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A new cannabigerol derivative, LE-127/2, induces autophagy mediated cell death in human cutaneous melanoma cells

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ABSTRACT

Despite the targeted- and immunotherapies used in the past decade, survival rate among patients with metastatic melanoma remains low, therefore, melanoma is responsible for the majority of skin cancer-related deaths. The ongoing investigation of natural antitumor agents, the nonpsychoactive cannabinoid, cannabigerol (CBG) found in *Cannabis sativa* is emerging as a promising candidate. CBG offers a potential therapeutic role in the treatment of melanoma demonstrating cell growth inhibition in some tumors. Its low water solubility and bioavailability hinder the potential effectiveness. To address these challenges, a modified CBG, namely LE-127/2 was synthesized by Mannich-type reaction. The aim was to investigate the effect of this novel compound on cell proliferation as well as the mechanism of cell death with a particular focus on autophagy and apoptosis. Human cutan melanoma cell lines, WM35, A2058 and WM3000 were utilized for the present study. Cell proliferation of the cells after the treatment with LE-127/2, parent CBG or vemurafenib was assessed by Cell Titer Blue Assay. Cells were treated with a 1.25–80 μM of the above-mentioned compounds, and it was found that at 20 μM of all drugs showed a comparable effective inhibition of cell proliferation, however, vemurafenib and CBG proved to be more effective than LE-127/2. In addition, clonogenic cell survival assays were performed to examine the inhibitory effect of LE-127/2 on the colony formation ability of melanoma cell lines. Cells treated with 20 μM of LE-127/2 for 14 days showed about a 50% suppression of clonogenic cell survival. LE-127/2 exerted the most intensive inhibition on A2058 cell colonies. Furthermore, notably, LDH cytotoxicity assay performed on HaCaT cell line, proved LE-127/2 to be cytotoxic only at higher concentration, such as 80 μM, while the parent CBG was cytotoxic at concentration as low as 5 μM, suggesting that the new CBG derivative as a drug candidate may be applied in human pharmacotherapy without causing a substantial damage in intact epidermal cells. Analysis of protein expression revealed the impact of LE-127/2 on the expression of basic proteins (LC-3, Beclin-1 and p62) involved in the process of autophagy in the three different melanoma cell lines studied. Elevated expression of these proteins was detected as a result of LE-127/2 (20 μM) treatment. LE-127/2 also induced the expression of some proteins involved in apoptosis, and it is particularly noteworthy the increased level of cleaved PARP. Based on the results obtained, it can be concluded that LE-127/2 induced autophagy could lead to the inhibition of cell proliferation and death in melanoma cells.

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1. Introduction

Melanoma is responsible for the majority of skin cancer-related deaths having a high propensity to metastasize (Patel et al., 1978; Silk et al., 2013; Tran et al., 2021). Prognosis and survival for malignant melanoma are highly depending on the early diagnosis and treatment (Davis et al., 2019).

Systemic and local therapies, including targeted therapies, have been expanded to previously used traditional therapeutic options (Mishra et al., 2018; Motofei, 2019). Despite many advances in the treatment of malignant melanoma, especially new targeted therapies, survival rate among patients with metastatic melanoma remains at low level (Motofei, 2019). Targeted therapy with BRAF inhibitors has proven to be effective in advanced BRAF-mutant melanoma, however, treatments with such BRAF inhibitors cause therapeutic resistance in half of the patients after about six months (Patel et al., 1978; Xie et al., 2006; Shepherd et al., 2010; Ascierto, et al. 2012). Among BRAF inhibitors, in a phase III study, vemurafenib showed a beneficial response in BRAF-mutated melanoma patients and the application of vemurafenib resulted in a better overall survival rate compared to dacarbazine (84% vs 64%), therefore, it was approved in 2012 for the clinical use in melanoma patients. However, the limitation of vemurafenib was the developed resistance in patients (Kugel and Aplin, 2014; Rajakulendran and Adam, 2014).

Another example of modern therapies concerns the administration of immune checkpoint inhibitors (ICIs) (eg., anti-CTLA-4 antibodies, anti-PD-1/PD-L1 antibodies), which have been successfully used in metastatic melanoma, however, the main untoward effects of ICIs are characterized by a series of immune-related adverse events, which in some cases can be potentially life-threatening (Orloff et al., 2016; Placzke et al., 2023).

To achieve more effective anti-tumor therapy, increased efforts have been directed to the research of herbal extracts and natural anti-tumor agents. A growing body of evidence suggests that many herbal extracts and bioactive phytochemicals show potential antitumor functions, thus, offering an alternative option for the treatment of malignant melanoma (Pal et al., 2016). For instance, luteolin, a natural flavonoid, exerts an anti-metastatic activity providing an alternative therapy for malignant melanoma. Another natural compound, polyphyllin I, extracted from *Paris polyphylla* plant, proved to induce apoptosis in melanoma cells, leading to cellular deaths (Li et al., 2019; Long and Pi, 2020). Recently, cannabigerol (CBG), one of the non-psychotropic cannabinoids presents in *Cannabis sativa (C.sativa)*, has been shown to exhibit anti-tumor activity in some kind of tumors including melanomas (Ligresti et al., 2006). In order to improve the biological properties and poor water solubility and bioavailability of cannabigerol, some semi-synthetic, nitrogen containing cannabigerol derivatives suitable for salt formation were synthesized previously by Mannich-type reaction, and these derivatives were tested in different tumorous cell lines (Lőrincz et al., 2023). One of the derivatives, namely, LE-127/2 (bis-*N*-butyl-dihydro-1,3-oxazine derivative of cannabigerol), showed a significant growth inhibition property in some tumorous cell lines (Lőrincz et al., 2023). Therefore, we decided to further investigate the effect and action mechanism of this compound on melanoma cells.

Autophagy is one of the main signal transduction processes in cells for the degradation of cellular debris and a hot topic in cancer research. Observations of autophagy deregulation in melanoma have brought to the forefront of melanoma research. Key regulatory proteins, including Atg8/microtubule-associated light chain 3 (LC-3I/II), Beclin- 1 and Sequestosome1/SQSTM1 (herein referred as p62) have been proposed as potential prognostic biomarkers (Uchiyama et al., 2008; Tang et al., 2016). Additionally, autophagy is interconnected closely to apoptosis by several molecular pathways, therefore, we aimed to examine the effect of LE-127/2 on melanoma cell proliferation, autophagy, and apoptosis, exploring the possible underlying mechanism examining main signaling pathways related to chemoresistance.

2. Materials and methods

2.1. Chemicals

Cannabigerol, CBG (CBDepot s.r.o., Teplice, Czech Republic) was used for comparison purposes as a parent compound. LE-127/2, the bis-*N*-butyl-dihydro-1,3-oxazine derivative of CBG (Fig. 1) was synthetized by the reaction of CBG with *n*-butylamine and formaldehyde in Mannich-type reaction by Lőricnz et al. (2023).

