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Promising Candidate As An Anticancer Agent For Childhood, A Sesquiterpene Lactone: Dehydroleucodine

A Sesquiterpene Lactone: Dehydroleucodine

Aylin Erol [1], Özde Gökbayrak[1], Prof.Dr.Safiye Aktaş[1], Prof.Dr.Nur Olgun[2]

1. *Dokuz Eylül University, Institute of Oncology, Department of Basic Oncology*
2. *Dokuz Eylül University, Institute of Oncology, Department of Clinical Oncology, Pediatric Oncology*

Corresponding Author: Aylin Erol, İnciraltı Mahallesi Mithatpaşa Cad, Dokuz Eylül Ünv. Hst. No:56/13, 35330 Balçova/İzmir, +90 554 593 56 83, aylin.erol.deu@gmail.com

Objective: *Despite emerging new treatments, there is a need for new anti-cancer agents for childhood solid tumors. Sesquiterpene lactones(SLs) are plant-derived secondary metabolites with anti-cancer properties. Dehydroleucodine(DhL) is a guaianolide-type SL isolated from Artemisia douglasiana (Asteraceae). In this study, the cytotoxic, antiproliferative, apoptotic effects of DhL on childhood solid tumors including Ewing sarcoma(ES), Hepatoblastoma(HBL), and Neuroblastoma(NB) were investigated in in-vitro conditions.*

Material and Methods: *In this study, NB cell line KELLY, ES cell line SK-ES-1, and HBL cell line Hep-G2 were used. Cell lines were cultured and LD50 doses of DhL and Cisplatin(CDDP) were calculated by MTT. Effective doses were applied to all cell lines, and percentages of apoptosis and necrosis were determined by Annexin V-PI test in flow cytometry. Morphological changes in cells were examined by toluidine blue staining. Immunocytochemical(ICC) examinations were performed with Caspase3,8, and 9 to determine the mechanism of apoptosis as Ki67 proliferation index. Statistical analysis was performed using the Mann-Whitney U test at a statistical significance level of $p<0,05$.*

Results: *LD50 doses of DhL and CDDP were determined as 75µM, 100µM for Kelly; 50µM, 100µM for HEP-G2; and 100µM, 300µM for SK-ES-1.*
The intrinsic pathway in all cell lines was more pronounced in DhL, which reduced proliferation leading to apoptosis. DhL reduced proliferation less than CDDP and resulted in similar apoptotic cell death.

Conclusions: *DhL showed a statistically significant anti-cancer activity similar to CDDP. It exerted this effect through apoptosis. Planning in-vivo experiments about this agent and explaining its molecular mechanisms, investigating the effects of other SL on childhood cancers are among our future goals.*

INTRODUCTION

Phytotherapeutic products and their semi-synthetic derivatives are widely and effectively used as anti-cancer agents. In the discovery of anti-cancer drugs associated with natural products; plant-based agents, including vinca bisindole alkaloids, epipodophyllotoxin analogs, taxanes, and camptothecins are among the most widely used cancer chemotherapeutics in clinical practice. Modern phytotherapeutic preparations are often prepared from plant extracts and unlike synthetics, a product contains a combination of a wide variety of plant extracts. Herbal therapy has the potential to interact with other treatments. The use of these medicinal plants for therapeutic purposes in childhood is a much more sensitive and careful issue.

Although very important developments have been achieved within the last 40 years in the treatment and survival of childhood solid tumors, new treatment strategies are still needed. Ewing sarcoma is a primitive neuroectodermal tumor most commonly found in long bones and pelvis. Neuroblastoma is the most common extracranial solid tumor of childhood. As a rare tumor, Hepatoblastoma is seen in the liver in childhood.

Sesquiterpene lactones (SLs) are active ingredients of various medicinal plants used in traditional medicine[1].SLs represents a large number of natural products containing 15 carbons, one or more lactone rings, with the joint combination of three isoprenyl groups arranged in several characteristic rings[2]. Although SLs are found in more than 100 flowering plant families, they are characteristically secondary metabolites found in plants belonging to the *Astraceae* family. Today, it is known that more than 5000 different SLs have been defined[3]. Plants containing SL are compounds frequently used in traditional medicine against inflammation and cancer[4]. Various SL derivatives used in adult cancers including breast, colorectal, non-small cell lung cancer, uveal melanoma, laryngeal squamous cell carcinoma, and acute leukemias have reached the clinical trial stage [5,6,7,8,9]. SLs named dehydrocostus lactone and costunolide isolated from *Saussurea lapa* plant were found to be cytotoxic in neuroblastoma cell lines (IMR-32, NB-39, SK-N-SH, and LA-N-1) [10]. However, DhL has not been

