Cancer Letters 325 (2012) 54–62

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Berry anthocyanidins synergistically suppress growth and invasive potential of human non-small-cell lung cancer cells

Hina Kausar [a], Jeyaprakash Jeyabalan [a], Farrukh Aqil [a], Deepika Chabba [c], Jasmeen Sidana [c], Inder P. Singh [c], Ramesh C. Gupta [a,b,*]

[a] James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA
[b] Department of Pharmacology and Toxicology, University of Louisville, Louisville, KY, USA
[c] Department of Natural Products, National Institute of Pharmaceutical Education and Research, S.A.S. Nagar, Punjab, India

ARTICLE INFO

Article history:
Received 13 April 2012
Received in revised form 21 May 2012
Accepted 23 May 2012

Keywords:
Anthocyanidins
Lung cancer
Synergism
Apoptosis
Metastasis
Blue berry
Bilberry
Jamun

ABSTRACT

Berry anthocyanidins (cyanidin, malvidin, peonidin, petunidin and delphinidin) have increasingly been explored for their anticancer effects; however, their combinatorial effects as a mixture, as present in blueberry, bilberry and Indian blackberry ('Jamun') remain untested. In this study, we demonstrate for the first time that the combination of suboptimal concentrations of equimolar anthocyanidins synergistically inhibited growth of two aggressive non-small-cell lung cancer cell lines, with minimal effects on non-tumorigenic cell viability. The induction of cell-cycle arrest, apoptosis and suppression of NSCLC cell invasion and migration were also significantly greater with the mixture than individual anthocyanidins. The superior effects of the combinatorial treatment presumably resulted from its effects on the oncogenic Notch and WNT pathways and their downstream targets (β-catenin, c-myc, cyclin D1, cyclin B1, pERK, MMP9 and VEGF proteins), enhanced cleavage of the apoptotic mediators Bcl2 and PARP and enhanced inhibition of TNFα-induced NF-kappa B activation. *In vivo*, both the native mixture of anthocyanidins from bilberry (0.5 mg/mouse) and the most potent anthocyanidin, delphinidin (1.5 mg/mouse) significantly inhibited the growth of H1299 xenografts in nude mice by ≈60%. Notably, the effective dose of delphinidin in the anthocyanidin mixture was 8-fold lower than delphinidin alone, further emphasizing synergism. Our results thus demonstrate therapeutic potential of berries rich in this mixture of diverse anthocyanidins for non-small-cell lung cancer treatment and to prevent its future recurrence and metastasis.

1. Introduction

Unlike significant progress made in the prognosis of certain cancers (breast, prostate, colon, etc.), the prognosis for lung cancer remains grim with the 5-year survival period still hovering around 15% [1]. Non-small-cell lung cancer (NSCLC) predominates (85%) all lung cancers and despite two decades of surgical, radio- and chemotherapeutic interventions, 35–50% of patients with stage I or II NSCLC still develop recurrence and metastasis [2]. Innovation therefore lies in the utilization of unconventional, user-friendly approaches to treat lung cancer and to prevent or delay the onset of its recurrence and metastasis.

Analogous to other solid organ tumors, lung tumorigenesis results via an accumulation of mutations in critical genes that regulate cell differentiation, proliferation, invasion, migration and apoptosis [3]. In addition, dysregulation of signaling pathways such as Notch, WNT, Raf/MEK/ERK, EGFR and PI3/Akt/mTOR, have also been reported to contribute to the progression of lung cancer [4–7]. A combination of agents targeting multiple cellular processes and signal transduction pathways therefore represents an ideal strategy for the prevention and treatment of this deadly disease.

Over the recent years various epidemiological studies have provided compelling evidence for the chemopreventive/therapeutic efficacies of various food and natural products. In this regard, berries have received great attention lately based on their potential to prevent chemically-induced colon [8] and esophageal [9] cancers in animal models. Our own studies have shown significant inhibition of breast cancer in the rat model by blueberry, black raspberry [10,11], and Indian blackberry "Jamun" (unpublished data). More data is now beginning to emerge indicating that berries and its phytochemicals also have significant therapeutic activity against lung [12], breast [13,14] and prostate cancer [15] *in vitro* and *in vivo*. The various biological activities of berries have been attributed to abundance of diverse phenolic constituents, particularly, the anthocyanins and their aglycones, anthocyanidins that cause

* Corresponding author at: James Graham Brown Cancer Center, University of Louisville, 304E Delia Baxter II, 580 S. Preston St., Louisville, KY 40202, USA. Tel.: +1 5028523682; fax: +1 5028523842.
E-mail address: rcgupta@louisville.edu (R.C. Gupta).

