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Cooperative antiproliferative and differentiation-enhancing activity of medicinal plant extracts in acute myeloid leukemia cells

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ABSTRACT

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy with poor prognosis and limited treatment options. Sea buckthorn (*Hippophae rhamnoides*) berries, dog rose (*Rosa canina*) rosehips, and garden sage (*Salvia officinalis*) and oregano (*Origanum vulgare*) aerial parts are widely used in traditional medicine and exhibit antitumor effects in preclinical models. However, these plants remain scarcely tested for antileukemic activity. Here, we show that their water-ethanol leaf extracts reduced the growth and viability of AML cells and, at non-cytotoxic doses, potentiated cell differentiation induced by a low concentration of 1α,25-dihydroxyvitamin D_3, the hormonal form of vitamin D, in a cell type-dependent manner. The latter effect was accompanied by upregulation of the vitamin D receptor protein components and its transcriptional activity. Furthermore, at minimally effective doses the extracts cooperated with one another to produce marked cytostatic effects associated with a partial S-phase arrest and a modest induction of apoptosis. In contrast, these combinations only slightly affected the growth and viability of proliferating normal human peripheral blood mononuclear cells. In addition, the extracts strongly inhibited microsomal lipid peroxidation and protected normal erythrocytes against hypoosmotic shock. Our results suggest that further exploration of the enhanced antileukemic effects of the combinations tested here may lead to the development of alternative therapeutic and preventive approaches against AML.

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1. Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and is characterized by the accumulation of highly proliferative immature leukemic cells in the bone marrow which suppresses normal hematopoiesis. Although 50–70% of younger patients with AML achieve complete remission following cytotoxic chemotherapy, most are expected to relapse and die [1]. Furthermore, due to its high general toxicity, standard aggressive treatment is largely unsuitable for elderly individuals representing the majority of patients with AML [2]. Despite a number of new compounds that have been in development for AML treatment, most have failed in clinical trials, and not a single new drug has yet been approved for AML in the last 40 years [3].

Phytochemicals (e.g., vinblastine, paclitaxel, topotecan, etoposide) have been successfully used for the treatment of lymphoid leukemias and solid malignancies [4]. Although the above drugs are not effective in AML therapy, other compounds, e.g., the alkaloid homoharringtonine from the Chinese coniferous tree *Cephalotaxus hainanensis* and the semisynthetic flavonoid flavopiridol from the Indian plant *Dysoxylum binectariferum* are currently evaluated in clinical trials in AML [5,6]. Extracts from various edible and medicinal plants possess therapeutic and preventive potential in hematopoietic cancers and represent rich sources of novel antileukemic drugs [7,8]. Furthermore, we and others have shown that both individual phytochemicals and whole plant extracts can cooperate with one another [9–14] and with

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other natural agents, such as vitamin D derivatives [15–20], in producing enhanced anticancer effects in preclinical models.

Here, we show that leaf extracts from several medicinal plants collected in the Trans-Ili Alatau region of Kazakhstan – sea buckthorn (*Hippophae rhamnoides*), dog rose (*Rosa canina*), garden sage (*Salvia officinalis*) and oregano (*Origanum vulgare*) – exhibited dose-dependent antiproliferative activity in AML cell lines. These extracts were also capable of potentiating the differentiation-inducing effects of 1α,25-dihydroxyvitamin D_3 (1,25D), the active form of vitamin D, which was accompanied by upregulation of the vitamin D receptor (VDR) levels and functional activity. In addition, different combinations of the extracts at minimally effective doses produced marked cooperative cell-type dependent cytostatic effects associated with a partial S-phase arrest and modulation of the levels of cell cycle regulatory proteins.

2. Materials and methods

2.1. Materials

RPMI 1640 medium and fetal calf serum (FCS) were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). Dulbecco's phosphate buffered saline (PBS), HEPES, penicillin and streptomycin were purchased from Biological Industries (Beit Haemek, Israel). Triton X-100, RNAse, Histopaque-1077 and other biochemical reagents were obtained from Sigma-Aldrich (Rehovot, Israel). 1,25D was a gift from Dr. A. Kutner (Pharmaceutical Research Institute, Warsaw, Poland). Antibodies to cyclins A and E, CDK2, $p27^{Kip1}$, VDR, RXRα and β-tubulin were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-PARP antibody was purchased from BioMol (Plymouth Meeting, PA, USA). Stock solutions of 1,25D ($\sim$0.2 mM) were prepared in absolute ethanol and the exact concentration of this agent was then determined spectrophotometrically at 264 nm (ε = 19000).