Vemurafenib (PLX4032, sold under the brand name, Zelboraf, inhibitor of the B-Raf enzyme, purity > 98%) was purchased from Merck (Merck, Darmstadt, Germany). The stock solutions of the compounds were prepared in DMSO (Sigma-Aldrich, St. Louise, MS, USA) and stored at − 20 °C. For each cell culture experiment, stock solutions were diluted with cell culturing media to obtain a final concentration of 1.25 μM to 80 μM, respectively, when added to cells.

2.2. Cell cultures

WM35, A2058 (CRL-3601) and WM3000 (WM3000–01–0001) human cutan melanoma cells were used for all experiments. To determine the cell cytotoxicity of LE-127/2, LDH cytotoxicity assay was used on non-malignant human epidermal keratinocyte (HaCaT) cell line.

To explore the cytotoxic effect of LE-127/2 on other non-malignant cells, rat-derived H9c2 cardiomyocyte and mouse-derived NIH-3T3 fibroblast cell lines treated with LE-127/2 were also tested using LDH cytotoxicity assay.

All cell lines were originally purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were cultured in DMEM (Dulbecco's Modified Eagle Medium, Biosera, Cholet, France) supplemented with 10% Fetal Bovine Serum (FBS), (Biosera, Cholet, France), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and maintained at 37 °C in a humidified atmosphere under 5% CO_2/95% air. The A2058 and WM35 cell lines carry a BRAF mutation, while WM3000 is a metastatic cell line that contains a Q61R mutation at position 61 of the N-RAS gene. All three cell lines are highly invasive, in terms of their growth, while WM35 growth is the slowest.

2.3. LDH cytotoxicity assay on non-malignant cells

To detect the cytotoxicity of LE-127/2 and CBG (parent compound of LE-127/2) non – malignant cell lines were used (human HaCaT cells, rat H9c2 and mouse NIH-3T3 fibroblast cell lines). Cells were plated at 6×10^3 density/well in a 96-well plate in the culturing medium supplemented with 10% FBS and incubated with increasing concentrations (1.25 μM to 80 μM) of LE-127/2 or CBG for 24 to 72 h.

The CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen, Carlsbad, CA, US) is based on a colorimetric method to quantify cellular cytotoxicity. Cell death is assessed by detecting lactate dehydrogenase (LDH) released into the cell culture medium. To perform the assay, aliquots of the culturing media from the drug-free control and drug treated groups, were collected at 24, 48 and 72 h and transferred to a new plate and the reaction mixture supplied in the kit was added. After a 30 min incubation, the reaction was terminated, and the absorbance was measured at 490 nm using a microplate reader.

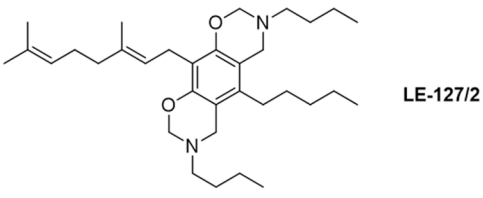

Fig. 1. Chemical structure of LE-127/2.

2.4. Detection of cell proliferation activity of WM35, A2058 and WM3000 cells treated with LE-127/2, CBG or vemurafenib

Cell proliferation activities of cells were assessed by Cell Titer-Blue® Cell Viability Assay (Promega, Madison, WI, USA), which is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent product (resorufin). Nonviable cells that lose their metabolic capacity do not generate a fluorescent signal (Stamatiou et al., 2009).

WM35, A2058 and WM3000 cells were seeded in 96-well plates at 6 × 10^3 cells /well density in their culturing media (DMEM) over 24 h before treatments. After 24 h cells were treated with increasing doses (2.5 μM to 80 μM) of LE-127/2, CBG or vemurafenib, respectively. DMSO was used as a solvent for all compounds, therefore the control groups were treated with medium containing DMSO instead of compounds. The treatment continued over 24 to 72 h at 37 °C in the cell culture incubator with 5% CO_2/ 95% air and the cell proliferation activity of the cells was measured after every 24-hour. At the termination of the reaction, the media was replaced with fresh culturing media without drugs, and then Cell Titer Blue reagent was added to the cells and all plates were incubated for 2 h at 37 °C in the cell culture incubator. Fluorescence intensity was measured at 560ex/590em nm by using Fluostar OPTIMA BMG Labtech (BMG Labtech, Offenburg, Germany).

2.5. Clonogenic cell survival assay (colony formation assay)

The clonogenic cell survival assay determines the ability of cells to proliferate indefinitely and measures its ability to form a large colony or a clone (Munshi et al., 2005). After the preparation of a single cell culture suspension, cells were seeded into 6-well plates (6 × 10^3 cells/well). Following the adhesion, cells were treated with gradually increasing concentrations of LE-127/2 (5–80 μM). The control group of cells was cultured in the complete cell culture media with 0.01% DMSO. The media on the cells containing LE-127/2 was replaced with fresh media including LE-127/2 every third day. The treatment was terminated after 14 days, and then, cells were washed with ice-cold phosphate-buffered saline (PBS). Afterwards, cells were fixed in the mixture of methanol and acetic acid (3:1), and then plates were stained with crystal violet (0.1% Crystal Violet solution, Sigma-Aldrich, St. Louise, MS, USA) for 10–15 min at 25 °C, washed with distilled water, and dried at room temperature. The pictures were taken of each plate, and cells were resuspended in 2% SDS solution for the quantification of the colonies. The absorbance was measured at 570 nm using Biotek plate reader system (BioTek, Winooski, VT, USA).

2.6. Protein expression analysis

2.6.1. Protein isolation and BCA assay for protein determination

For protein extraction the cells were collected with cell scrapers in ice cold cell lysis buffer (M-PER tissue lysis kit (Thermo Fisher Scientific, Waltham, MA, USA)) supplemented with a protease and phosphatase inhibitor cocktail. The lysis was performed on ice for 30 min, afterwards the samples were centrifuged at 20,000 rpm at 4 °C for additional 20 min. Supernatants were collected for protein concentration measurements with BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), using bovine serum albumin (BSA) as a standard. The aliquots of protein samples were applied for Western blots and autophagic array analysis.

2.6.2. Detection of autophagic proteins by human autophagy array

Regulation of any kind of the cellular processes is a complex mechanism, frequently involving multiple proteins. Recently, as a result of the developing technology, it is common to examine the mechanisms affecting multiplex proteins with array technology, the main advantage of which is several proteins can be detected at the same time, and then, based on the results, the desired proteins can be selected for verification by classical Western blotting.

