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Original Articles

Dehydroleucodine inhibits tumor growth in a preclinical melanoma model by inducing cell cycle arrest, senescence and apoptosis

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ABSTRACT

Malignant melanoma represents the fastest growing public health risk of all cancer types worldwide. Several strategies and anti-cancer drugs have been used in an effort to improve treatments, but the development of resistance to anti-neoplastic drugs remains the major cause of chemotherapy failure in melanomas. Previously, we showed that the sesquiterpene lactone, dehydroleucodine (DhL), promotes the accumulation of DNA damage markers, such as H2AX and 53BP1, in human tumor cells. Also DhL was shown to trigger either cell senescence or apoptosis in a concentration-dependent manner in HeLa and MCF7 cells. Here, we evaluated the effects of DhL on B16F0 mouse melanoma cells *in vitro* and in a pre-clinical melanoma model. DhL inhibited the proliferation of B16F0 cells by inducing senescence or apoptosis in a concentration-dependent manner. Also, DhL reduced the expression of the cell cycle proteins cyclin D1 and B1 and the inhibitor of apoptosis protein, survivin. In melanomas generated by subcutaneous injection of B16F0 cells into C57/BL6 mice, the treatment with 20 mg DhL /Kg/day in preventive, simultaneous and therapeutic protocols reduced tumor volumes by 70%, 60% and 50%, respectively. DhL treatments reduced the number of proliferating, while increasing the number of senescent and apoptotic tumor cells. To estimate the long-term effects of DhL, a mathematical model was applied to fit experimental data. Extrapolation beyond experimental time points revealed that DhL administration following preventive and therapeutic protocols is predicted to be more effective than simultaneous treatments with DhL in restricting tumor growth.

Introduction

Sesquiterpene lactones (SLs) are a large and structurally diverse group of plant metabolites, which includes many members with anti-tumor effects, and one of these has been shown to reduce rat melanoma growth [1]. SLs of the guaianolide group are of particular interest as anti-tumor agents because each chemical substitution to the guaianolide skeleton confers a distinct biological activity to the resulting compound [2].Our group is particularly interested in the lactone, dehydroleucodine (DhL), a SL of the guaianolide group with an alpha-methylene butyrogamma-ring connected to a seven-atom ring that is fused to another exocyclic alpha, beta-unsaturated cyclopentenone ring (for structure see [3] and Fig. 1).

DhL can be isolated and purified to concentrations over 1%, starting from the above-ground parts of *Artemisia douglasiana* (Besser), a widespread and readily available medicinal herb that is commonly used in Argentina [4]. In a recent study [5], we demonstrated the anti-proliferative effects *in vitro* of DhL in human cancer cells. Our analysis of the accumulation of DNA damage response (DDR) markers revealed a striking correlation between the extent of DNA damage and the activation of senescence and apoptosis programs, which were selectively stimulated by lower and higher DhL concentrations, respectively. Clonogenic assays in the presence of DhL revealed that proliferating cells were very effectively eliminated by DhL due to the induction of apoptotic and senescence programs. Further analysis of the novel role of DhL in cellular senescence showed that the anti-proliferative effect was associated with a delay in progression through the G2 phase that preceded cell cycle arrest in the

Abbreviations: FBS, fetal bovine serum; HE, hematoxylin-eosin; SA- β -Gal, senescence-associated β-galactosidase; IC50, half maximal inhibitory concentration; gv, tumor growth velocity; DDR, DNA damage response.

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following G1 phase. This phenomenon was accompanied by reduced cyclin B1 and higher p53 levels, suggesting that p53 might be responsible for promoting cell cycle withdrawal.

Cyclin B1 is the regulatory subunit of CDK1, which plays a pivotal role in the transition of the cell cycle from the G2 phase to mitosis [6]. The inhibitor of apoptosis protein (IAP) survivin, implicated in the inhibition of apoptosis, also promotes transition through the G2/M checkpoint of the cell cycle. Expression of this protein is controlled by a variety of mechanisms, including transcriptional mechanisms via β-catenin/Tcf-Lef, known to be highly relevant in many cancer types [7]. In melanomas, the loss or inactivation of cell death control contributes to resistance to chemotherapeutic drugs. This desensitization process involves a combination of strategies, including activation or upregulation of anti-apoptotic survival proteins (e.g. survivin) and inactivation of pro-apoptotic effectors. In many tumor cells, aberrant expression of cell cycle regulatory proteins (cyclins, CDKs, p21) also contributes to uncontrolled cell cycle progression [8].

In this study, we sought to obtain *in vivo* evidence for the efficacy of DhL in the treatment specifically of melanomas. To that end, we evaluated in a pre-clinical murine melanoma model whether DhL reduced the proliferation and growth of B16F0-derived melanomas in syngenic C57/BL6 mice. Our results confirmed that DhL was also anti-proliferative *in vitro* in B16F0 mouse melanoma cells. This phenomenon was accompanied by upregulation of p21, reduced cyclin D1, cyclin B1 and survivin levels, as well as reduced β-catenin/Tcf-Lef dependent transcription. Also, DhL induced either premature senescence or apoptosis in these cells depending on the concentration used.

Collectively, our findings indicate that DhL treatments lead to a significant reduction in the volume of tumors formed by B16F0 melanoma cells and do so by promoting senescence and apoptosis. Moreover, we determined using mathematical modeling that treatments with DhL for extended periods of time following preventive and therapeutic protocols were more effective than simultaneous treatments in reducing tumor volume.

