΔCt values were examined for significance using a linear model adjusting for the effects of clinical stage of disease, body mass index (BMI), smoking status, and age.

Results
BRB composition and dosing regimen
Polyphenolic profiles were obtained for the BRB powder (BRB-P) used for pharmaceutical compounding of the BRB troches (BRB-T) as well as the BRB-T (Table 1). Total anthocyanin, ellagitannins (ET), methyl ellagic acid, and quercetin glycoside levels were determined, as well as measurements for free ellagic acid and individual phytochemical components. Anthocyanins were the dominant polyphenol class with the disaccharide and trisaccharide substitutions most abundant. Because of the complexity of the ellagitannins and difficulty of their analysis, it is common to hydrolyze extracts to liberate ellagic acid and report total ellagic acid. The various species were quantified individually as ellagic acid and ellagitannins could demonstrate distinct bioactive activities in vivo. Sanguin H-6 and lambertianin C were the two most prominent ETs and tentatively identified according to their parent masses and liberation of ellagic acid in the MS² experiment. The m/z 783 ET species are likely pedunculagin or structural isomers as found in pomegranate (39). The total ellagitannins represent a very large class of polyphenols in BRBs at 24% of the total polyphenols. Although essentially nonbioavailable as ETs, once ingested, ellagitannins can hydrolyze in the gut to give free ellagic acid, which in turn can be absorbed or metabolized to urolithins by gut bacteria. ETs may also impact gut physiology locally without being absorbed. Quercetin glycosides are another bioactive class of polyphenols and they are substituted with sugars similar to the anthocyanins with rutinoside and xylosylrutinosides predominating. They are not absorbed intact but rather must be deglycosylated, absorbed as aglycone quercetin, and reconjugated to glucuronide and sulphate species in the enterocytes and in circulation. Total anthocyanin and free ellagic acid levels are consistent (<10% difference) with previous estimates reported by Stoner and colleagues across multiple BRB harvest years and studies (40).

Participation and adherence of OSCC patients
Sixty-two eligible newly diagnosed OSCC patients were successfully identified by systematic medical records review for the Head and Neck Oncology Clinic at The Ohio State University, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute (Table 2). After obtaining an informed consent in accordance with Institutional guidelines, 38 OSCC patients were enrolled into this phase 0 study with intent to complete the NCT01465776 protocol for short-term administration of BRB troches. Following consent, two patients declined to provide presurgical biopsy tissues. An additional three patients provided presurgical tissues, but declined to consume the BRB troches. Consequently, 33 patients fully initiated the NCT01465776 protocol by providing tissues and administering BRB troches. As the study was contoured to the patient’s existing standard-of-care
Adherence to the study protocol was assessed with respect to self-reported BRB administration. Adherence to BRB troche administration and documentation was 97.2% in the 30 OSCC patients completing the protocol during an average two-week (13.9 ± 1.3 days, 165.6 ± 15.3 troches) period of administration. To support the self-reported compliance, analytic measurements of BRB components were performed in OSCC tissues following BRB troche administration. In vitro dissolution kinetic studies demonstrated that 55.4% of the total phenolics present in the BRB troches were released within 25 minutes (Fig. 2), a duration that mimicked the patient-driven “tumbling” times for the current protocol. Furthermore, analytic measurements using HPLC Electrospray Ionization/Tandem Mass Spectrometry (HPLC ESI-MS/MS) demonstrated the presence of established BRB components in the OSCC tissues (N = 15) following completion of the study protocol (Fig. 3). The dominant anthocyanins, cyanidin-3-rutinoside and cyanidin-3-sulfonylrutinoside (Fig. 3A and 3B), were readily detectable in all tested OSCC tissue biopsies obtained at the time of surgical resection for cure. Furthermore, the minor content anthocyanins cyanidin-3-glucoside and cyanidin-3-sambubioside (Fig. 3A and 3B) were detected in 100% and 93%, respectively, of the tissue samples following short-term BRB troche administration.

