Therapeutic Effect of Blueberry Extracts for Acute Myeloid Leukemia

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Abstract

Acute Myeloid Leukemia (AML) is an aggressive hematological malignancy with high incidence in the aging population. In addition, AML is one of the more common pediatric malignancies. Unfortunately, both of these patient groups are quite sensitive to chemotherapy toxicities. Investigation of blueberries specifically as an anti-AML agent has been limited, despite being a prominent natural product with no reported toxicity. In this study, blueberry extracts are reported for the first time to exert a dietary therapeutic effect in animal models of AML. Furthermore, in vitro studies revealed that blueberry extracts exerted anti-AML efficacy against myeloid leukemia cell lines as well as against primary AML, and specifically provoked Erk and Akt regulation within the leukemia stem cell population. This study provides evidence that blueberries may be unique sources for anti-AML biopharmaceutical compound discovery, further warranting fractionation of this natural product. More so, blueberries themselves may provide an intriguing dietary option to enhance the anti-AML efficacy of traditional therapy for subsets of patients that otherwise may not tolerate rigorous combinations of therapeutics.

Keywords

Acute myeloid leukemia; Blueberry; Mouse models

Introduction

Natural product research has described numerous plant-derived compounds and extracts with beneficial health effects. Blueberries are rich sources for polyphenols, and are generally recognized for their health benefits arising from studies in models of aging, inflammation, diabetes, and cancer [1-5]. In contrast, the efficacy of blueberries or their components in cellular models of Acute Myeloid Leukemia (AML) and other leukemia’s has minimally been explored [6]. AML is a heterogeneous group of hematological cancers defined by clonal expansion of immature myeloid progenitors in the blood and bone marrow [7-9]. It is an aggressive disease that is defined by various potential cytogenetic and molecular alterations and immunophenotypes [10]. While the incidence of AML is greatest in the aging population, it can occur at any age and is one of the more common pediatric malignancies [7-9]. The prognosis for most AML is poor, with minimal clinical advances being made over the past few decades [10]. Remission is sometimes achieved, but only after aggressive chemotherapy that can be detrimental to pediatric and elderly patients. Therefore, studies aimed at identifying bioactive compounds with anti-AML efficacy from blueberries and other natural products are of interest for biopharmaceutical development given the need for effective and minimally toxic therapies. In the current study, we evaluated an anti-AML effect for blueberry extracts in both cellular and animal models of AML. By defining a role for blueberries as an anti-AML natural product this study may also lead to dietary interventions to augment the treatment of AML in pediatric or elderly patients that otherwise may not respond well to aggressive chemotherapy.

Materials and Methods

Cell culture

Murine C1498, 32D-p210-GFP, and 32D-FLT3-ITD cells, and human HL-60/vcr cells were maintained at 37°C and 5% CO2 in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Human KG-1 cells were likewise maintained, in IMDM supplemented with 20% FBS and 1% penicillin/streptomycin. Patient AML samples were obtained using informed consent approved by the Penn State College of
In Vitro Assays

Cellular viability assays were performed as previously described using a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay according to the manufacturer’s instructions ([Promega, Madison, WI] [11,12]). Colony forming assays were performed as previously described using MethoCult H4434 according to the manufacturer’s instructions (Stem Cell Technologies, Vancouver, BC, Canada) [11]. Apoptosis assays were performed as previously described using Annexin V and 7-aminonactinomycin D from BD Biosciences (San Jose, CA), and a fluorophore-conjugated antibody targeting CD45 from Biolegend (San Diego, CA) to gate the blast population [11,12]. Phosflow analysis was performed by using the BD Cytofix/Cytopermit kit according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). Briefly, cells were fixed following treatment and then their membranes were permeabilized to permit intracellular staining with fluorophore-conjugated antibodies targeting phosphorylated Akt and Erk (BD Biosciences, San Jose, CA). Cells were subsequently stained to evaluate leukemia stem cells with fluorophore-conjugated antibodies targeting CD34 and CD38 (BD Biosciences, San Jose, CA) as well as CD96 (eBioscience, San Diego, CA). Flow cytometry was performed at the Penn State College of Medicine Flow Cytometry Core using a BD Biosciences LSR II flow cytometer and BD FACS Diva software.