studied in childhood cancers

Dehydroleucodine (DhL), a guaianolide-type sesquiterpene lactone was obtained from the extract of the *Artemisia douglasiana* plant. Anti-inflammatory, anti-tumorigenic, antiproliferative effects of *Artemisia douglasiana* have been found[11]. DhL was found to be cytotoxic to human leukemia cells (B16), astrocytoma cells (D384) in *in-vitro* conditions in studies performed on DhL[12], [13], [14].

In this study, it was aimed to investigate under *in-vitro* conditions the cytotoxic, antiproliferative, and apoptotic effects of DhL, an SL obtained from *Artemisia douglasiana* plant on NB, ES, and HBL, which are childhood cancers.

MATERIALS AND METHODS

The study was approved by the Non-Invasive Research Ethics Committee of Dokuz Eylül University on 05.17.2018 with the protocol number 2018 / 12-30.

Reagents: Dehydroleucodine, ≥98% (HPLC) (Sigma Aldrich, D4196-5MG), and Cisplatin (Koçak) 50 mg / 100 ml I.V. were purchased as a vial containing a concentrated solution for infusion.

Cell culture: Human Neuroblastoma cell line KELLY (DSMZ, ACC355), Ewing Sarcoma cell line SK-ES-1 (DSMZ, ACC518), and Hepatobalstoma cell line Hep-G2 (DSMZ, ACC180) were used for experiments. In KELLY and SK-ES-1 cell lines, 10% fetal bovine serum(FBS) was added to RPMI 1640 culture medium for cell culture. [RPMI 1640 + 2mM Glutamine + 1% penicillin / streptomycin + 10% FBS].

DMEM culture medium with added 10% FBS for HEP-G2 cell line [DMEM + 2mM Glutamine + 1% penicillin / streptomycin + 10% FBS] was used. All cells were passaged 2 times a week with Trypsin/EDTA at a concentration of 0.25% by chemically removing them from the culture dish. All stages of cell culture processes were carried out in laminar flow type-two cabinets and the cells were cultured in an incubator at 37 ° C containing 5% CO2. Cells were removed with Trypsin/EDTA and 1000 cells were seeded in each well in 100 µL complete medium, and 6 wells were planned for each run, while each experiment was repeated three times.

When the cells in 96 wells became confluent after 24 hours for NB and SK-ES-1 cell lines, and after 48 hours for HEP-G2, applications of agents were initiated. Doses of 10,25,50,75,100 µM for DhL and 50,100,150,200,300 µM for CDDP were used.

Cell Viability Assay: The effect of DhL and CDDP on cell proliferation was analyzed by MTT[15] (AppliChem, Lot 5H008900). At the end of the experiment period of cells treated with agents in 96 well-plates, 10 µL MTT (AppliChem, Lot 5H008900) was placed in each well. Cells were removed and left in the incubator at 37 ° C for 4 hours in the dark.

At the end of the incubation period, DMSO was washed out. A 50 µL of content was removed from each well and 50µL of DMSO was added in its place. The well-plate was left on the orbital shaker for 15 minutes. After DMSO was washed out, the well-plate was read at 562 nm in an ELISA reader and the absorbance values were obtained. The viability of the samples determined based on their absorbance values obtained was compared to the control.

Flow Cytometric Determination of Apoptosis using Annexin-V: Staining with Annexin V-FITC / Propidium Iodide (PI) [16] (BD apoptosis detection kit: 556547) was performed for the analysis of apoptotic cell death rates related to the use of DhL and CDDP. 6 well-plates were inoculated with 1 x 10^5 cells per well. After 24 hours of applications with agents at LD50 doses determined by MTT, cells were removed from the plates with a cell scraper. Then cells were centrifuged at 1300rpm for 7 minutes. The supernatant was removed. Pellets were washed twice with PBS, then by the buffer solution contained in the kit, according to the protocol of the kit. After pipetting 100 µL of the solution, 5 µL AnnexinV FITC, and 5 µL PI on the pellets, they were incubated in the dark for 15 minutes. AnnexinV-PI positivity were checked on a flow cytometry device (BD Accuri C6) and rates of cellular viability, early and late apoptosis was determined.