Adverse experiences and toxicity of BRB troches
In accordance with NCI Data and Safety Monitoring Guidelines, events were monitored and reported consistent with “the anticipated level of risk involved” in the study at the Investigator’s discretion. Adverse events were monitored and reported to ensure participant safety and to facilitate identification of emerging differences within the study population. There were no grade 3–4 adverse experiences reported in association with the short-term administration of BRB troches. OSCC patients most commonly reported some mild irritation or soreness in the oral cavity (grade 1, “related”: 6/38, 15.8%), typically proximal to the surgical biopsy site and not necessitating clinical intervention, while self-administering BRB troches. One patient reported pain or swelling (grade 2, “unrelated”: 1/38, 2.6%) near the surgical biopsy site and was similarly determined by the attending physician to be independent of BRB troche administration and “unlikely” related to the BRB intervention. Three study participants reporting an adverse experience of grade 1 or grade 2 (3/38, 7.9%) eventually discontinued BRB troche use, while five additional study participants (5/38, 13.2%) voluntarily discontinued BRB troche administration without any indication of an adverse experience.

Downregulation of antiapoptotic transcriptional biomarkers following short-term delivery of BRB troches
Predictive cancer biomarkers are surrogates for measuring potential responsiveness to a treatment or intervention in patients already presenting with disease. A prognostic 7-gene panel of apoptosis-associated molecular biomarkers (AURKA, BAX, BCL2, BIRC5, CASP3, CASP14, EGFR) was used to explore relevant gene expression changes in OSCC tissues after short-term delivery of BRB troches. Five apoptosis-associated genes (AURKA, BAX, BIRC5, EGFR, and NFKB1; Table 3) demonstrated significant (P < 0.05) overexpression in OSCC tissues with respect to patient-matched HARM tissues. Importantly, five genes (AURKA, BCL2, BIRC5, CASP3, EGFR) further demonstrated a significantly
Reduced ($P < 0.05$) level of expression in oral tumor tissues following short-term administration of BRB troches (Table 3), with three of these genes (AURKA, BIRC5, EGFR) remaining significant following multiple-comparison correction.

Inhibition of proinflammation transcriptional biomarkers following short-term administration of BRB troches

A prognostic 4-gene panel of inflammation-associated molecular biomarkers (NFKB1, PTGS1, PTGS2, and TBXA2R) was used to investigate gene expression changes in oral tumors following BRB troche administration. All four genes (Table 3) demonstrated significant ($P < 0.05$) overexpression in OSCC tissues with respect to patient-matched HARM tissues, and three genes (NFKB1, PTGS1, and PTGS2) demonstrated a significantly reduced ($P < 0.05$) level of expression in tumor tissues following short-term BRB administration (Table 3). BRB effects for NFKB1 and PTGS1 remained significant following Bonferroni correction.

While eight candidate genes for BRB-driven molecular efficacy demonstrated significant expression changes (AURKA, BAX, BIRC5, EGFR, NFKB1, PTGS1, PTGS2, and TBXA2R) following short-term BRB troche administration, only five retained significance subsequent to multiple comparison adjustments. These five genes (AURKA, BIRC5, EGFR, NFKB1, PTGS1) represent a signature for BRB exposure and potential molecular efficacy in oral malignant tissues.

Patient stratification by BRB molecular efficacy

Normalized gene expression profiles revealed that prognostic proinflammatory and prosurvival biomarkers were overexpressed in OSCC tissues compared with patient-matched noninvolved HARM tissues. Notably, 11 of 12 (91.7%) biomarkers demonstrated a decrease in expression in OSCC tissues following short-term delivery of BRB troches (Table 3). Patient-level response profiles for each molecular biomarker demonstrate a significant (Fig. 4A) or general trend (Fig. 4B) in decreased expression in OSCC patients following administration of BRB troches. On average each molecular biomarker demonstrated a BRB response (transcriptional inhibition) that would favor cancer prevention in 79% of the OSCC patients. Furthermore, patient-level response profiles reveal that there is a minority of cases that demonstrate an increase in transcriptional expression in individual molecular biomarkers, suggesting potential for the predictive value of these biomarkers.

