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Biochemical mechanisms underlying the pro-apoptotic activity of 7,8-dihydroxy-4-methylcoumarin in human leukemic cells

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ABSTRACT

The search for new drugs requires a deep understanding of the molecular basis of drug action, being necessary the elucidation of the mechanism of action with the understanding of the relationship between structure and activity. In the present study, we evaluated the pro-apoptotic activity of 7,8-dihydroxy-4-methylcoumarin (DHMC) and its underlying mechanisms in human leukemic cells. Here, we present evidence that DHMC induced selective and concentration-dependent apoptosis in human leukemic cells. The pro-apoptotic effect of DHMC was mediated by activation of the JNKs and inhibition of the ERK1/2 and PI3K/Akt pathways, with no participation of the p38 cascade after 24 h of treatment. Indeed, down-regulation of the proto-oncogene c-myc as well as induction of the cell cycle inhibitor $p21^{WAF1/CIP1}$ through a p53 independent mechanism were observed in U-937 cells. These findings suggest that DHCM may have a potential therapeutic role in the future treatment of hematological malignancies.

1. Introduction

In leukemias early hematopoietic progenitors fail to respond to external stimuli within the bone marrow microenvironment. They do not undergo programmed cell death (apoptosis) and fail to differentiate into discrete types of blood cells, acquire immortality, self-perpetuate at the expense of other cells, and then spill into the peripheral blood [1]. Despite the numerous progresses achieved in the treatment of leukemias over the last years, many problems as cellular heterogeneity, development of multidrug-resistance, heterogeneous molecular abnormalities, and lack of selective action of antineoplastic agents still remain [2]. Nowadays, a variety of anticancer drugs including ara-C, cisplatin, etoposide, and taxol which interact with diverse intracellular molecular targets have been shown to engage the final common pathway

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Abbreviations: DHMC, 7,8-dihydroxy-4-methylcoumarin; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; TBE buffer, tris-borate–EDTA; NAC, N-acetyl-L-cysteine; IC_{50}, inhibitory proliferation concentration 50; CC_{50}, cytotoxic concentration 50; HO, Hoechst 33342; PI, propidium iodide.

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of programmed cell death, resulting in the apoptosis of human leukemic cells [3–5]. These conventional therapies cause serious side effects and, at best, merely extend the patient's lifespan by a few years, supporting the necessity to develop alternative therapeutic strategies. In our effort to discover and develop new potential and selective anti-leukemic compounds, we continue our studies using coumarins as leader molecules.

Coumarins are a group of heterocyclic compounds synthesized by numerous green plant species as well as by some bacteria and fungi [6], that have been widely used as a fragrance in food and cosmetic products [7]. Numerous biological activities have been attributed to simple coumarins and analogues as anti-coagulant [8], anti-inflammatory [9,10] anti-viral [11] and antimicrobial effects [12,13], enzyme inhibition [14,15], scavenging of reactive oxygen species [16,17] and central nervous system actions [18].

Different natural and synthetic coumarins are of great interest due to their described anti-tumoral properties in sensitive neoplasm. Several authors reported that numerous nitrated, hydroxylated or isopentenylated coumarins and derived pyrano- and furanocoumarins show strong anti-proliferative activity and induce apoptosis in various cancer cell lines [19–22]. The hydroxylated coumarin, esculetin, exhibits anti-tumoral effects by inhibiting cell cycle progression and inducing programmed cell death in HL-60 cells [23,24]. Lopez-Gonzalez et al. [25] showed that coumarin and its metabolite 7-hydroxycoumarin have cytostatic and pro-apoptotic actions in non-small cell lung carcinoma. On the other hand, several coumarins show synergistic effects with retinoic acid on the differentiation of human leukemic HL-60 cells [26]. Furthermore, daphnetin behaves as a potent anti-proliferative and differentiation agent in human renal cell carcinoma [27]. We have previously reported that a group of natural coumarins exhibit anti-proliferative and differentiation activity in the promonocytic U-937 cell line [28] and more recently we have observed that hydroxylated coumarins induce apoptosis in human leukemic cells (unpublished observations). These studies strongly support the potential therapeutic applications of coumarins, making these molecules attractive for their further evaluation as novel therapeutic agents for cancer treatment.