Immunocytochemical (ICC) Staining: The cells were treated for 24 hours with determined doses of DhL and CDDP in 25 cm2 flasks obtained from the prepared suspension. Then 10 µL aliquots of suspension were spread on slides treated with lysine. To evaluate the antibodies, the necessary protocol was developed in the Ventana Discovery device, which performs automatic ICC staining[17]. Slides stained with toluidine blue were examined to check cellularity. Meanwhile, the antibodies Caspase3 (Elabscience-E-AB-30756), Caspase9 (Elabscience-E-AB-30760), Caspase 8 (Elabscience-E-AB-30759), and Ki67 (abcam, ab15580) were prepared in appropriate dilutions and 100µL aliquots were placed on each slide. Coloring was performed with OmniMap anti-rabbit

HRP and DAB as secondary antibodies (Roche-Ventana 760-4311). After staining with hematoxylin, an evaluation was made by light microscopy. All cells on the slides were counted and the percentage of positively stained cells were recorded.

Observation of Changes in Cell Morphology: Toluidine blue is a vital and basic dye. It has been used in the morphological evaluation of cells after the applications of different agents. Cells were spread on slides and fixed with methanol. After the slides were stained with an automated stainer Aerospray (Wescor, 09149), the morphological evaluation was performed using a microscope (Olympus B50).

Statistical Analysis: The data were evaluated with the SPSS 22.0 program using the nonparametric Mann-Whitney-U test, $P < 0.05$ was considered statistically significant.

RESULTS:

Results of the Cell Viability Test: A single dose was not determined for all three cell lines. LD50 of CDDP was determined at a dose of 100 µM for N-MYC positive Kelly cell line, 100 µM for HEP-G2 cell line, 300 µM for ES cell line SK-ES-1. It was decided that the most effective LD50 dose was achieved at 24 hours rather than at 24, 48.and 72 hours. LD50 values for DhL were 75 µM for the Kelly cell line, 50 µM for the HEP-G2 cell line, and 10 µM for the SK-ES-1 cell line.