Molecular–clinical correlations in OSCC patients after short-term administration of BRB troches

Molecular biomarkers were interrogated against common clinical features following BRB troche delivery to reveal potential associations between transcriptional regulation and clinicopathologic modifiers (Table 4). The biomarkers BAX, BCL2, BIRC5, PTGS1, and TBXA2R demonstrated a negative correlation with molecular efficacy; in other words, the greatest molecular efficacy (inhibition of gene expression) following BRB administration was demonstrated in younger patients. Similarly, PTGS2 molecular efficacy was more pronounced in OSCC patients with lower tumor staging, while higher BMI indices dampened the BRB effects in AURKA and EGFR.

Discussion

Chemoprevention and nutritional prevention strategies seek to reduce cancer risk and/or inhibit carcinogenesis through the administration of agents and modification of lifestyle behaviors. The ideal paradigm strategy would be effective, safe, easy, cost-effective, and nominally alter existing lifestyle behaviors. Early-phase trials of novel delivery systems of food-based products, such as the current study protocol, are challenged by conducting the intervention in partnership with the highest at-risk or actively manifest cancer patient populations in a manner that does not
compromise existing standard-of-care practices. As recently discussed by Brenner and Hawk (41), the necessary clinical design of early-phase cancer risk reduction interventions imposes many restrictions on the ability to obtain biomarker data efficiently.

Combining an appropriately targetable patient population and cancer with a pertinent cancer prevention strategy remains an ongoing clinical challenge. In contrast to definitive therapeutic strategies for malignant disease, cancer prevention actions require relatively long-term administration in advance of overt disease in recognized at-risk populations to reduce the risk of future carcinogenesis.

Importantly, it has been estimated that a modifiable factor, diet, contributes to nearly 35% of all cancers (22). It is hypothesized that food-based cancer prevention strategies can leverage the complex interactions and complementarity of multiple bioactive phytochemicals to reduce the risk for cancer development. Physiologic targets of these strategies include curbing deregulated cell growth/death and alleviating chronic proinflammatory...
responses within the solid tumor microenvironment. The current phase 0 trial protocol explored the potential for repeated low-dose, short-term BRB troches to successfully deliver bioactive components to OSCC tissues and to effect relevant transcriptional changes in those tissues. By design, the short-term duration of the intervention, the single low daily dose of BRBs, and the selected cohort of current OSCC patients awaiting curative surgical therapy, acknowledges that there is no reasonable expectation of disease preventive efficacy. However, this study does demonstrate that hallmark features of oral carcinogenesis are modifiable targets of BRB phytochemicals, and that the transcriptional changes that occur for the BRB intervention support a role for these phytochemicals in a food-based oral cancer prevention strategy. These long-term goals include incorporation of BRBs or their components and metabolites into primary chemopreventive practices, as well as to define the potential utility of BRB phytochemicals towards secondary/tertiary prevention and cancer control measures in oral cancer patients. Importantly, our analysis of molecular efficacy and clinical correlates provides the opportunity for identifying specific BRB-responsive surrogate endpoints for primary prevention trials in populations, such as cigarette smokers, who are high at-risk for subsequently developing oral cancer.

The aerodigestive tract, especially the oral cavity, is compromised by contact with several significant risk factors for cancer development, predominantly exposure to alcohol and tobacco smoke. These insults confer a persistent field of damage (15, 42, 43) in the epithelial cells and subsequently emerge as malignant lesions (OSCCs) and nonmalignogenic but HARM (37, 38). These OSCCs and HARM tissues further afford the unique opportunity for minimally invasive surveillance as well as active targeting of an intervention, such as demonstrated with our BRB troches.

