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Centaurea albonitens extract enhances the therapeutic effects of Vincristine in leukemic cells by inducing apoptosis

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ABSTRACT

Drug-induced toxicities and dose-related side effects are the major challenges in the conventional cancer therapy by the chemo drugs. On the other hand, herbal derivatives have obtained a great research interest in the field of therapeutic applications because of their more favorable specifications including less toxicity, cost-effective and more physiologically compatible than the chemical drugs. For this purpose, we evaluated methanolic extract prepared from *Centaurea albonitens* Turrill alone and in combination with Vincristine (VCR) for its potential cytotoxic effects in NALM-6, REH, NB4 and KMM-1 cell lines by using the various approaches. *Centaurea* genus is one of the current medicinal plants, which has used in traditional medicine, However, there are rare studies to examine its anticancer properties against hematologic malignant cells. In this study, we demonstrated *Centaurea albonitens* extract (CAE) induces cytotoxicity through G0/G1 phase arrest followed by apoptosis in a dose- and time- dependent manner, although with varying efficiency. Interestingly, normal cells didn't exhibit significant cytotoxicity after CAE treatment. Moreover, we found that low dose of CAE enhances anti-cancer effects of VCR in pre-B ALL cell lines (NALM-6 and REH). Further investigations validated synergistic anticancer activities of VCR and CAE through inducing apoptosis without significant cell cycle arrest. Taken together, our results demonstrated for the first time that the methanolic extract of *Centaurea albonitens* can be considered as a potential anticancer agent and/or an enhancer of chemotherapeutic sensitivity of VCR.

1. Introduction

Chemotherapy-related adverse effects are considered as treatment limitations in the cancer therapy. The administration of different chemotherapeutic drugs for cancer treatment induces a variety of side effects, especially on blood cells [1–3]. Additionally, extensive irradiation usually causes other difficulties in patients, such as mucositis, hematopoietic syndrome and other disasters [4–7]. Regarding to the disadvantages of the conventional treatment methods, developing more effective drugs with less toxicity and side effects is one of the most important requirements. Irrespective of the power of synthetic chemistry as an approach to discover and develop new therapeutic agents, the bioactive plant extracts and their derivatives would be very worthwhile for detecting efficient and safe anti-tumor therapeutics [8,9]. Today, more researchers have focused on the utilization of phytochemicals such as curcumin, genistein, resveratrol, epigallocatechin-3-gallate and sulforaphane for the managing cancer [10–14]. Additionally, the role of the positive synergistic effects of secondary metabolites and chemo drugs should not be ignored as one of the rational reasons for paying attention to the plant extracts in the recent chemotherapeutic and chemoprevention investigations. Since many reliable investigations have defended the combination strategies to improve the health status of patients and access to the appropriate therapeutic efficacy, hence, the usage of medicinal plants as a collection of bioactive phytochemical should be optimized [15].

Centaurea is the largest genus in the Asteraceae family, has been close to 600 species and is generally found in the Mediterranean area and west Asia [16]. The genus *Centaurea* is known for its various medicinal properties such as antioxidant, antimicrobial, anti-fungal and anti-inflammatory [17–22]. Recent studies exhibited cytotoxic effects of various species of *Centaurea* genus and their components against various cancer cell lines including CaCo2, MCF-7, MIA PaCa-2, A431,

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Hela, PAM212, SP1 and COLO320, although at varying efficiency [21,23–28]. In the present study, we investigated the cytotoxic effects associated with methanolic extract of aerial parts of *Centaurea albonitens* Turrill, one of the less investigated *Centaurea* species, in a panel of hematological cell lines including pre-B acute lymphoblastic leukemia (ALL), multiple myeloma and acute promyelocytic leukemia (APL) derived cells. The present study showed for the first time that *Centaurea albonitens* extract (CAE) reduced cell viability in hematologic malignant cell lines, however, some of the cell lines tend to be more sensitive to the extract. The complex synergistic interactions between the drugs and herbs are supposed to be able to reduce toxicity and/or enhance and promote therapeutic effects. Our study demonstrated that CAE not only is effective as a single agent and induces a caspase 3-dependent apoptosis in pre-B ALL derived cells, but also enhances chemotherapeutic sensitivity of Vincristine (VCR).

2. Material and method

2.1. Preparation of the CAE and determination of secondary metabolite profiles

The aerial parts of *Centaurea albonitens* were collected from Hamedan province, Iran. A voucher specimen was deposited at the Herbarium of Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (No. 3234). Crude extraction was performed by maceration for 24 h with continuous shaking at room temperature. The filtrate was evaporated to dryness and used for in vitro assays. One way of plant component determination is the use of reagents like NaOH (for flavonoids), Wagner and Mayer (for alkaloids), sulfuric acid (for sesquiterpene lactones) and etc. For this purpose, we added 1 ml NaOH to 2 ml extract and 2 drops Mayer, 3–5 drops Wagner and 2 drops sulfuric acid each to 1 mL extract. Precipitation is a sign of the presence of mentioned components in this extract, although depending on the severity.