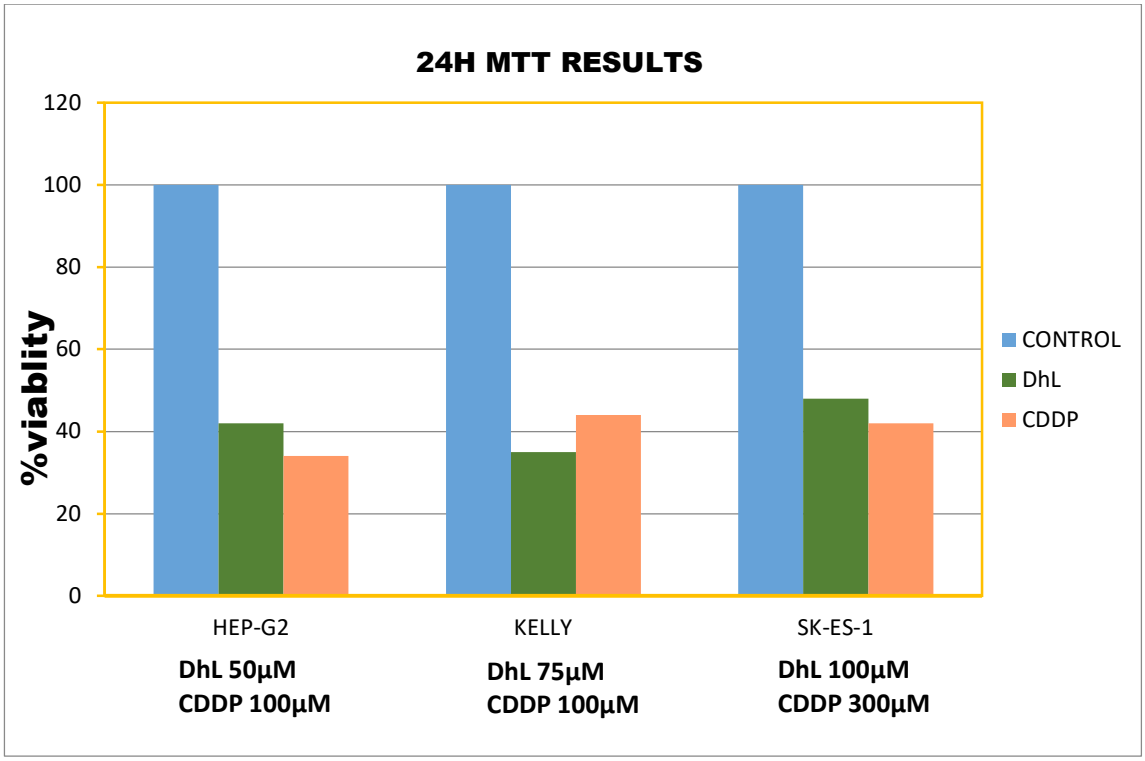

Fig.1: MTT results

Results of Flow Cytometric Determination of Apoptosis using AnnexinV-PI: When compared with the control group both early (31.4%) and late apoptotic (59.2%) effects were detected for CDDP regarding KELLY cell line. When DhL and CDDP were compared, it was found that DhL has both early apoptotic (48.4%) and late apoptotic (50.9%) effects. For the HEP-G2 cell line; control viability (70.4%) was found to be significantly reduced with the applied agents. It was determined that the effect of CDDP on the decrease in vitality occurred through apoptosis (93.4%) which was also true for DhL. For the SK-ES-1 cell line, it was determined that the effective dose of CDDP (300mm)exerted this effect through apoptosis. (75.2%). It was observed that DhL(86.5%) also significantly caused cell death through apoptosis.