In the present study multiple proteins of autophagy Human Autophagic Array was used (Assaygene (SARB0023), Dublin, Ireland). Based on the results of the cell proliferation assays, all human cutan melanoma cells were treated with 20 μM LE-127/2 for 48 h and isolated proteins+ were used for array analysis carried out according to the manual provided by the manufacturer's instructions. The intensity of each spot was measured using ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

2.6.3. Analysis of protein expression by Western blotting

After determination of protein concentration, the samples were diluted with 4xLaemmli buffer and were boiled at 95 °C for 8 min, and then, total of 40 μg protein was separated on 12% sodium dodecyl sulfate-polyacrylamide gel by SDS-PAGE. As a molecular-weight size marker Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories #1,610,374, Hercules, CA, USA) were used. After SDS-PAGE run proteins were transferred onto PVDF membranes (Immuno-Blot® PVDF Membrane, Bio-Rad Laboratories, Hercules CA, USA), which were then blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature. Membranes were then incubated with specific primary antibodies at 4 °C overnight against the following proteins: LC-3I/II, p62, Beclin-1, Atg12, caspase-3, Tom20, Heme-oxigenase-1(HO-1), p53, PDCD-4 (Programmed cell death protein 4), bax (bcl-2 associated x protein), bcl-2 (B-cell lymphoma 2), (PARP) ((Poly (ADP-ribose) polymerase) (diluted at 1:1000). Anti-HPRT antibody against housekeeping protein was applied to ensure equal loading of the samples. The following day the membranes were washed three times with TBST and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Thermo Fisher Scientific, San Diego, CA, USA). The signal was detected by chemiluminescence using an enhanced chemiluminescence system by Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) and the signal was visualized by using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The level of the protein of interest was calculated and then normalized against HPRT using the Bio-Rad Image Lab 5.2.1 software (Bio-Rad Laboratories, Hercules, CA, USA). All Western blot analyses were carried out in triplicates. Finally, the calculated band intensity of the treated samples was normalized over the untreated control samples.

2.6.4. Detection of LC-3 expression in melanoma cells by immunofluorescence

Immunofluorescence detection of the expression of LC-3 in WM35, A2058 and WM3000 cells was performed after the treatment with 20 μM of LE-127/2 for 48 h. All cells were cultured on coverslips placed into 6 well plates. Cells were fixed with methanol, washed with PBS, and then, permeabilized using 0.1% TRITON-X. Cells were incubated overnight at 4 °C with the primary antibody against LC-3 (Cell Signaling Technology, Danvers, MA, USA) diluted at 1:100 in 1% BSA with 0.1% Triton-X solution. The next day the slides were washed in 1xPBS with 0.05% Triton-X solution and incubated for 1 hour with fluorescence anti-rabbit secondary antibody (Alexa-488-conjugated) (Thermo Fisher Scientific, San Diego, CA, USA) diluted in 2% BSA-0.1% with Triton X solution, 1:1000. The slides were washed again three times, fixed in 1% paraformaldehyde solution, and 0.5 μg/ml DAPI was added to the coverslides, for observation under inverted fluorescence microscope (Carl Zeiss AG, Jena, Germany).

2.6.5. Statistical analysis

Results were calculated from three independent experiments. Data from the cell proliferation and cytotoxicity assays and Western blot results were analyzed using the unpaired Student's *t*-test. The statistical analyses were performed using GraphPad Prism (version 5.01; San Diego, CA). A p value of 0.05 was set as the threshold for statistical significance.

3. Results

3.1. LDH cell cytotoxicity of LE-127/2 compared to CBG parent compound

LDH is the most frequently used marker to investigate the cytotoxic effect of new potential drug candidates. The damage of the plasma membrane results in a release of LDH into the cell culture medium. Thus, the extracellular LDH content can be quantified by a coupled enzymatic reaction, in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH.

LDH release was calculated as the percentage absorbance at 490 nm of each sample compared to the drug-free control. Three independent experiments were carried out with three replicate wells for each concentration of LE-127/2 or CBG. Remarkably, CBG significantly reduced HaCaT cell growth at 10 μM of CBG, showing that it was toxic for intact non-tumor cells. Drastic reduction of cell growth particularly was observed at 72 h after CBG treatment (Fig. 2A). It is very noteworthy that, in contrary to CBG, no significant cell damage was measured in the growth of HaCaT cells, if the cells were treated with LE-127/2 up to 80 μM (Fig. 2B). Morphological observation using an inverted microscope (Nikon Eclipse TS 100) (Minoto City, Tokio, Japan) did not show an extremely pronounced effect of LE-127/2 on HaCaT cells morphology.

Cytotoxic effect of LE-127/2 detected on other non-malignant cells (H9c2 and NIH-3T3 fibroblast cells) is shown in Fig. S1 (Supplementary Information). H9c2 cells showed significant cell death only at 48–72 h after LE-127/2 treatment, at 40 μM of the compound, however, CBG showed toxicity at concentrations as low as 20 μM (Fig. S1 A and B, Supplementary Information).

Remarkably, fibroblast cells were the least sensitive to LE-127/2. Similarly to HaCaT cells, these cells were sensitive to LE-127/2 treatment only at 80 μM, and no significant cytotoxic effect was observed until 48 h after treatment, while CBG was toxic on fibroblast cells already at 20 μM concentration (Fig. S1 C and D, Supplementary Information).

In addition, the half-maximal inhibitory concentration (IC_{50}) was determined using GraphPad Prism Nonlinear regression analysis (Fig. S2, Supplementary Information).

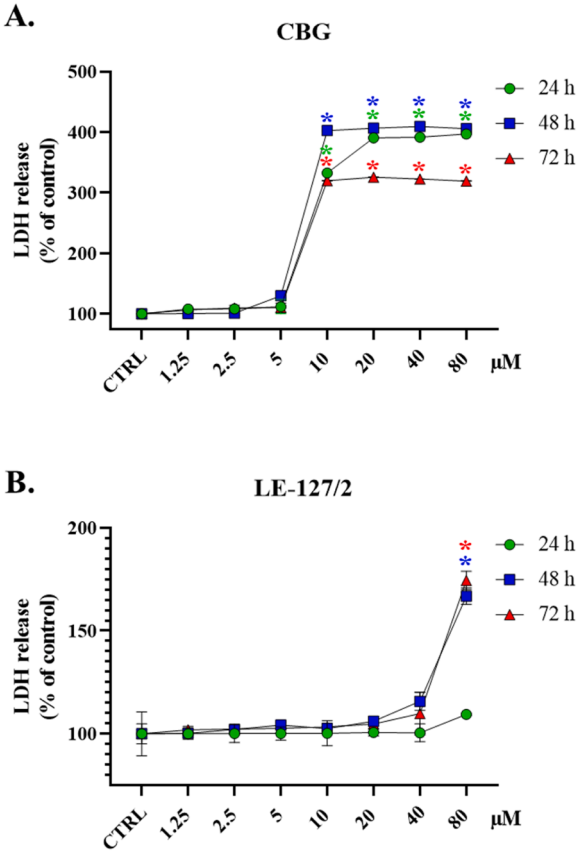

Fig. 2. Cytotoxic effect of CBG and LE-127/2 on LDH release in HaCaT cells. (A, B). 6×10^3 cells were plated into 96-well plates and treated with increasing doses of CBG or LE-127/2 (1.25 μM to 80 μM over 24 to 72 h. DMSO (CTRL) was used as a vehicle. LDH activity was measured at 490 nm in the aliquots obtained from the culturing media of drug treated cells $^*p < 0.05$ - mean significant effect of LE-127/2 or CBG. Data expressed as mean ($n = 3$) ± *S*.D. of the absorbance ratio normalized to untreated cells.