2.2. Cell culture and treatment

Human pre-B ALL cell lines (NALM-6 and REH), human APL cell line (NB4), human multiple myeloma cell line (KMM-1) and normal cell line (MDBK) were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C under standard cell culture conditions. VCR (Sigma-Aldrich, St Louis, MO, USA) powder and CAE were dissolved in DMSO as stock solutions and then diluted with fresh RPMI 1640 medium. All of cell lines were treated with relevant amounts of CAE, to attain concentration of 40–100 µg/ml for up to 48 h. For drug combination experiments, NALM-6 and REH cells were co-treated with the desired concentration of VCR and CAE for 48 h. The final concentration of DMSO in treated cells was less than 0.1%.

2.3. Trypan blue dye exclusion assay

To examine the cytotoxic effect of CAE on cell viability and logarithmic growth, cells were seeded at 1×10^5 cells per ml and incubated in the presence of various concentrations of CAE up to 48 h. Then, the cell suspension was mixed with 0.4% trypan blue solution at a 1:1 ratio and after incubating time of 1–2 min at room temperature, the mixture was loaded into the chamber of Neubauer hemocytometer. Finally, the cell viability index was assessed as follows: viability (%) = viable cell count/total cell count × 100.

2.4. MTT assay

Metabolic activity of treated cells was measured using the MTT method. Briefly, cells were seeded in 96-well plates at a density of 1×10^4 (for NALM-6, REH and KMM-1) and 5×10^3 (for NB4) cells per well and incubated with increasing concentrations of CAE for 24 h and 48 h. Then, 100 µl of MTT (5 mg/ml in PBS) was added to the wells and incubated for 4 h at 37 °C. The mixture was removed and 100 µl DMSO was added to the wells for dissolving formazan crystals. Untreated cells were used as control groups. The OD values of the wells were detected at 570 nm by ELISA microplate reader.

2.5. DAPI staining

To detect morphological evidence of apoptosis, cells were washed once with DAPI (Sigma-Aldrich)-methanol solution, suspended in DAPI-methanol for 15 min at 37 °C protected from light and washed twice with PBS, and then examined in fluorescent microscope.

2.6. Cell cycle analysis

To cell cycle distribution analysis, we used Propidium Iodide (PI) staining. NALM-6 and REH cells were seeded into six-well plates at the concentration of 1×10^6 cells per well and incubated with different concentrations of CAE, VCR and CAE plus VCR for 48 h. The cells were then harvested, washed twice with PBS and fixed with 70% ethanol at −20 °C overnight. Afterward, cells were treated with 0.5 g/ml RNase in PBS and incubated at 37 °C before staining with 50 g/ml PI for 30 min. Finally, cellular DNA content was analyzed using flow cytometry. The distributions of the cell cycle were calculated using the FlowJo software version 7.6.1.

2.7. Annexin V-FITC/PI double staining

Flow cytometry was carried out to evaluate apoptosis in NALM-6 and REH cells. Briefly, the cells were cultured at 5×10^5 cells per well into 6-well plates, collected and washed with PBS and binding buffer. Next, 5 µl of fluorochrome-conjugated Annexin V (Annexin V-FITC/PI kit, eBioscience, USA) were added to 100 µl of the cell suspension and after 10–15 min incubation in the dark, 5 µl of Propidium Iodide staining was added to each microtube. The events for live, early and late apoptotic cells were counted with the Attune NxT acoustic focusing cytometer Analyzer (Thermo Fisher, USA) and illustrated as dot plot using FlowJo software version 7.6.1. Annexin V-positive and PI-negative cells were considered to be in early apoptotic phase and cells having positive staining for both Annexin-V and PI were deemed to undergo late apoptosis.

2.8. RNA extraction, reverse transcription and quantitative real-time RT-PCR

Cells were collected and total RNA was extracted using a Hybrid-R RNA purification kit (GeneAll, Korea) following the manufacturer's instructions. RNA quantity and quality were evaluated using NanoDrop TM 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis respectively. Reverse transcription was performed using the multiScript™ RT High-Capacity cDNA Reverse Transcription kit (Thermo Fisher scientific, USA). The cDNA template was amplified by Rotor-Gene Q real time RT-PCR System (Qiagen, Valencia, CA) using the RealQ Plus Master Mix (Ampliqon, DK). The cycling condition was as follows, 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s and 60–67 °C (primer TM) for 60 s. The primer sequences are presented in Table 1. All data were controlled for the quantity of RNA input by performing measurements on the endogenous reference gene ABL. As well as, RNA results from treated samples were normalized to results obtained using RNA from the control, untreated sample. Hence, the mean CT of the target genes was calculated from triplicate measurements and then normalized with the mean CT of ABL gene. The Pfaffl method was used to calculate the relative expression of each gene (Pfaffl, 2001).