0304-3835/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canlet.2012.05.029

intense coloration. In separate studies, the berry extracts and its active constituents both the anthocyanins and anthocyanidins have been shown to inhibit malignant cell survival and confound many signaling events involved in oncogene expression, cellular transformation, cell-cycle regulation, apoptosis, metastasis and angiogenesis [16–19] through their effects on a myriad of signaling molecules, including DNA repair genes, the transcription factor NF-kappa B, c-myc, Bcl-2, COX-2, NOS, Cyclin D1, Notch, WNT/β-catenin, MAPK, EGFR, PI3/AKT pathways and the metastatic and angiogenic mediators VEGF, uPAR and MMPs [14,17–28], thus making them ideal allies in the fight against cancer.

The present studies were promulgated on the hypothesis that since most of the anthocyanidins are present as a complex mixture in berries like blueberry, bilberry and Indian blackberry, they might exhibit better effects in concert rather than when used individually. We therefore tested these anthocyanidins [cyanidin (Cy), malvidin (Mv), peonidin (Pe), petunidin (Pt)] and delphinidin (Dp)] individually and as a mixture for their potential to inhibit NSCLC cell growth and metastasis in culture and delineate the underlying mechanisms. Since the anthocyanidin composition varies with the type of berries, a combination of anthocyanidins at equimolar ratio was tested *in vitro* in this study for proof of concept. Furthermore, *in vivo* antitumor effects of the native anthocyanidin mixture and the most potent anthocyanidin, Dp isolated from bilberry and blackcurrant, respectively were also determined using nude mouse xenograft model. We found significantly higher anticancer effects of the anthocyanidins in the mixture form than the individual compounds against both tumor cell proliferation and metastasis and in modulation of various molecular targets mediating the antiproliferative, antimetastatic and apoptotic effects.

2. Materials and methods

Anthocyanidins (chloride forms of Cy, Mv, Pe, Pt and Dp) at 96–98% purity were purchased from Chromadex (Irvine, CA). Cell culture medium (DMEM, DMEM/F12), and other supplements were purchased from Invitrogen (Grand Island, NY). All other reagents and compounds used were of analytical grades.

2.1. Cells, culture conditions and treatments

The non-tumorigenic human bronchial epithelial cells (Beas2b) and the tumorigenic NSCLC H1299 (p53null/EGFRWT) and A549 (p53WT/EGFRWT) cells were a kind gift from Drs. Wolfgang Zaccharias and Paula Bates (University of Louisville) and Dr. C. Gairola (University of Kentucky), respectively. The cell lines were authenticated by short tandem repeat sequencing and mycoplasma detection analysis performed at the John Hopkins genetic resources core facility (Baltimore, MD). Beas2B cells were maintained in DMEM/F12 medium, while both the A549 and H1299 cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. For cell proliferation and mechanistic studies, cells at 60–75% confluency were treated with individual anthocyanidin (12.5–100 μM) or their equimolar mixture (12.5–100 μM) in dimethyl sulfoxide (DMSO) or vehicle (DMSO) alone for different times. For metastasis studies, cells were treated with non-cytotoxic concentrations of individual anthocyanidins (3.125–12.5 μM) or their equimolar mixture (3.125–12.5 μM) for 24 h. Post treatment with test agents, cells were harvested and subjected to further analysis as described below.

2.2. Preparation of anthocyanidin test solution

To minimize concerns regarding the stability of anthocyanidins, fresh solutions of test anthocyanidins were prepared in DMSO for each experiment and then diluted to the final concentration in tissue culture media immediately before adding to the cells. Equimolar anthocyanidin mixture was prepared by mixing the five individual anthocyanidins in 1:1 ratio. Furthermore, experiments were also carried under subdued light to minimize any photo degradation of the test agents.

2.3. Measurement of cell viability

Cytotoxicity of anthocyanidins in lung cell lines was assessed by the enzymatic reduction of MTT. Briefly, 4.5×10^3 cells/well grown in 96-well tissue culture plates were exposed to indicated concentrations of test agents (12.5–100 μM) or the equimolar mixture containing 12.5–100 μM of each anthocyanidin for 72 h. Post treatment, cells were incubated for 2 h with 0.5 mg/ml of MTT reagent and the absorbance of resulting formazan product was measured at 570 nm in a microplate reader (Bio-Rad, Philadelphia, PA, USA). Calcusyn software (Biosoft, Ferguson, MO) which is based on the median effect model of Chou and Talalay [29] was used to calculate the combination index values for each concentration tested, whereby combination index values of <1 indicate synergistic, =1 indicates additive, and >1 indicate antagonistic effects.