3.2. Antiproliferative effect of LE-127/2 in comparison with CBG and vemurafenib

Three different (WM35, A2058 and WM 3000) human cutaneous melanoma cell lines were utilized to investigate the inhibitory effect of LE-127/2 (Lőrincz, Tóth et al., 2023) in comparison with parent CBG and vemurafenib. Increasing concentrations (2.5 μM to 80 μM) of the drugs were applied to detect the proliferation activity of the cells followed over 24 to 72 h by using Cell Titer Blue Assay (Fig. 3).

Proliferation activities measured as fluorescence were plotted against increasing concentrations of the drugs and normalized over the drug-free control group (DMSO), presented in Fig. 3 (A to I). The results show that vemurafenib exhibited more pronounced and more effective antiproliferative effect on each melanoma tumor cell line than LE-127/2 and CBG, since significantly decreased the proliferation of all the cells at 2.5 μM concentration (Fig. 3G, H, I). Particularly, the effect of vemurafenib on WM35 cells was proved to suppress significantly the cell proliferation as early as 24 h after the treatment, almost 90% of the cells were detected dead by the assay used above (Fig. 3G).

The parent CBG compound proved to be more effective than LE-127/2. It showed similar effect in all the cells, and significantly inhibited cell proliferation already as low as at 5 μM concentration, and even lower concentration (2.5 μM) suppressed the growth of WM3000 cells (Fig. 3D, E, F).

In comparison to vemurafenib and CBG, LE-127/2 showed lower intensity to inhibit the cell proliferation in each cell line. In all three cell lines the significant inhibitory effect of LE-127/2 was measured, if 20 μM of the drug was applied. Some effect of LE-127/2 was already detectable at 24 and 48 h, however, it reached a significant level ($p <$ 0.5) at 72 h after the treatment. The only exception was the A2058 cell line, which most likely proved to be more sensitive to LE-127/2 than the other two melanoma cell lines. Thus, LE-127/2 showed a dose-dependent and time-dependent effect in all of the three examined melanoma cells (Fig. 3A, B, C).

Altogether, comparing the effectiveness of vemurafenib, CBG and LE-127/2, it is clear from the presented bar graphs (Fig. 3) that at 20 μM concentration all three drugs exert significant inhibition on all of the utilized human melanoma cell lines. It is very important to emphasize that although LE-127/2 was effective only at higher concentrations than vemurafenib and CBG, it proved to be toxic on HaCaT cells only at higher concentration, such as 80 μM, indicating that non-malignant intact cells much less sensitive than the effective concentration of the compound.

3.3. Results of clonogenic cell survival assay

Clonogenic cell survival assays were used to examine the inhibitory effect of LE-127/2 compound on the clone formation ability of human cutaneous melanoma cancer cells, WM35, A2058 and WM3000 cell lines. Based on the proliferation activity assay results, cells were treated with increasing concentrations of LE-127/2 (5–80 μM) for two weeks and subjected to a cell colony formation assay at 37 °C in a 5% CO_2 incubator with 95% air. Fig. 4 shows that LE-127/2 effectively suppressed colony formation in all three cell lines. WM3000 and A2058

