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Glucopsychosine increases cytosolic calcium to induce calpain-mediated apoptosis of acute myeloid leukemia cells

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ABSTRACT

To identify novel anti-cancer agents, we created and screened a unique nutraceutical library for activity against acute myeloid leukemia (AML) cells. From this screen, we determined that glucopsychosine was selectively toxic toward AML cell lines and primary AML patient samples with no effect toward normal hematopoietic cells. It delayed tumor growth and reduced tumor weights in mouse xenograft models without imparting toxicity. Glucopsychosine increased cytosolic calcium and induced apoptosis through calpain enzymes. Extracellular calcium was functionally important for glucopsychosine-induced AML cell death and surface calcium channel expression is altered in AML cells highlighting a unique mechanism of glucopsychosine's selectivity.

1. Introduction

Acute myeloid leukemia (AML) is an aggressive malignant disease characterized by the clonal expansion of myeloid precursors that fail to terminally differentiate (i.e., blasts) [1]. Front-line AML therapy consists of 3 days of daunorubucin (45–90 mg/m^2) and 7 days of continuous intravenous infusions of cytarabine (100 mg/m^2) and results in complete response rates up to 80% [2]. However, within 2 years there may be a reoccurrence of disease that is resistant to induction chemotherapy [3]. Although recent advances have improved therapies for other hematological malignancies, AML therapy has remained essentially unchanged for 30 years [4]. Thus, there is a critical need for new, more efficacious AML therapies.

Nutraceuticals are food-derived bioactive compounds with physiological activity and belong to a larger group of molecules known as natural health products. While the role of nutraceuticals in cancer treatments is largely unknown, recent studies have demonstrated that the natural health products kinetin riboside [5], an adenosine derivative from coconut milk, and parthenolide [6], an extract from the medicinal herb feverfew, possess anti-leukemia activity. Given these findings, we hypothesized that nutraceuticals would possess similar anti-leukemia activity. Since nutraceuticals are under-represented in large commercially available libraries, we compiled a unique nutraceutical-specific library consisting of 30 compounds and screened this library against AML cell lines to identify novel anti-leukemia nutraceuticals. From this screen, we identified glucopsychosine (GLU), a lipid derived from bovine milk.

2. Materials and methods

2.1. Reagents

Glucopsychosine was obtained from Matreya, Inc. (Pleasant Gap, PA) and reconstituted in 100% ethanol. The stock solution (5 mM) was diluted in phosphate buffered saline (PBS), aliquoted and stored at −20 °C to prevent excessive freeze thaw cycles. Fluo-3AM (Life Technologies; Grand Island, NY), z-VAD-FMK (Z-VAD; R&D Systems, Minneapolis, MN), Verapamil, Q-VD-OPh, 8-(N,N-Diethylamino)-octyl-3,4,5-trimethoxybenzoate HCl (TMB-8) and cyclosporin (Sigma Chemical; St. Louis, MO) and MDL28170 (Tocris; Bristol, UK) were purchased and reconstituted according to manufacturer's protocols.

2.2. Cell culture

Acute myeloid leukemia (OCI-AML2, KG1A, U937) cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies; Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and antibiotics

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(100 units/mL of streptomycin and 100 μg/mL of penicillin; Sigma Chemical). RWPE-1, normal prostate cells were incubated in keratinocyte serum free media (Life Technologies). Cells were incubated in a humidified air atmosphere containing 5% CO_2 at 37 °C.

Primary human AML samples, obtained from Dr. Mark Minden, Princess Margaret Cancer Center, were cultured at 37 °C in IMDM, 20% FCS and antibiotics. These cells were isolated from the peripheral blood of consenting AML patients who had at least 80% malignant cells among the mononuclear cells. Normal bone marrow-derived $CD34^+$ hematopoietic cells were purchased from Stem Cell Technologies (Vancouver, BC). The collection and use of human tissue for this study was approved by institutional ethics review boards (University Health Network, Toronto, ON, Canada and University of Waterloo, Waterloo, Ontario).

2.3. Cell growth and viability

Cell growth and viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) reduction assay (Promega, Madison, WI), according to the manufacturer's protocol and as previously described [7,8]. Cells were seeded in 96-well plates and treated with compound for 72 h. Optical density was measured at 490 nm. Cell viability was also assessed by Annexin V and PI (ANN/PI) staining (Biovision, Mountainview, CA) and flow cytometry, as previously described [8].

2.4. Nutraceutical screen

Nutraceuticals were obtained from commercial sources (NeaLanders Inc., Frutarom, Matreya and Sequoia Chemical Inc.). The unique nutraceutical library was created and screened similar to previously described methods [7–9]. Briefly, AML cells (1.5×10^4/well) were seeded in 96-well polystyrene tissue culture plates. After seeding, cells were treated with aliquots (10 μM final concentration) of library compound with a final dimethyl sulfoxide (DMSO) concentration of less than 0.05%. After 72 h, cell growth and viability were measured by the MTS assay.

2.5. Cytosolic calcium measurements

Cytosolic calcium was measured using fluo-3AM, which is a highly charged fluorescent dye that accumulates in the cytosol and emits green fluorescence following subsequent binding of calcium ions, similar to methods described previously [10]. Briefly, a stock solution of 1 mM fluo-3AM was diluted to 5 μM in fluo-3AM loading buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 137 mM NaCl, 5nM KCl, 1 mM Na_2HPO_4, 5 mM glucose, and 0.5 mM $MgCl_2$ (pH 7.4)]. Treated cells (with glucopsychosine or vehicle control) were incubated with fluo-3AM for 30 min at 37 °C with gentle agitation. After incubation, cells were washed twice in a calcium estimation buffer [fluo-3AM loading buffer and 1 mM $CaCl_2$] to remove and inactivate any extracellular fluo-3AM. Cells were then incubated with propidium iodide (PI) for 15 min at room temperature. The shift in green fluorescence was measured by flow cytometry (Guava 8HT; EMD Millipore, Billerica, MA). Cytosolic calcium was quantified by taking the mean fluorescence values of live gated cells and normalizing them to the zero control.