However, the anti-tumor mechanisms of coumarins are not well understood. Several researchers have investigated different intracellular events in an attempt to elucidate the mechanism of action of active coumarins in cancer cells. Cellular events as modulation of mitogen-activated protein kinases (MAPKs) [29–31], inhibition of protein kinases including EGF receptor tyrosine kinase, PKA and PKC [32] or topoisomerase II activity [30] have been evaluated. Chu et al. [23] reported that esculetin induces apoptosis by the mitochondrial pathway in the HL-60 cell line. Moreover, in the last years it has been proposed that the pro-oxidant action of plant derived phenolics rather than their antioxidant action may be an important mechanism for their apoptosis-inducing properties in cancer cells [33]. In scopoletin-induced apoptosis in HL-60 cells it has been suggested that the generation of reactive oxygen species may contribute to the induction of cell death [34].

In the present study, we focused on the effect of DHMC on the major stress signaling pathways: the mitogen-activated protein kinases, phosphoinositide-3-kinase/Akt signaling cascade (PI3K/Akt) and c-myc proto-oncogene, which are intimately involved in the regulation of cell growth, differentiation and apoptosis in hematopoietic cells.

In this work, we present evidence that DHMC induced selective apoptosis in human leukemic cells mediated by activation of the JNKs pathway and inhibition of the ERK1/2 pathway, with no participation of the p38 cascade after 24 h of treatment. Cell exposed to DHMC for 18 h showed inhibition of the PI3K/Akt pathway, an important survival pathway in leukemic cells. Furthermore, down-regulation of c-myc proto-oncogene and induction of the cell cycle inhibitor $p21^{WAF1/CIP1}$ in a p53 independent mechanism was also observed.

2. Materials and methods

2.1. Reagents and antibodies

Cell culture medium, antibiotics, bovine serum albumin (BSA), phosphate-buffered saline (PBS), dibutyryl cyclic adenosine monophosphate (dbcAMP), N-acetyl-L-cystein (NAC), dimethyl sulfoxide (DMSO), proteinase K and 4β-phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Company (St. Louis, MO). Fetal calf serum was purchased from Natocor (Argentina). LY294002 was from Tocris Cookson (Ellisville, MO). All other chemicals used were of analytical grade. Anti-c-Myc, -phospho-Akt1/2/3, -ERK1, -caspase 3, -PARP, -actin, rabbit antibodies; anti-p21, -phospho-p38, -phospho-ERK1/2, -phospho-JNK, -Akt1, -p38, -JNK1 mouse antibodies and anti-CD88 goat antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

7,8-Dihydroxy-4-methylcoumarin (DHMC) was synthesized through the Pechmann reaction between a properly substituted phenol and ethyl acetoacetate. DHMC was dissolved in 0.01% (v/v) DMSO and stored at −20 °C.

2.2. Cell culture

The human promonocytic U-937 cell line and the human promyelocytic HL-60 cells (American Type Culture Collection, Rockville, MD) were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. The cell suspension was split at the third day and diluted 1 day before experimental procedures.

Peripheral blood mononuclear cells from heparinized samples of healthy donors isolated by centrifugation on Ficoll-Hypaque were provided by Dr. Mónica Vermeulen (IIHEMA; Academia Nacional de Medicina, Buenos Aires, Argentina). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal calf serum and 50 μg/ml gentamicin.

2.3. Assessment of cell viability

Cell viability was monitored by the trypan blue exclusion assay. Cells growing in exponential phase were seeded at 10^5 cells in 1 ml of RPMI 1640 in a 24 well culture plate and incubated in a 5% CO_2 atmosphere. Cells were exposed to