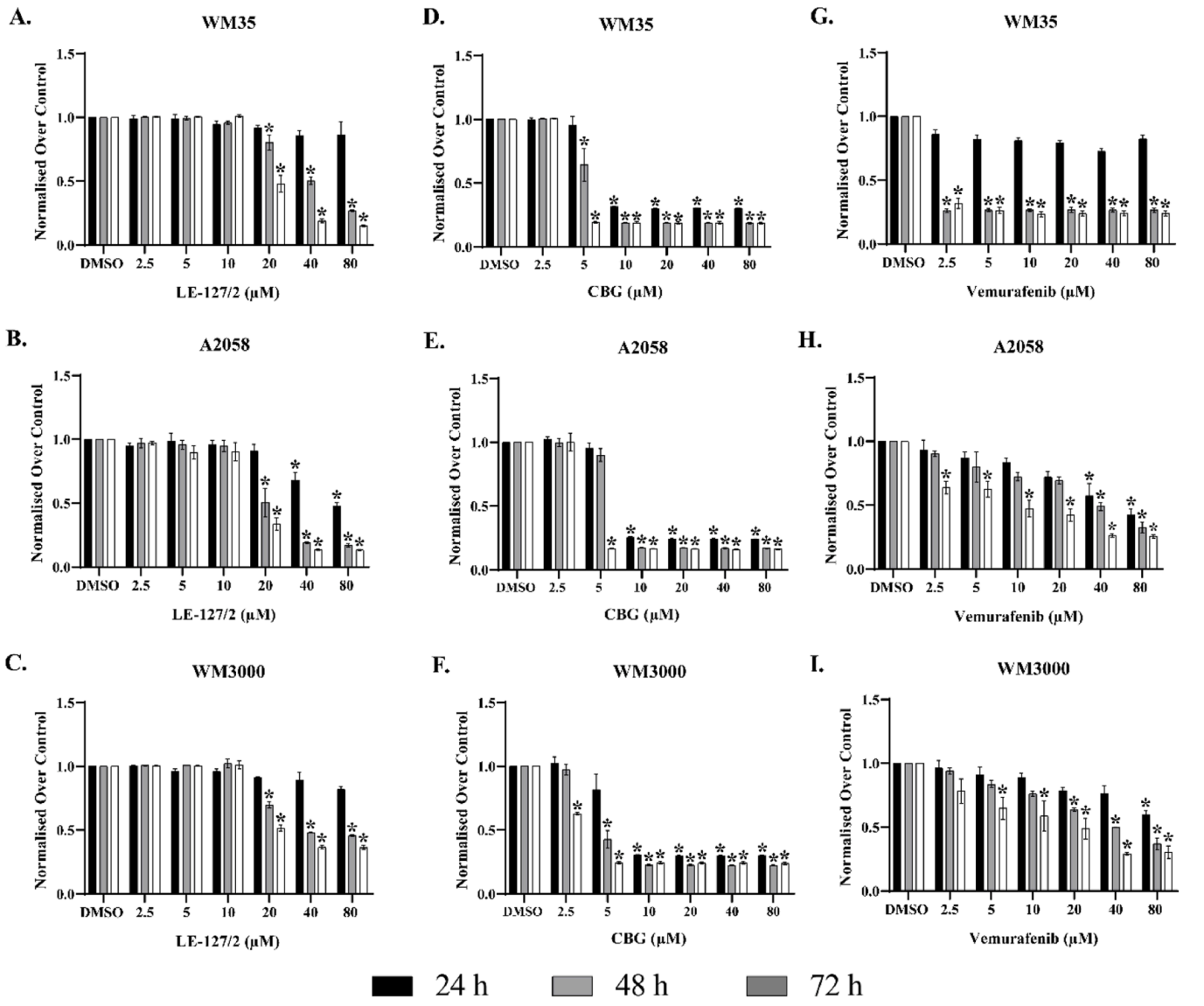

Fig. 3. Cell proliferation activity of A2058, WM35 and WM3000 cells treated with LE-127/2 (A-C), CBG (D-F), or vemurafenib (G-I). Melanoma cells were treated with increasing concentrations of LE-127/2, CBG or vemurafenib (2.5 μM to 80 μM) for 72 h and the cell proliferation activity was measured. Two-way ANOVA analysis showed significant differences were, * $p < 0.05$. Data expressed as mean ($n = 3$) ± *S*.D.

cells showed almost the same ability to form colonies, and in both of the cell lines LE-127/2 profoundly affected the growth of the colonies, at the lower concentrations of the drug, at concentrations, of 10 to 20 μM. However, more effectively LE-127/2 suppressed colony formation in A2058 and WM 30,000 cells at 10 μM concentration than in WM35 (Fig. 4). There was a decrease of about 2–3-fold in the number of colonies if 20 μM of LE-127/2 was applied in comparison to the drug-free control group in all the examined cell lines. The results revealed by clonogenic formation assay are in harmony with the results of cell proliferation assay; the compound expressed similar intensive inhibition on cell growth and colony formation at 20 μM (Fig. 4) and the effect of LE-127/2 exerted on human cutaneous melanoma cells has dose- and time-dependent manner (Fig. 4).

3.4. Protein expression analysis

The programmed cell death (PCD) includes three major types: apoptosis, autophagy and necrosis (Shen et al., 2023). The connection and transformation between cell death modes are closely related to human diseases; both autophagy and apoptosis are fundamental in the development of melanoma and the formation of metastases, therefore, our goal was to identify the effect of LE-127/2 on main autophagy and apoptosis related proteins.

3.4.1. Evaluation of the human autophagy array data

Monitoring the change of multiple protein expression on LE-127/2 treatment, Human Autophagy Array was used for the analysis of protein samples obtained from the cells treated with 20 μM of LE 127/2 for 48 h (description in the "Materials and Methods"). Array membranes with specific antigens (Fig. 5) were probed with proteins from the cells and then all steps were carried out as provided by the manufacturer. Results of array analyses are presented in Fig. 6. Spot intensities show the expression level of specific proteins placed on the array in the control and treated cells (Fig. 6). The spot density was analyzed with the Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA), and all signals were normalized to the spots corresponding to the duplicate Positive Controls on the array (arranged in the upper left and lower right corners in the arrays) (right panel in Fig. 6A, 6B and 6C). The signal intensity for each individual antigen-specific antibody spot is proportional to the relative expression of the antigen in the examined sample, thus, by

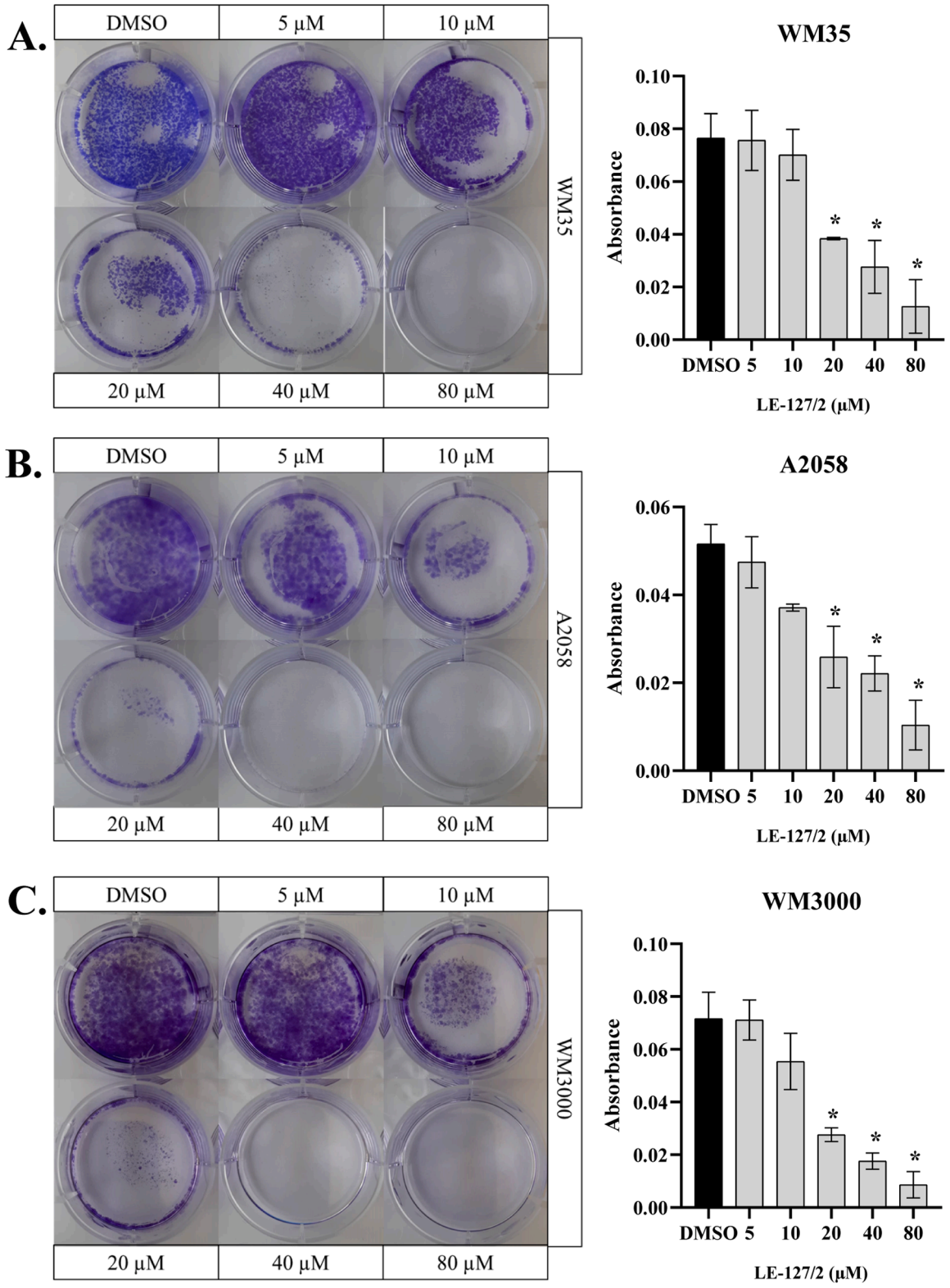

Fig. 4. Clonogenic cell survival of A2058 (A), WM35 (B) and WM3000 (C) induced by LE-127/2 treatment. Cells were treated with LE-127/2 and maintained at 37 °C in a 5% CO_2 incubator with 95% air. At the termination of the experiment the colonies of survived cells were fixed and visualized by 0.1% crystal violet staining. The colonies were resuspended in 2% SDS solution and quantified by determination of absorbance at 570 nm. Data expressed as mean ($n = 3$) ± *S*.D, significant differences (*$p < 0.05$) were calculated with two-way ANOVA analysis.