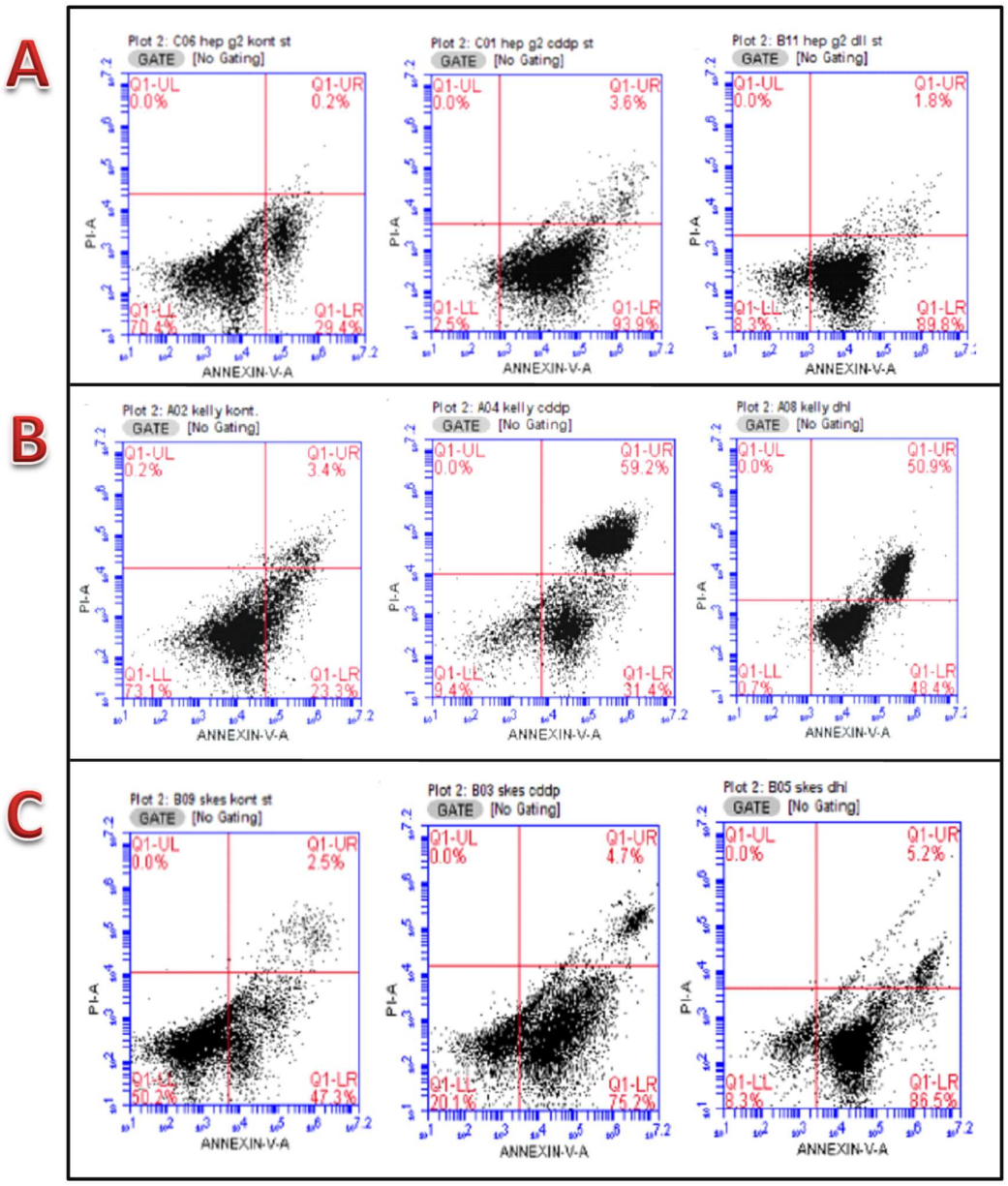

Fig.2: All three cell lines AnnexinV-PI results**, A;** HEP-G2 control, CDDP, DhL AnnexinV-PI**, B;** KELLY control,CDDP,DhL AnnexinV-PI**, C;** SK-ES-1 control, CDDP, DhL AnnexinV-PI

Results of Immunocytochemical (ICC) Staining:

With ICC staining, KI67 proliferation index marker, Caspase 8 extrinsic pathway apoptosis marker, Caspase 9 intrinsic pathway apoptosis marker, Caspase 3 typical common pathway apoptosis marker were evaluated. If we look at the Ki67 proliferation index for Kelly, it has been thought that our candidate agent reduced proliferation less than CDDP and has the main effect by dragging cells into apoptosis. The fact that proliferation indices are lower in comparison with CDDP suggests that it will be more effective in combined treatment models with chemotherapy. When we question the pathway through which the apoptotic effect occurs, CDDP uses both intrinsic and extrinsic pathways following the literature. It was determined that DhL uses the extrinsic apoptotic pathway.

When we look at the Ki67 proliferation index for HEP-G2, a similar proliferative decrease was observed compared to the control agents. When the evaluation was made for Caspase 3, 8, and 9, it was observed that DhL used the intrinsic apoptotic pathway.

When we looked at the Ki67 proliferation index for the SK-ES-1 cell line, it was observed that CDDP reduced cell proliferation, exerted its apoptotic effect more frequently by using the intrinsic pathway, and DhL decreased proliferation compared to control, but it did not decrease Ki67 proliferation index as much as CDDP, and it induced cell death through the intrinsic apoptotic pathway.

ICC		*Ki67*	*Caspase3*	*Caspase8*	*Caspase9*
Hep-G2	**DhL**	20%	40%	30%	70%
	CDDP	10%	45%	20%	80%
SK-ES-1	**DhL**	30%	20%	30%	90%
	CDDP	20%	50%	60%	90%
KELLY	**DhL**	20%	60%	60%	25%
	CDDP	3%	90%	90%	80%

Table 1. Expression levels of Ki67, Caspase 8, Caspase 9, Caspase 3 determined after applications of control CDDP and DhL.

Results of Morphological Changes: Necrotic cellular changes were observed morphologically in the CDDP and DhL groups in all three cell lines compared to the control group.

Results of the Statistical Analysis: When the DhL and CDDP groups were compared non-parametrically with the control group, statistically significant levels of cell death, an increasing amount of apoptosis, and a decrease in the proliferation index were found ($p < 0.05$). DhL did not show a statistically significant difference when compared to CDDP at the effective doses determined.

DISCUSSION:

In this study, the effect of SL derivative DhL, which has reached the stage of clinical trials in adult cancers from in-vitro studies and is an anticancer agent candidate, on three different childhood periods was questioned under *in-vitro* conditions. Cell death and apoptosis pathways of DhL have been studied, and anticancer properties of DhL has been shown in NB, HBL, ES. DhL decreased cell viability and proliferation index and led to apoptosis and necroptosis predominantly through intrinsic pathways.

Cancer in childhood is rare compared to cancers in adults, but it causes more deaths among children from infancy to 15 years of age than any other factor except injuries. Annual childhood cancer incidence has increased slightly over the past 30 years; however, mortality rates have decreased significantly for many cancers, largely due to therapeutic improvements in therapy. SLs are secondary metabolites of plants frequently used in studies due to their strong bioactivity, including cytotoxicity and antineoplastic activity on cancer cells in *in-vitro* and in *in-vivo* studies.