Material and methods

Reagents

Dehydroleucodine (DhL) (structure: Fig. 1) at 93% purity, obtained as described in our previous study [5], was used from a stock solution of 0.4 M in DMSO. Peroxidase-labeled streptavidin was from Dako Denmark S/A (Glostrup, Denmark). Antibodies against cyclin B1, p21, p53 and survivin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against beta-actin were from Sigma-Aldrich (St. Louis, MO, USA).

Animals

The animals used were 8–12-week-old C57BL/6 mice, born and housed in our animal facility, with a 12L:12D cycle and food and water *ad libitum* until they were sacrificed following CO_2 intoxication. Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In each experiment, 8 male and 8 female mice from different cages were combined respectively. All procedures were approved by the Animal Research Committee of the Universidad Nacional de Cuyo (CICUAL). Authorization # 0028741/2010.

Cell lines and treatments

Murine melanoma B16F0 cells provided by Drs I. Hart, G. Moore and P. Parsons, were grown in RPMI1640 medium with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator with 5% CO2 at 37 °C. The cells were harvested after reaching 70–80% confluence and were plated either for subsequent passages or treatments with DhL. 2×10^5 cells were seeded in 6-well plates in RPMI1640 medium with 10% FBS for 12 h. Then the existing medium was replaced by fresh medium (RPMI1640, 10% FBS) containing DMSO or DhL (defined as time 0) and cultured for the specified periods of time. DMSO was used as a vehicle control in all experiments.

Cell proliferation assays

2×10^5 cells were treated with 0–25 μM of DhL for 72 h. To determine cell numbers, cells were trypsinized, suspended in regular medium, and counted with a Neubauer chamber.

Tumor growth assays

3×10^5 B16F0 cells in 200 μl PBS were injected subcutaneously into the right flank of C57BL/6 mice. After the indicated periods, 20 mg DhL in 20 μl DMSO/Kg/day or an equivalent volume of DMSO were injected daily into the right flank following three different protocols (preventive, simultaneous and therapeutic). In the preventive protocol, DhL or DMSO was injected 15 days prior to injection of B16 F0 cells. Alternatively, in the simultaneous protocol, DhL or DMSO was injected the same day as B16F0 cells, while in the therapeutic protocol DhL or DMSO was injected 8 days after the B16F0 cells. In each experiment, groups of 8 animals were used per treatment. The appearance of tumors was monitored by palpation. The tumor length and width were measured with a caliper and tumor volume was calculated using the following equation: $\text{volume} = \text{width}^2 \times \text{length} \times \pi/6$ [9]. According to established bioethical regulations, tumors were measured until they reached a maximum of 2500 mm^3, at which point the animals were euthanized.

Towards the end of treatments, animals were sacrificed, and tumors were excised and prepared for histological analysis. For paraffin sections, tumors were fixed in 0.1 M phosphate buffer (pH 7.3)/formaldehyde 10% v/v for 48 h, dehydrated in alcohol, clarified in xylene, embedded in paraffin, and microtome sectioned at 5 μm. Paraffin-embedded histological slices were stained with hematoxylin-eosin (HE) for histological analysis.

Apoptosis assays

In vitro experiments: To determine early stage apoptosis, cells were stained with Annexin V using the Annexin V-FITC fluorescence detection kit (BD Biosciences San Jose, CA, USA) according to the manufacturer's instructions as described [5]. Briefly,

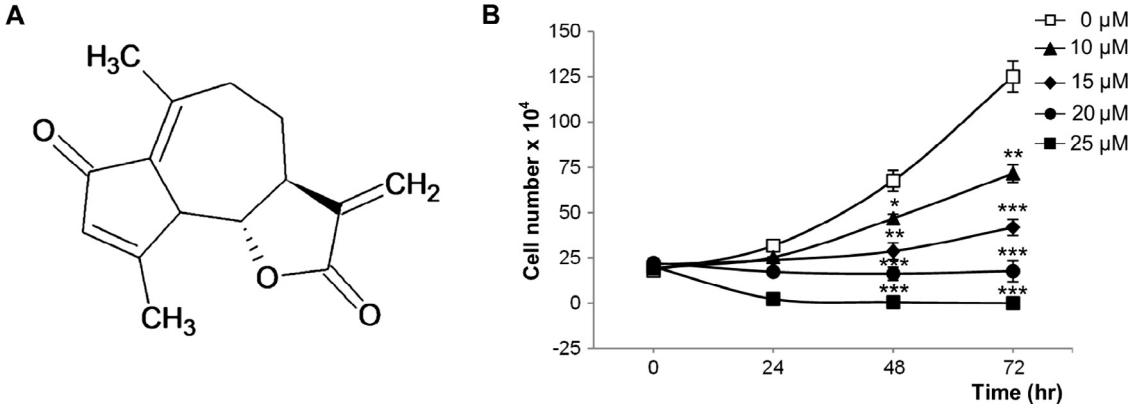

Fig. 1. DhL treatment inhibits cell proliferation in a dose-dependent manner.
The chemical structure of dehydroleucodine (left). B16F0 cells were treated with 0, 10, 20, or 25 μM DhL for 72 h and counted every 24 h (right). Data are expressed as the mean ± SEM of three independent experiments. * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$ vs. control group (0 μM DhL).