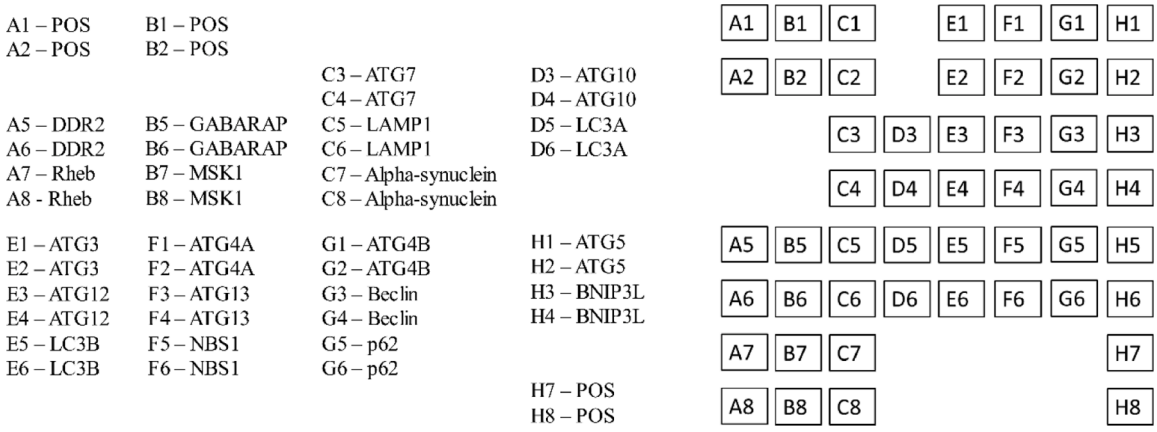

Fig. 5. Map of autophagy array with the spots of detectable proteins (from the manual provided in the kit).

comparison of signal intensities among array images of untreated drug-free control samples and array images of LE-127/2 treated samples, the relative differences in expression levels of each protein was determined. In Fig. 6 black columns indicate the expression of basic autophagic markers, such as Atg12, LC-3 (LC3A and LC3B).

It is worthy to note that LC3A usually shows a perinuclear and nuclear localization, while LC3B is distributed throughout the cytoplasm and localized in the nucleolar regions, both widely used as biomarkers of autophagy (Koukourakis et al., 2015). According to array analysis LC-3 (LC3A and LC3B), Beclin-1 and Sequestosome 1/SQSTM1/p62 (p62) compared to the untreated control cells showed increased expression in all examined cells, if treated with LE-127/2 compound. Remarkable, that Beclin-1 showed higher expression in LE-127/2 treated A2058 cells than in the other two cell lines (Fig. 6B).

3.4.2. Verification of LC-3, p62, beclin-1 and atg12 expression by western blot

3.4.2.1. LC-3II expression as a hallmark of autophagy is increased upon LE-127/2 treatment. To date, LC-3 is identified as one of the novel prognostic biomarkers for early-stage melanomas (Tang et al., 2016). Under physiological conditions of the skin LC-3 is cytoplasmic and shows lower level than in malignant melanomas where it is induced and forms the membrane bound form that is LC3-II, thus acting as a marker of autophagy induction (Tang et al., 2016). Fig. 7 depicts changing of LC-3 protein expression at the given time points in response to treatment with LE-127/2. The results presented clearly show that the level of LC-3 protein changed as a result of the treatments (Fig. 7A-C). As it is shown in Fig. 7B, LC-3II showed elevated expression in WM35 and A2058 melanoma cells after 24 h treatment but was increased in WM3000 cell at 48 h after the onset of the treatment. It indicates that a cytosolic form of LC-3 (LC3-I) is conjugated to phosphatidylethanolamine LC-3II, which level is increased, and a hallmark of autophagy induction. Therefore, it is possibly to induce autophagy with LE-127/2, taking about 24–48 h in examined melanoma cell lines. In summary, increase in LC-3 (Fig. 7A and B) protein expression detected by Western blot at 48 h after the LE-124/2 treatment is comparable with the results obtained by array analysis, according to which expression of LC-3II was increased in drug treated cells (Fig. 6). This was supported by the immunofluorescence staining, showing a slightly more intensive of the green color in the cytoplasm of the LE-127/2 treated cells than in the untreated control cells. Probably, it is due to the appearance of increased “LC-3 puncta” (dots) as a result of autophagy induction. (Fig. 7C).

3.4.2.2. Expression levels of other autophagic proteins on LE-127/2 treatment. The cargo receptor of autophagy, p62 was proved to be overexpressed in metastatic melanomas with poor prognosis and described as a multifunctional protein, as one of the candidate drivers in melanoma tumor progression (Lai et al., 2018; Karras et al., 2019). p62 protein expression showed a sharp increase at 24 h following the LE-127/2 treatment in all used melanoma cells and a gradual decrease in WM35 and WM3000 cells at 48 to 72 h. However, the treatment in A2058 cells resulted in a gradual increase during all over the 72 h (Fig. 8 upper panel and Fig. 8b).

The expression of Beclin-1 showed only a slight non-significant increase during the treatments, except in the A2058 cell line, where the level of Beclin-1 was significantly increased at 72 h (Fig. 8 upper panel and Fig. 8a). This result is fully comparable with those of the array analysis (Fig. 6).

The expression of Atg12 protein showed a decrease only at 24 h after the treatment with LE-127/2, and then, followed an increased expression of the protein at 48–72 h (Fig. 8 upper panel and Fig. 8c). These results are consistent with the results obtained in the array study (Fig. 6).

We also examined the expression of Tom20 protein that has been described as an important protein in melanoma cell death (Zhou et al., 2018). Tom20 transmits ROS signaling to mitochondria therefore plays a fundamental role in mitophagy. Treatment with LE-127/2 resulted in significantly decreased level of Tom20 at 24 h after the treatment, and then an increased level at 48 and 72 h in WM35 and A2058 cells comparable to the amount of Tom20 protein level measured in the drug free control cells (0 h). Tom20 expression in W3000 cells showed different trend and was significantly decreased in time-dependent manner if compared to the control cells (Fig. 8 upper panel and Fig. 8d).