2.6. Western blotting

Whole cell lysates were prepared from treated cells, heated for 5 min at 95 °C, and subjected to gel electrophoresis on 7.5% SDS–polyacrylamide gels at 150 V for 85 min. The samples were then transferred at 25 V for 45 min to a PVDF membrane and blocked with 5% milk in Tris-buffered saline-tween (TBS-T) for 1 h. The membrane was incubated with the primary antibody, PARP-1 or calpain antibody (1:1000; Santa Cruz Biotechnologies), overnight at 4 °C. Membranes were then washed and incubated with the appropriate secondary antibody (1:10000) for 1 h at room temperature. Enhanced chemiluminescence (ECL) was used to detect proteins according to the manufacturer's instructions (GE Healthcare; Baie d'Urfe, Quebec) and luminescence was captured after 5 min using the Kodak Image Station 4000MM Pro and analyzed with a Kodak Molecular Imaging Software Version 5.0.1.27. All membranes were stained with Ponceau S and imaged to ensure equal loading, as described by Dam et al. [11].

2.7. Calpain activation assay

The Calpain-Glo Protease Assay (Promega, Madison, WI) was used to measure calpain activity through luminescence. A proluminescent calpain substrate was added to treated cells in white-walled, 96-well plates and measured at 10 min intervals according to manufacturer's instructions. The experiment was performed in triplicate with the mean luminescence value and standard deviation shown for each sample treatment.

2.8. Xenograft animal model

NOD/SCID ($N = 24$, Jackson Laboratory, Bar Harbor, ME) mice were injected subcutaneously in the left flank with OCI-AML2 leukemia cells (2.5×10^6). Mice were then randomly assigned to receive glucopsychosine (20 or 40 mg/kg/every other day; in 0.9% NaCl and 0.01% tween-80) or vehicle control (0.9% NaCl and 0.01% tween-80) intraperitoneally. Tumor volumes (tumor length $\times$ width^2 $\times$ 0.5236) were monitored every other day using a caliper. At the end of the experiment (18d), mice were sacrificed by sodium pentobarbital injection and cardiac puncture, blood collected and tumors excised and weighed. Blood was sent to the Animal Health Laboratory, University of Guelph for blood biochemistry analysis. All animal studies were carried out according to the regulations of the Canadian Council on Animal Care and with the approval of the University of Waterloo, Animal Care Committee.

2.9. Interrogation of publically available databases

The expression profile of the set of known calcium channel genes was assessed in publicly available datasets of AML patients compared to normal hematopoietic cells [12]. Mean fold-change was computed for the AML vs. normal comparison, and statistical significance (>1.25-fold; $p < 0.001$) was assessed by ANOVA to derive the set of differentially expressed calcium channel genes.

2.10. Statistical analysis

Unless otherwise stated, the results are presented as mean ± SD. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software, USA). $p \leqslant 0.05$ was accepted as being statistically significant.

3. Results

3.1. A nutraceutical screen for novel anti-cancer compounds identifies glucopsychosine

To identify nutraceuticals with anti-cancer activity we compiled a unique library consisting of 30 nutraceuticals with previously uncharacterized anti-cancer activity from commercially available sources. The library was screened against the AML cell lines, OCI-AML2 and KG1A, and the "normal" prostate cell line RWPE using the MTS assay following a 72 h incubation period. The compound which imparted the greatest reduction in viability in both AML cell lines without affecting normal cell viability was glucopsychosine (Fig. 1A and B). Compound 4 (toosendanin) demonstrated toxicity in all cell lines, including the control cell line, and was therefore not analyzed further and compound 21 is currently being evaluated in our laboratory. Glucopsychosine's activity was validated by generating dose response curves on a panel of available AML ($n = 3$) cell lines using the MTS assay following a 72 h incubation period. It reduced the viability in a dose dependent manner in all AML lines tested (Fig. 1C, left panel; see Supplementary Table 1 for confirmation of AML surface markers). Cell lines of breast, colon and cervical cancer origin were also tested and showed varying degrees of sensitivity to glucopsychosine; ALL, CML and myeloma cells were not sensitive and AML cells were consistently the most sensitive (Supplementary Fig. 1). Glucopsychosine's cytotoxicity was confirmed using the Annexin/PI assay following 72 h of incubation (IC50: 4.2 ± 0.5 μM; Fig. 1C, right panel).

Given the cytotoxicity of glucopsychosine in AML cell lines, we tested its activity in primary AML patient samples and in normal hematopoietic cells using the ANN/PI assay. Glucopsychosine reduced the viability of primary AML patient samples ($n = 3$) but had no effect on the viability of normal hematopoietic cells ($n = 3$) (Fig. 1D). Together, these results demonstrate glucopsychosine's selective toxicity toward AML cells.

3.2. Glucopsychosine induces caspase-independent apoptosis

Time course analysis using the ANN/PI assay demonstrated that glucopsychosine-induced apoptosis (i.e., ANN^+/PI^-), which was initiated after 12 h of treatment with 10 μM glucopsychosine