2.4. Cell-cycle analysis

H1299 cells treated with vehicle or test agents were fixed gently in 70% ethanol overnight at 4 °C. After resuspending in PBS containing 10 μg/ml propidium iodide (PI), 0.1 mg/ml RNase and 0.1% Triton X-100, the cells were incubated in dark for 30 min and analyzed on a flow cytometer (BD Biosciences, San Jose, CA). The population of cells in each cell-cycle phase was determined using Flow Jo 7.2.5 for Windows (Tree Star, Ashland, OR).

2.5. Assays for apoptosis

Flow cytometric analysis: Apoptotic populations of H1299 cells treated with vehicle or test compound(s) were quantified using the dual staining annexin V-FITC/PI apoptosis detection kit (Invitrogen, Grand Island, NY) as per manufacturer's instructions.

Morphological examination: H1299 cells treated with vehicle or test compound(s) were stained with 1:1 mixture of ethidium bromide (EB) and acridine orange (AO) (5 μg/ml each) for 5 min, and then analyzed for morphological changes under a fluorescence microscope (Nikon Eclipse TS100) at 20× magnification.

2.6. Assays for metastasis

Cell migration and invasion assays: The *in vitro* cell migration and invasion assays were performed using 24-well transwell unit with polycarbonate filters that have a diameter of 6.5 mm and a pore size of 8.0 μm (Corning Costar, Cambridge, MA). For cell invasion assays, the filter membranes were coated with Matrigel (Becton Dickinson, San Jose, CA) and air dried under the hood overnight, before seeding the cells. Briefly, H1299 cells (4×10^4) suspended in 200 μl of serum-free medium in the presence and absence of test agent(s) were seeded onto the upper compartment of the transwell chamber. The lower chamber was filled with complete medium containing 10% FBS. Post 24 h treatment with test agents, the medium in the upper chamber was removed and the filters were fixed with methanol for 10 min. The cells remaining on the upper surface of the filter membrane were then completely removed by wiping with a cotton swab, and the cells on the opposite surface of the filter membrane were stained with 0.2% toluidine blue for 5 min. After a series of washes in water, the stained cells were solubilized in 20% acetic acid and absorbance read at 570 nm.

Cell motility assay: H1299 cells were seeded at a density of 3×10^4 cells/well in 24-well culture plates and allowed to form a confluent monolayer. A layer of cells was then scraped with a 1000 μl micropipette tip to create a wound of ~1–2 mm width and photographed. Cells were then treated with vehicle or test compound(s) for 24 h, and then imaged again to determine their effects on cell motility.

2.7. Western-blot analysis

Western-blot analysis was done as described previously [30] and blots were probed for cleaved Notch1, Wnt1, β-catenin and MMP9 antibodies (Assay biotech, Sunnyvale, CA), Notch1, cyclinB1, cyclin D1, c-myc, bax, bcl2, pERK and VEGF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). β-Actin (Sigma, St Louis, MO) was used as loading control.

2.8. Electrophoretic mobility shift assay (EMSA)

For the analysis of NF-kappa B activation, nuclear extracts from H1299 cells pretreated with vehicle or test agents at 37 °C for 12 h and then challenged with TNFα (1 ng/ml) for 30 min were prepared using the nuclear isolation kit from Pierce (NE-PER Nuclear and Cytoplasmic Extraction kit, Pierce Biotech., Rockford, IL). Extracts were then incubated with ^{32}P-end labeled double-stranded NF-kappa B (wild-type) oligonucleotide for 30 min at 37 °C, and the DNA–protein complex formed was separated from free oligonucleotide on 6% native polyacrylamide gels. Radioactive bands were visualized by Packard InstantImager.

2.9. Isolation of anthocyanidin mixture from bilberry and Dp from black currant

Enriched bilberry powder (36% anthocyanins) (Indena, Seattle, WA, USA) was extracted with 75% aqueous ethanol containing 0.1% HCl and enriched by loading the concentrated extracts on an XAD-761 column. The polyphenolics, including anthocyanins were eluted with methanol containing 0.1% HCl under gravity. Pooled eluates were concentrated and hydrolyzed with 2 N HCl for 2 h at 110 °C. Hydrolysates were purified on an Amberlite XAD-7/HP-20 (1:1) column (Sigma–Aldrich, St. Louis, MO) by washing with water to remove residual sugar and organic acids and