Several research groups prior studies exploring the tolerability and feasibility of BRB bioactive components on reducing the malignant phenotype in the aerodigestive tract. Stoner and colleagues (32) conducted an early-phase pharmacokinetic study in 11 healthy participants who received 45 g of BRB powder once daily for 7 days and were assessed for safety, tolerability, and presence of systemic BRB phytochemicals. Urine and plasma samples demonstrated the presence of ellagic acid, and the anthocyanin metabolites cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-rutinoside, and cyanidin-3-sulfoxybutinoside. Importantly, this study demonstrated that there were nominal safety/tolerability concerns following daily administration of 45 g of BRB powder, more than 10 times the amount delivered in our current protocol. Kresty and colleagues (33) similarly administered 32–45 g of BRB powder daily for 26 weeks to 10 Barrett esophagus patients. Patient compliance to the BRB protocol was reported overall as extremely good with no significant adverse events, and demonstrated that high levels of compliance could be achieved during a 6-month BRB powder intervention. Systemic metabolic biomarkers of DNA damage (8-epi-prostaglandin F2α, 8-hydroxy-2-deoxyguanosine) were decreased in the urine of patients following the 6-month BRB intervention.

Work by Mallery and colleagues (36) extended the potential uses of BRBs into the oral cavity and established the utility of a "mucodelivery gel" formulation for focused delivery within the oral cavity of healthy volunteers. The differences in strategy between these prior studies and the current clinical trial are significant. The mucodelivery patch is used as a very focal mechanism to deliver BRB bioactives to a known lesion. The surrounding HARM is not targeted or treated by design. In contrast, our troches will ultimately be used in a different scenario where manifest lesions are not present, but rather we are treating the at-risk mucosal field by accessing the entire oral mucosa. For example, after successful treatment of a tobacco-related oral cancer, an incidence rate for a second tobacco-related cancer can be 15%–20% per year in high-risk populations. This would be an ideal target population for an intervention that accesses the entire HARM field of carcinization. In the work by Ugalde and colleagues (44) and Mallery and colleagues (45) 0.5 g of a 10% freeze-dried BRB mucodelivery gel was applied to premalignant lesions for 12 weeks while undergoing clinical observations for progression. This corresponds to an estimated 50 mg of freeze-dried BRB powder delivery to the defined premalignant tissues of interest. In the current biomarkers study, each troche contained 360 mg of freeze-dried BRB powder (7× the mucodelivery gel dosage) delivered by oral tumbling to the entire mucosal tissues of the oral cavity for an average of two weeks. While studies by the Mallery and colleagues targeted discrete premalignant lesions at a lower relative dose, the current study attempted to deliver a larger total phytochemical dose to the complete oral carcinization field (HARM and OSCC tissues). Consequently, these studies are distinct but complementary in approach and intent.

Local and systemic detection of anthocyanins strongly supported the functional effectiveness of a low-dose targeted topical delivery of BRB phytochemicals in the oral cavity. Shumway and

Table 3. Fold differences in expression for prognostic molecular biomarkers of oral carcinogenesis and BRB-predictive molecular biomarkers in OSCC patients

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<tr>
<th>Prognostic molecular biomarkers</th>
<th>Predictive molecular biomarkers</th>
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<td>OSCC1 – HARM1</td>
<td>OSCC2 – OSCC1</td>
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<td><strong>Fold change</strong></td>
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<td><strong>CI</strong></td>
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Abbreviation: CI, upper and lower limits for 90% confidence interval. ^BRB effect is > 2 SEM. ^BRB effect is > Bonferroni bound.
colleagues (46) showed the potential molecular efficacy of topical BRBs within the oral cavity following application of a 10% BRB powder bioadhesive gel four times a day to established premalignant lesions. Despite appreciable interpatient variation, topical application of BRBs significantly reduced the prevalence of LOH in a subset of IEN patients, with 41% demonstrating a favorable response (decreased lesion grade) following BRB gel application. While complicated by the dynamic progression/regression profile of IEN, these studies reveal the potential of BRBs to favorably mediate molecular profiles in at-risk oral tissues and demonstrated a BRB delivery system that allowed rapid local transmucosal and systemic delivery of BRB phytochemicals. The current study established that BRB phytochemicals were: (i) readily dispersed from the troche delivery system with kinetics that allowed 55.4% of total phenolics to be released within 25 minutes (Fig. 2) and (ii) incorporated into the target tumor tissues in the oral cavity (Fig. 3). Consequently, measurements of protocol adherence were assessed using both self-reported compliance documentation and analytical profiling for BRB phytochemicals. Importantly, the presence of BRB