Members of the genus Inula are popularly known as a medicinal plant and have been used since ancient times. Members of this genus have a long list of medicinal uses, including antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, antitumor, antidiabetic, antiasthmatic, anti-allergic, and cytotoxic properties CDDP is a well known chemotherapeutic drug. It is being used for the treatment of many human cancers, including bladder, head and neck, lung, ovarian and testicular cancers. It is effective against various types of cancer,

including carcinomas, germ cell tumors, lymphomas, and sarcomas. CDDP cross-links with purine bases in DNA, interfering with DNA repair mechanisms, causing DNA damage, and then inducing apoptosis in cancer cells.

However, its numerous undesirable side effects such as drug resistance, severe kidney problems, allergic reactions, immunity to infections, gastrointestinal disorders, bleeding, and hearing loss are known[18]. In this study, the effect of DhL was compared with CDDP because CDDP is the most widely used agent with a proven established therapeutic effect of childhood cancers.

In previous studies, DhL was found to be cytotoxic to human leukemia cells in *in-vitro* conditions[12]. The cytotoxic effect of dehydroleucodine (DhL), an SL, on human leukemia cells was investigated. Two SLs were isolated from the extract showing activity against these leukemia cells: the first was DhL which showed pronounced cytotoxic activity. The second SL, leucodine, was found to have no discernible activity. In our study, it was found to be effective in childhood cancers *in-vitro*.

SLs called dehydrocostus lactone and costunolide derived from *the Saussurea lapa* plant were found to have cytotoxic activity in neuroblastoma cell lines (IMR-32, NB-39, SK-N-SH, and LA-N-1) [10]. Both compounds showed cytotoxic activity against neuroblastoma cell lines. Evidence of cellular apoptosis such as nuclear condensation and membrane inversion has been observed as a result of treatment with SL. Western blotting tests performed have demonstrated that both compounds stimulate Caspase-7 activation and PARP cleavage. Also, it has been observed that SLs suppressed the invasion and migration abilities of the cells. These results show that Dehydrocostus Lactone and Costunolide are promising candidates to be developed as new anti-cancer agents effective against NB.
In 2013 Martin Chadwick et al. investigated the benefits of different SLs on plants and humans[3]. In their study, it was stated that JNK and p38 activation pathways were not blocked by an SL, parthenolide, but the MAPK / MEKK1 signaling pathway was blocked by an SL. This is an important discovery as approximately 30% of mammalian tumors contain Ras proto-oncogene mutations that act via the MAPK / MEKK1 pathway.

In a study by Katarzyna Gach et al. conducted in 2012, the relation of anti-cancer activity of SLs with oxidative stress was investigated[19]. Reactive oxygen species (ROS) that initiate apoptosis in a mitochondria-dependent pathway are thought to be useful to kill cancer cells if they can be produced in cancer. One of the most important regulators of redox balance in cells is reduced glutathione (GSH). GSH levels in cancer cells are higher than normal cells. Therefore, SL can induce apoptosis of cancer cells by decreasing intracellular GSH levels. It has been explained that the use of SL, which can affect intracellular redox signaling pathways, is acceptable as an interesting approach for cancer treatment.

DhL and dehydroparishin-B, a new guaiane-type sesquiterpene acid, were obtained from chloroform extract prepared from *Artemisia douglasiana*. Studies have shown that both dehydroparishin-B and DhL block the cellular proliferation of B16 melanoma cells and inhibit cell migration. These results have demonstrated that DhL and dehydroparishin-B may become potential candidates for the treatment of metastatic melanomas. It has been found that the DHL-containing extract applied to human cerebral astrocytoma cell D384 causes cell death by triggering cell cycle arrest, apoptosis and DNA damage. It has also been found that cell death results in increased expression of CDKN1A and BAX proteins.

In D384 cells exposed to DhL, a significant induction was observed in total TP73 and phosphorylated TP53, TP73 and γ-H2AX protein levels, without any increase in total TP53 levels. Overall, these studies reveal that DhL has a significant effect on human astrocytoma cells, reduction in survival through the expression of TP73 and phosphorylation of TP53. In 1999, in a study by Peter Rüngeler et al., it was determined that SLs inhibit NF-κB activation, and SLs with different structures showed different inhibitory activities[20].

The strength of our study is that DhL is being studied for the first time in childhood cancers. Our weakness is that its mechanism of action has not been studied at the molecular level. In our future studies, *in-vivo* experiments with this agent should be planned and its molecular mechanisms should be fully explained.

In conclusion; DhL is thought to be a candidate anti-cancer agent since it shows similar anti-cancer activity to CDDP. Our future study targets are to investigate the effects of this agent and other SLs in *in-vivo* conditions by creating NB, HBL, ES cancer models in nude mice.

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