2.2. Plant materials

Aerial parts of *H. rhamnoides* L. and *R. canina* L. were collected in the foothills of the Trans-Ili Alatau mountains (Almaty region, Kazakhstan) during July 2013. The plants were identified, and voucher specimens No. 5470 (*H. rhamnoides* L.) and No. 3389 (*R. canina* L.) were deposited at the herbarium of the Institute of Botany and Phytointroduction (Almaty, Kazakhstan). Commercial samples of dried leaves of *S. officinalis* and aerial parts of *O. vulgare* (TES, Almaty, Kasakhstan), collected in the Almaty region, were purchased from a local pharmacy.

2.3. Preparation of plant extracts

Crushed dried leaves of *H. rhamnoides*,*R. canina* and *S. officinalis* or aerial parts of *O. vulgare* were extracted with 50% (v/v) aqueous ethanol, at room temperature for 20 h in the dark, as described previously [21]. The mixture was then centrifuged at 20,000*g* for 10 min and the supernatant was dried at 37 °C in a rotary evaporator. Stock solutions of the dried extracts (100 mg/ml) were freshly prepared in 50% ethanol before experiments.

2.4. Determination of total polyphenol and flavonoid content in dried plant extracts

Total phenolic content was determined by the standard Folin-Ciocalteu assay [22]. The absorbance of the samples was measured at a wavelength of 765 nm and the values were expressed as gallic acid equivalents (GAE, μg per mg dried extract). The total flavonoid content was determined using a colorimetric method described by Heimler et al. [23]. The absorption was measured at 510 nm and the amount of total flavonoids was expressed as quercetin equivalents [QE, μg per mg dried extract].

2.5. Preparation of rat liver microsomes

Healthy Wistar rats (12–14 weeks, 300 $\pm$ 50 g) were housed under standard conditions of light and dark cycle with free access to food and water. The experimental protocols were approved by the Institutional Ethical Committee of the Institute of Human and Animal Physiology (Almaty, Kazakhstan). The animals were killed under light ether anesthesia. The liver was isolated, washed, and perfused with chilled saline. The minced tissue was homogenized (1:10 w/v) in 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA on ice. The homogenate was centrifuged at 10,000*g* at 4 °C, for 20 min. The supernatant was further centrifuged at 100,000*g*, for 60 min, to obtain the microsomal fraction. Microsomes were suspended in a buffer containing 10 mM histidine (pH 7.2), 25% (v/v) glycerol, 0.1 mM EDTA and 0.2 mM $CaCl_2$, and were kept at −20 °C. The protein content was measured by the Lowry assay using bovine serum albumin as a standard.

2.6. Isolation of rat erythrocytes

Erythrocytes were obtained by centrifuging blood for 10 min at 1000*g*. Plasma and white blood cells were removed, erythrocytes were washed twice with a buffer containing 5 mM Na_2HPO_4 (pH 7.4) and 150 mM NaCl, and used fresh.

2.7. Determination of lipid peroxidation in liver microsomes

Lipid peroxidation (LPO) was assessed by measuring malondialdehyde (MDA) content in the form of thiobarbituric acid-reacting substances (TBARS) by the method of Ohkawa et al. [24]. Briefly, liver microsomes were preincubated with tested agents in a buffer containing 50 mM KH_2PO_4 (pH 7.2) and 145 mM NaCl at 37 °C, for 10 min, under constant stirring. The basal and 0.02 mM Fe^{2+}/0.5 mM ascorbate-induced microsomal LPO was then determined in a reaction mixture containing 0.9 M sodium acetate buffer (pH 3.5), 0.4% SDS, 20 mM thiobarbituric acid and 15 mM phenazine methosulfate following incubation at 95 °C for 60 min. After cooling to room temperature, the mixture was extracted by *n*-butanol:pyridine (15:1, v/v) and centrifuged at 3000*g*, for 5 min. The organic layer was collected and its absorbance was measured at 532 nm. The MDA concentration was expressed as nmol of TBARS per mg protein.

2.8. Determination of osmotic resistance of erythrocytes

Osmotic resistance of erythrocytes (ORE) was measured as described previously [25]. Briefly, isolated erythrocytes were preincubated with test agents at 37 °C, for 10 min, and then subjected to hypotonic solution of NaCl (0.4%), for 20 min at 37 °C and centrifuged. Hemoglobin absorbance was then measured in the supernatant at 540 nm. The level of hemolysis was calculated as the percentage of total hemolysis caused by 0.1% Na_2CO_3.

2.9. Cell culture

HL60 myeloblastic leukemia cells (ATCC-CCL-240) and U937 promonocytic leukemia cells (ATCC-CRL-1593.2) were purchased from American Type Culture Collection (Rockville, MD, USA). Samples of human peripheral blood were collected from healthy donors at Soroka University Medical Center (Beer-Sheva, Israel) after informed consent (Ben-Gurion University Helsinki Committee for Protection of Human Subjects; approval #3587). Peripheral blood

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