**Figure 4.**
Patient-level transcriptional responses in OSCC biopsy tissues following short-term BRB troche administration. A, significant reduction in gene expression levels was evident for AURKA, BIRC5, and EGFR ($P < 0.05$). B, the molecular biomarkers NFKB1, PTGS1, and PTGS2, which demonstrated similar transcriptional inhibition but did not reach statistical significance following Bonferroni correction, are included for comparison. x-axis, OSCC patient ID and self-reported compliance to the BRB protocol; y-axis, fold-change in gene expression.
The acquisition of deregulated cell growth by cancer cells is a defining trait early in tumorigenesis. Cancer cells readily lose responsiveness to inhibitory proliferative signals, while gaining the capacity to enhance the contribution of growth promoting factors. In OSCCs, the overexpression and deregulated expression of the EGFR is an early and common feature of neoplastic progression (16, 52, 53). The vast majority (85%–100%) of head and neck squamous cell carcinomas (HNSCCs) overexpress EGFR (54, 55), and it has represented an important preventive and therapeutic target for several years (17). However, despite promising indicators of therapeutic effectiveness and increased survival times in some studies, mitigating the overexpressed status of EGFR alone using tyrosine kinase (e.g., erlotinib, gefitinib) or mAb (cetuximab, panitumumab) inhibitors has demonstrated important but limited efficacy (17). While it is noteworthy that short-term administration of BRB troches significantly decreased EGFR expression in OSCC tissues, it is also important to acknowledge that the true value of this inhibition may be in conjunction with concurrent modifications in parallel signaling pathways (56).

Oral cancer progression is also marked by the ability of localized cells to survive in a chronic proinflammatory microenvironment, as well as evade immune surveillance and counter immune response mechanisms (16, 19, 21, 51). Recently, we described how an BRB extract could modulate the immunosuppressive activity of myeloid-derived suppressor cells often found upregulated in cancer patients, as well as inhibit regulatory T-cell survival/proliferation and subsequent immunosuppressive activities (57). Furthermore, NF-κB is at the crossroads of inflammatory, immune response, proliferative, and apoptotic signaling events, and its overexpression fosters a tissue microenvironment promoting carcinogenesis. Consequently, it is fundamentally important to be able to arbitrate its expression in a manner that favors cancer prevention strategies. NF-κB induces key components within the arachidonic acid metabolism pathway, including COX enzymes as well as cell survival factors such as B-cell CLL/lymphoma 2 (BCL2). The presence and overexpression of proinflammatory mediators, such as PTGS1 (COX-1), PTGS2 (COX-2), and NOS2 (iNOS) in the tumor microenvironment are consistent events within a deregulated chronic immune response. Abnormal NF-κB signaling contributes to avoidance of immune surveillance, diminished apoptotic cell removal, and increased chronic oxidative stress as a consequence of elevated COX-1 and COX-2 expressions. Short-term administration of BRB troches to OSCC patients was able to significantly decrease tumor expression levels of PTGS1, PTGS2, and NFKB1, and consequently provide a window of opportunity for an intervention strategy that reduces the chronic proinflammatory signaling environment intrinsic to the OSCC tumor microenvironment.