Animal trials

NOD-Scid and C57BL/6j mice were bred from founders obtained from the Jackson Laboratory (Bar Harbor, ME). Female NOD-Scid mice were xenografted with HL-60/vcr (2x10⁶ cells/mouse) or KG-1 (2.5x10⁶ cells/mouse) cells by tail vein injection. After one week, mice were randomized and given ad libitum access to either control drinking water or crude blueberry extract dissolved in drinking water (1% w/v). Male and female C57BL/6j mice were engrafted with C1498 cells (1x10⁶ cells/mouse) by retro-orbital injection. After one week, mice were randomized and given a crude blueberry preparation to eat (25 mg/mouse/week), ad libitum access to chloroquine in the drinking water (0.288 mg/ml sweetened with 15 mg/ml glucose to alleviate bitterness), a combination of both, or control water (sweetened with 15 mg/ml glucose) [13]. Mice were euthanized once they reached moribund status. All procedures were approved by the Institutional Animal Care and Use Committee of the Penn State College of Medicine.

Blueberry extraction

Solvents and reagents were obtained from VWR (Radnor, PA) and Sigma (St. Louis, MO). Briefly, whole blueberries were lyophilized, crushed to powder, and a crude extract was prepared by extracting for 30 minutes with agitation using aqueous acetone (70/30 acetone/water), and dried by rotary-evaporation and lyophilization [3,4]. As these were whole extracts, the mass yield was slightly over 50% of the original starting material. Standardized blueberry powder was alternatively obtained from FutureCeuticals (Mornence, IL). No differences were noted in cell culture studies between the blueberry crude extracts, and the standardized blueberry powder.

Statistical analysis

Colony-forming and apoptosis assay comparisons of untreated controls and treatments with blueberry extracts were made using an unpaired t-test. For in vivo studies, survival analysis was performed using the Mantel-Cox Logrank test. All experiments were performed with an n = 3 biological replicate sample size, and graphical averages are depicted +/- standard deviation from the mean.

Results and Discussion

Initially, blueberry extracts were evaluated for anti-AML therapeutic efficacy using cell lines and patient samples. Crude preparations were observed to diminish the viability of the AML cell lines HL-60/vcr, C1498, and 32D-FLT3-ITD, as well as the Chronic Myeloid Leukemia (CML) cell line 32D-p210-GFP (Figure 1A). Anti-AML efficacy for crude blueberry extracts was further demonstrated using a colony forming assay with a poor prognosis patient sample (#329: i/m3, -7) (Figure 1B), as well as several patient samples using short-term apoptosis assays (Figure 1C). Apoptosis assays used different patient cases including three poor prognosis AML (#651: -7, -5, #652: -7, #658: complex cytogenetics), one intermediate prognosis AML (#657:+8), one favorable prognosis AML (#661: #661: #657, #658, and #661) and patient CML (case #653) were determined in the presence of 500 µg/ml crude blueberry extract. (C) Colony forming capacity of a poor prognosis patient AML case #329 was determined in the presence of 500 µg/ml crude blueberry extract (t-test, p = 0.0245, n ≥ 3). (B) Colony forming capacity of a poor prognosis patient AML case #329 was determined in the presence of 500 µg/ml crude blueberry extract (t-test, p = 0.0245, n ≥ 3). (C) Ficol-prepared WBC fractions of patient AML (case #651, #652, #657, #658, and #661) and patient CML (case #653) were exposed to 500 µg/ml crude blueberry extracts for 48 hours followed by flow cytometry to determine percent apoptosis (Annexin V+) in the CD45-gated blast population (t-test, p = 0.0098, n = 6).
There is an urgent need for more effective and less toxic therapies for the treatment of AML. Many patients are not effectively treated with current standard of care therapies due to severe toxicity. Many times treatment will not commence or will be halted before completion.