It can be noted that during 72 h treatment, the expression of autophagic proteins did not show a clear increase or decrease, rather a fluctuation was characteristic for expression of different proteins examined by the two methods. Based on the array analysis, in addition to the proteins detected by Western blotting, more autophagy related proteins also showed change at their expression level, however, studying these proteins are beyond the scope of the present work.

3.4.3. Effect of LE-127/2 treatment on the expression level of HO-1

Enhanced HO-1 expression promotes resistance and activates protective autophagy responsible in the development of chemoresistance (Was et al., 2020; Wang et al., 2023). Interestingly, although HO-1 overexpression in melanoma cells enhances tumor progression can reduce growth and metastasis of melanoma (Was et al., 2020). Because of this importance of HO-1, we aimed to examine the effect of 20 μM LE-127/2 treatment on HO-1 expression in WM35, A2058 and WM3000 cells. Fig. 9. shows the expression of HO-1 in all examined melanoma cells, WM35, A2058 and WM3000, respectively. Protein expression detected by Western blot for HO-1 protein (Fig. 9A) revealed that no significant change was observed in the expression of HO-1 in the cells treated with LE-127/2 compared to drug free control groups, and the

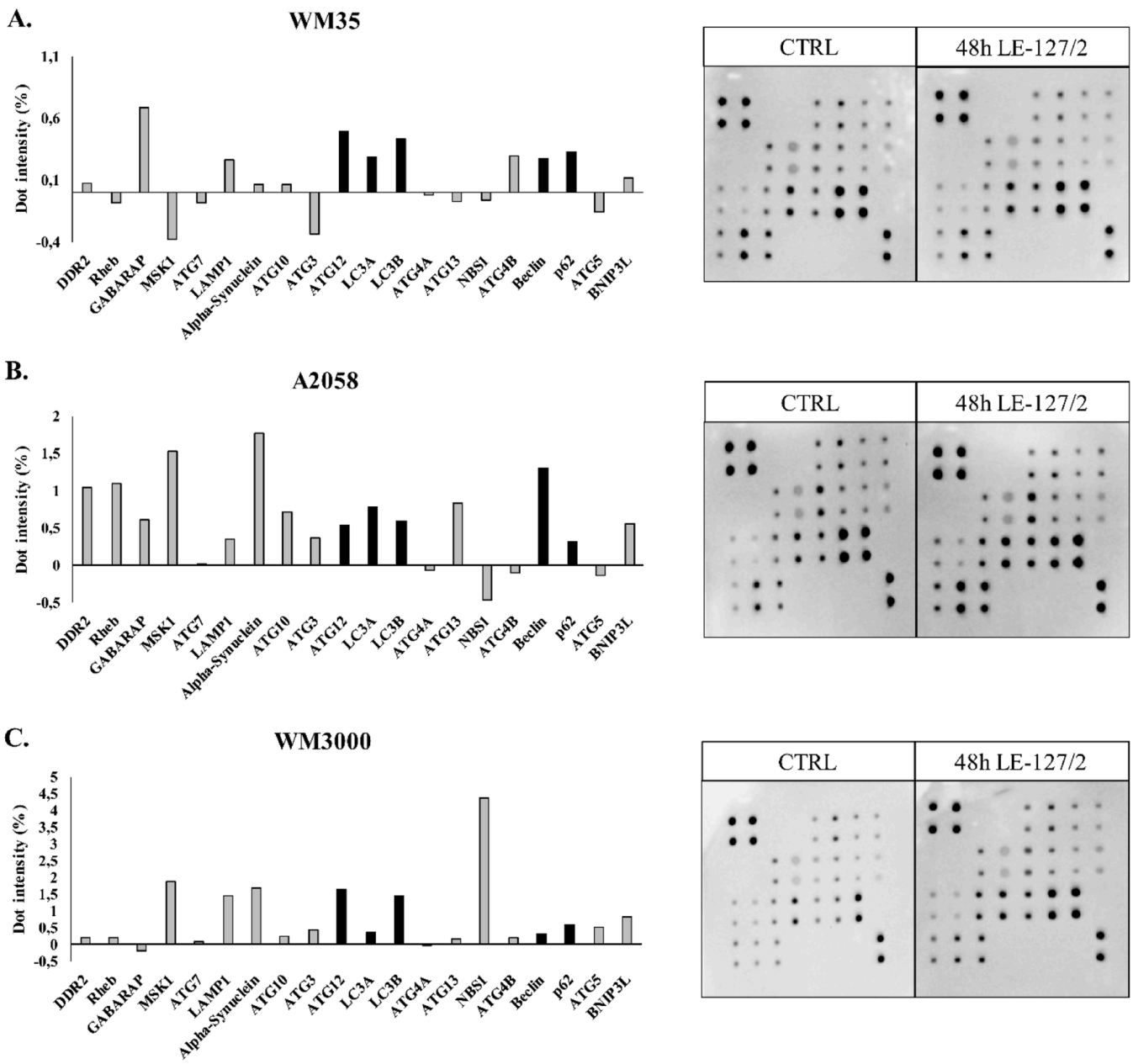

Fig. 6. Results obtained with Human Autophagy Arrays (Assay Genie). Membranes were probed with the LE-127/2 (20 μM) treated and untreated cell lysates from WM35, A2058 and W3000 cell lines. The signal intensity for each antigen-specific antibody spot (right side) is proportional to the relative expression of the antigen of detected protein in that sample (left side). A representative sample was applied to each array (because of the limited number of the array).

expression of HO-1 was only slightly elevated at 72 h after the treatment (Fig. 9B).

3.4.4. *Expression of apoptotic proteins*

Detecting apoptosis related proteins, we tried to clarify whether cell death was caused by apoptosis or not. Thus, cell lysates obtained from cells treated with 20 μM of LE-127/2 for 24 to 72 h were used for Western blot to examine the expression of several apoptosis related proteins.

3.4.4.1. Expression of p53, bax, bcl-2, PDCD4 and pten. The p53 tumor suppressor protein is a major hub in a molecular network controlling cell death and its activation results in a stop in growth of tumor cells either by p53-dependent or p53-independent pathway of apoptosis (Loureiro et al., 2021). After the quantification of p53 protein expression detected by Western blot (Fig. 10 upper panel and Fig. 10a), p53 was found to be expressed at different levels in the three cell lines and the level of p53 protein was decreased but not significantly as a result of LE-127/2 treatment. Expression of bax and bcl-2 protein was increased during the treatment compared to the drug-free control group, however, only bax protein showed a significant increase detected at 48 h after the treatment (Fig. 10 upper panel and Fig. 10b and c).

The expression of PTEN protein was investigated, and the data revealed that A2058 cells missing PTEN expression that is connected to the more aggressive and intensive cell proliferation of A2058 cells than the other two, WM35 and WM3000 cell lines (Fig. 10 upper panel and Fig. 10d).