Apoptotic programmed cell death and control of the cell-cycle restriction point are essential for maintaining cellular homeostasis and genomic integrity. As progressively advanced tumorigenic cells are increasingly effective at avoiding these mechanisms, interventions that arrest cell-cycle progression and allow DNA damage discrimination and removal of field-defective cells continue to represent promising preventive or therapeutic strategies. The Aurora kinase family of serine/threonine kinases plays instrumental roles during mitotic assembly and progression. Deregulation of AURKA results in aberrant cyclin B/CDK1 control, defective G2/M checkpoint transition, chromosomal segregation instabilities, and malignant transformation, and overexpression is common in many solid tumors, including HNSCCs (48, 50, 58). Inappropriate overexpression of AURKA can direct p53 phosphorylation, obstruct its transactivation activities, and supereude cell proliferation and apoptotic control measures (59), while suppressing overexpression can reintroduce apoptotic selection and growth control (48). AURKA can also positively regulate NF-κB activities by phosphorylating IκBα and Ras signaling via RalA phosphorylation. Evaluation of small-molecule inhibitors MK8745 (60), MLN8237 (61, 62), TC-28 (63), MLN8054 (47), and VX680 (47) has demonstrated some efficacy in inhibiting AURKA-associated mitotic transition defects, but several have been subsequently discontinued following clinical evaluation (VX680, phase II; MLN8054, phase I; ref. 64). In partnership with deregulated cell-cycle checks, the loss of apoptotic control

### Table 4. Clinicopathologic features demonstrating a dampened BRB effect following short-term topical administration in OSCC patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>OSCC2–OSCC1</th>
<th>Age</th>
<th>Stage</th>
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<tbody>
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<td>AURKA</td>
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<td>TBX2A2R</td>
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Table 4. Clinopathologic features demonstrating a dampened BRB effect following short-term topical administration in OSCC patients.
during carcinogenesis results in the inability to remove (and persistence of) genetically compromised cells. In OSCCs, deregulation of the apoptotic machinery is commonly associated with disease progression and tumor formation. Overexpression of prosurvival biomarkers BCL2 and BIRC5 (Survivin) are hallmark features of HNSCCs. BCL2 is the canonical member of the BCL2 family of intrinsic mitochondrial prosurvival proteins, while Survivin (BIRC5) is an inhibitor of apoptosis protein (IAP) thatorchestrates cytoprotective activities by inhibiting caspase signaling events. Short-term administration of BRB troches was able to successfully downregulate the expression of these prosurvival factors in a manner consistent with suppressing the “resisting cell death” hallmark of cancer (16). Therapeutic interventions have explored modulation of BCL2, mostly in hematopoietic malignancies, via BH3 antagonistic mimetics, small molecules, antiangiogenic agents, and natural compound derivatives (reviewed in ref. 65), and Survivin using the small-molecule inhibitor YM155. However, BH3 mimetic peptides demonstrate suboptimal pharmacologic properties, antimitotic agents show a broad specificity, promote resistance selection, and demonstrate significant toxicities, while the targeted efficacy of YM155 remains under active investigation (66).