Dietary interventions may offer alternative or adjuvants that are minimally toxic if at all. In the present study, blueberry extracts were evaluated for the first time for anti-AML efficacy in both cellular and animal models of AML. In addition to a demonstration of therapeutic efficacy in these models, the present study further demonstrated that blueberry extracts could regulate the AML stem cell. Therefore, constituent components of the blueberry hold promise not only as anti-AML therapeutics, but as anti-AML stem cell therapeutics. As part of an extract, or isolated and in combination with cytotoxic chemotherapeutics, blueberry components hold the potential to eradicate the specific cells that are primarily responsible for AML development, progression, relapse, and therapy resistance. More so, the success of the in vivo studies further showed good bioavailability for the components of the blueberry that exert anti-AML efficacy.
Altogether suggesting that blueberries could be a profound source for anti-leukemia biopharmaceutical discovery and development.

Finally, we have demonstrated that blueberry extracts can impede pro-inflammatory signaling pathways mediated by a neutral sphingomyelinase [3,4]. These particular pathways may augment leukemogenic signaling by redox-mediated inactivation of tyrosine phosphatases, which can lead to increased tyrosine kinase-mediated signaling [18,19]. In addition to the prevalence of dysregulated growth factor signaling

Pathways in AML and other myeloid hematological disorders, inflammatory cytokines have been associated with relapsing AML and AML secondary to Fanconi anemia [10,20-24]. Altogether, the persistence of these conditions in AML suggests that novel anti-inflammatory agents such as those derived from blueberry extracts may be of particular benefit for the treatment of AML. In conclusion, this study demonstrated anti-AML efficacy for blueberry extracts. This is of significance because natural products have long served as natural products have long served as a source for drug discovery.

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Figure 3: Survival extended in mice with AML following dietary blueberry extract supplementation
A. NOD-Scid mice were xenografted with the human AML cell line HL-60/vcr (2x10^6 cells/mouse by tail vein injection) and survival was monitored after mice were given crude blueberry extract (1% w/v) (dark line with diamonds) *ad libitum* in the drinking water, or control water (light gray line with squares), starting one week following engraftment (Mantel-Cox Logrank test, p=0.0017, n=7/group).

B. NOD-Scid mice were xenografted with the human AML cell line KG-1 (2.5x10^6 cells/mouse by tail vein injection) and survival was monitored after mice were given crude blueberry extract (1% w/v) (dark line with diamonds) *ad libitum* in the drinking water, or control water (light gray line with squares), starting one week following engraftment (Mantel-Cox Logrank test, p=0.0382, n=7/group).

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Figure 4: Dietary blueberry extract supplementation in combination with chloroquine extended the survival of mice with AML
C57BL/6J mice were engrafted with the murine AML cell line C1498 (1x10^6 cells/mouse by retro-orbital injection) and survival was monitored after mice were given a crude blueberry preparation to eat (25 mg/mouse/week) (line with upside down triangles), chloroquine in the drinking water (25 µM sweetened with 1% w/v glucose to alleviate bitterness) *ad libitum* (line with right side up triangles), or a combination of both (line with circles), or control water alone (line with squares), starting one week following engraftment (Mantel-Cox Logrank tests: Males & Females together, p = 0.0263, n ≥ 14/group; Males only, not significant at p=0.1225, n ≥ 7/group; Females only, p = 0.0479, n ≥ 7/group).
sources and blueprints for the discovery and development of novel biopharmaceuticals [25-27]. Therefore, the present study may hold promise to identify minimally toxic and potentially combinatorial anti-AML compounds that can be utilized in anti-AML biopharmaceutical development.

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