The ability of BRBs to modulate signaling events associated with neoplastic progression in preclinical and clinical models is well documented (28, 40, 67); however, the translation of these findings to human oral carcinogenesis is only beginning to be addressed. The current study demonstrates for the first time that short-term administration of BRB troches to current OSCC patients results in a significant reduction of keystone molecular features of oral cancer progression in a manner that favors chemoprevention. In addition, this predictive biomarker signature illustrates that there are patient-level variations that can assist in identifying potential “responder” and “nonresponder” populations for molecular efficacy. Previously, Mallery and colleagues described potential responder categories following a BRB topical intervention in patients with premalignant lesions, in part by individual patient gene expression profile changes (68). Although it may seem that there should be an a priori argument for the benefit and safety of food-based chemopreventive strategies, the decision to participate in such interventions must be evaluated for patient-level responses in concert with epidemiologic population level data. Recently, Tsimeridou and colleagues (69) described the clinical utility of a personalized medicine approach using a nonrandomized phase I trial. Despite the latitudes allowed in patient tumor type and prior therapeutic interventions, the study was able to demonstrate the translational value in identifying molecular alterations and structuring subsequent patient care practices using molecular efficacy profiles. An overall favorable risk-to-benefit ratio can be greatly enhanced if appropriately (and inappropriately) targeted patient categories can be identified. It has been proposed previously that interpatient variation in response to BRB-based interventions may correspond to differential profiles for absorption, processing, and localized tissue uptake of BRB phytochemicals (44). The complex microenvironmental parameters of the oral cavity include interaction networks between the pharmacologic agents contained in BRBs, BRB-derived metabolites, the host oral epithelium, resident bacterial biofilms, and insults such as alcohol and tobacco smoke exposure. Recently, emerging criteria have identified targetable populations demonstrating differential responses based upon oral microbiome–associated anthocyanin degradation capacities (70, 71). Despite these variabilities and the intrinsic heterogeneity of cancers, 64.3% of 28 OSCC tissues demonstrated a favorable response (reduced precancerous gene expression) in all five molecular biomarkers in the BRB effects signature, while only 6 of 28 tumors showed a favorable response in ≥2 molecular biomarkers in the transcriptional signature. These “poor” response profiles were not associated with low self-reported compliance measurements or lack of BRB components when assessable.

Food-based aerodigestive cancer prevention strategies using BRBs have demonstrated both clinical and molecular efficacy in a series of early-phase clinical trials, and have established favorable responses in both precursor and malignant epithelial lesions. Seminal translational studies by Stoner and his collaborators have demonstrated the consistent efficacy of BRBs and their bioactive components in esophagus and colon cancers (28, 40). We demonstrate for the first time that short-term, low-dose administration of BRB troches to current oral cancer patients is well tolerated, without significant adverse experiences, and that patients exhibit high levels of protocol compliance (97.2% self-reported, 100% analytic measurements) and study completion (79.2%). Furthermore, bioactive components of BRBs, namely the anthocyanins cyanidin-3-rutinoside, cyanidin-3-sambubioside, cyanidin-3-glucoside, and cyanidin-3-sambubioside, were retained and readily detected in the target tumor tissues despite a presurgical fasting period in excess of 8 hours. BRB troche administration resulted in a significant reduction of canonical biomarkers of oral carcinogenesis in malignant tissues and defined a BRB signature of molecular exposure and efficacy incorporating the AURKA, BIRC5, EGFR, NFKB1, and PTGS1 genes. These transcriptional biomarkers represent established key mediators in epithelial carcinogenesis and rational targets for chemoprevention strategies. BRBs represent a food-based approach to cancer prevention that leverages favorable bioactivities of well-known compounds such as anthocyanins, as well as potential interactive effects of other phenolic components, including ellagic acid and quercetin, to provide a strategy to reduce molecular risk factors for oral cancer.

Disclosure of Potential Conflicts of Interest
Y. Vodovotz has ownership interest (including patents) in U.S. patent 7,592,028. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: T.J. Knobloch, L.K. Uhlrig, B.M. Warner, S.K. Clinton, K. Riedl, Y. Vodovotz, A. Agrawal, C.M. Weghorst
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.J. Knobloch, L.K. Uhlrig, S.K. Clinton, C.L. Sardo-Molmenti, Y. Vodovotz, D.E. Schuller, E. Ozer
Study supervision: T.J. Knobloch, L.K. Uhlrig, S.K. Clinton, Y. Vodovotz, A. Agrawal, C.M. Weghorst
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References

Black Raspberries Suppress Biomarkers of Oral Carcinogenesis


Suppression of Proinflammatory and Prosurvival Biomarkers in Oral Cancer Patients Consuming a Black Raspberry Phytochemical-Rich Troche

Thomas J. Knobloch, Lana K. Uhrig, Dennis K. Pearl, et al.


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