PDCD4, a novel tumor suppressor, was described to have an impact on overall survival of patients with metastatic melanoma (Tran et al., 2021), therefore, we studied the effect of LE-127/2 on the expression of PDCD4 protein. Following 24 h of LE-127/2 treatment, the level of PDCD4 was significantly dropped and it returned to the level same as the

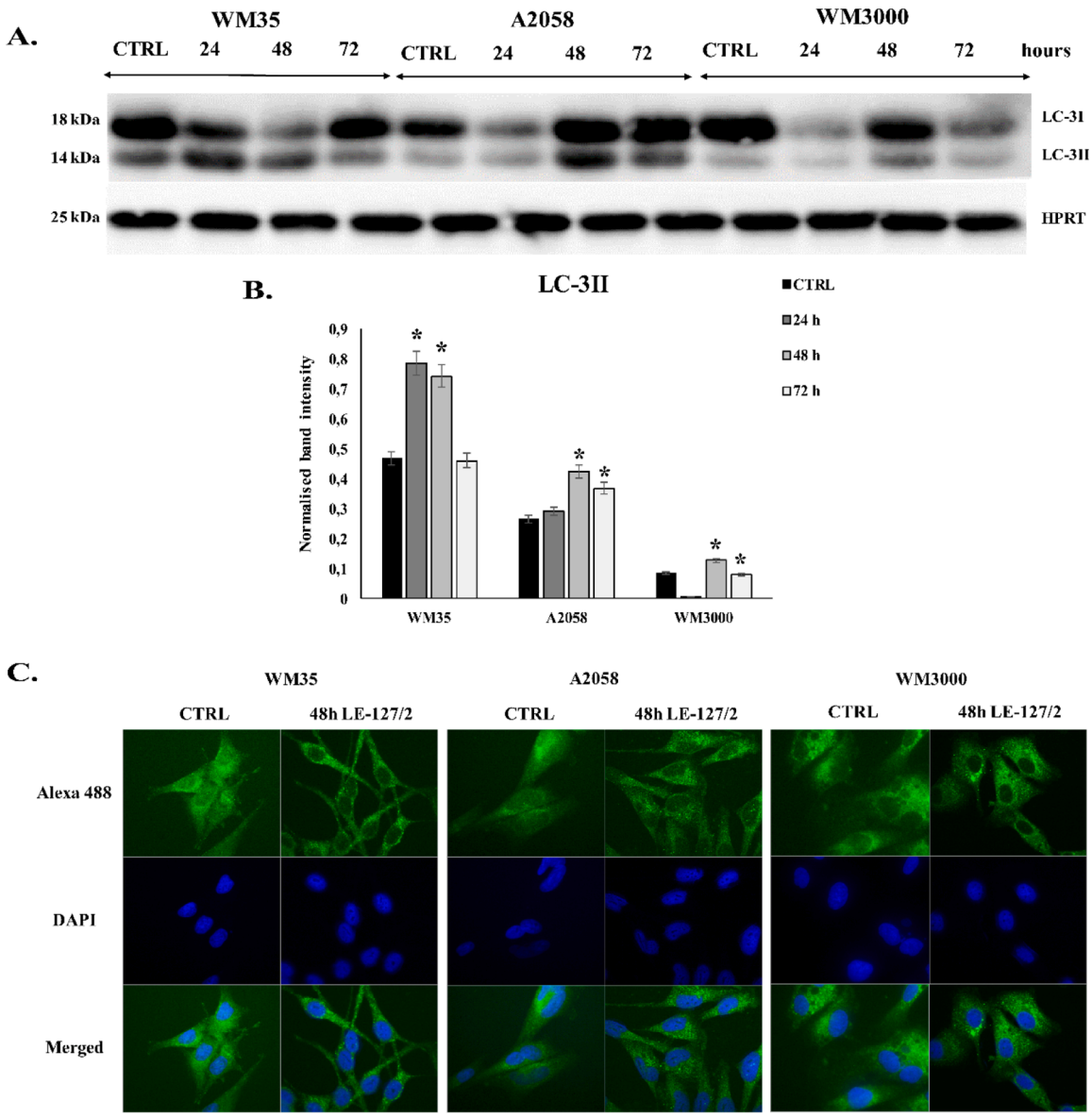

Fig. 7. LC-3 protein expression in melanoma cells treated with LE-127/2. Protein lysates were analyzed for LC-3 protein by Western blot (A and B). **Immunofluorescence detection of LC-3** (C). Cells were grown on coverslips and treated with LE-127/2 for 48 h, and then, fixed in 4% paraformaldehyde; were incubated overnight at 4 °C with the primary anti - LC-3 antibody, diluted at 1:100 in 1% BSA-0.1% Triton X 100. Alexa-488 anti-rabbit antibody was used against primary antibody to detect LC-3 puncta in the cells (green), and DAPI (blue) was added to detect cell nuclei (C panel). Pictures were taken under fluorescence microscope (Zeiss Axioscope), magnification was 200x.

drug-free control group after 48–72 h treatment. Interestingly, it was almost 1.5-fold elevated in WM35 cells at 48 h compared to drug-free control value (Fig. 10 upper panel and Fig. 10e).

3.4.4.2. PARP activation by LE-127/2. Increased expression of cleaved-PARP in all three LE-127/2 treated melanoma cell lines compared to the control group was revealed (Fig. 11). PARP (116 kDa) cleavage was activated upon treatment of the cells, and the level of the cleaved PARP (89 kDa) was increased. Significantly increased level of cleaved-PARP in LE-127/2 treated cells showed a time-dependency, and also cell-dependent expression of PARP and cleaved PARP was observed in all three different melanoma cell lines. The most intensive and earliest PARP activation was detected in WM35 cells.

Caspase-3 protein expression showed a significant change at 24–48 h following the treatment in WM35 and A2058 cell lines, however, it did not change significantly in WM3000 upon the treatment.

4. Discussion

Therapeutic options of human cutan melanoma to eradicate malignant cells primarily include surgery, and in the past ten years such as adjuvant treatments, immunotherapy and targeted therapy (van Zeijl et al., 2017). As it was mentioned in the introduction, various therapeutic combinations are used to increase clinical responses and overall survival in patients (Soengas and Lowe, 2003; Flaherty, 2012; Flaherty et al., 2012; Luke and Schwartz, 2013; Austin et al., 2017; van Zeijl et al., 2017; Mattia et al., 2018). Among four genomic subgroups of melanoma, such as BRAF, NRAS, NF1, and triple wild-type, mutation of the BRAF oncogene in exon 15/codon 600 (BRAFV6.00E) present in about 50% of patients, thus, representing the most critical therapeutic target for melanoma therapy (Shaw and Nathan, 2013; Wilson and Schuchter, 2016), therefore, it is a good reason that vemurafenib as the first approved BRAF inhibitor was chosen for the treatment of patients suffered from BRAF metastatic melanoma. Vemurafenib inhibits V600E

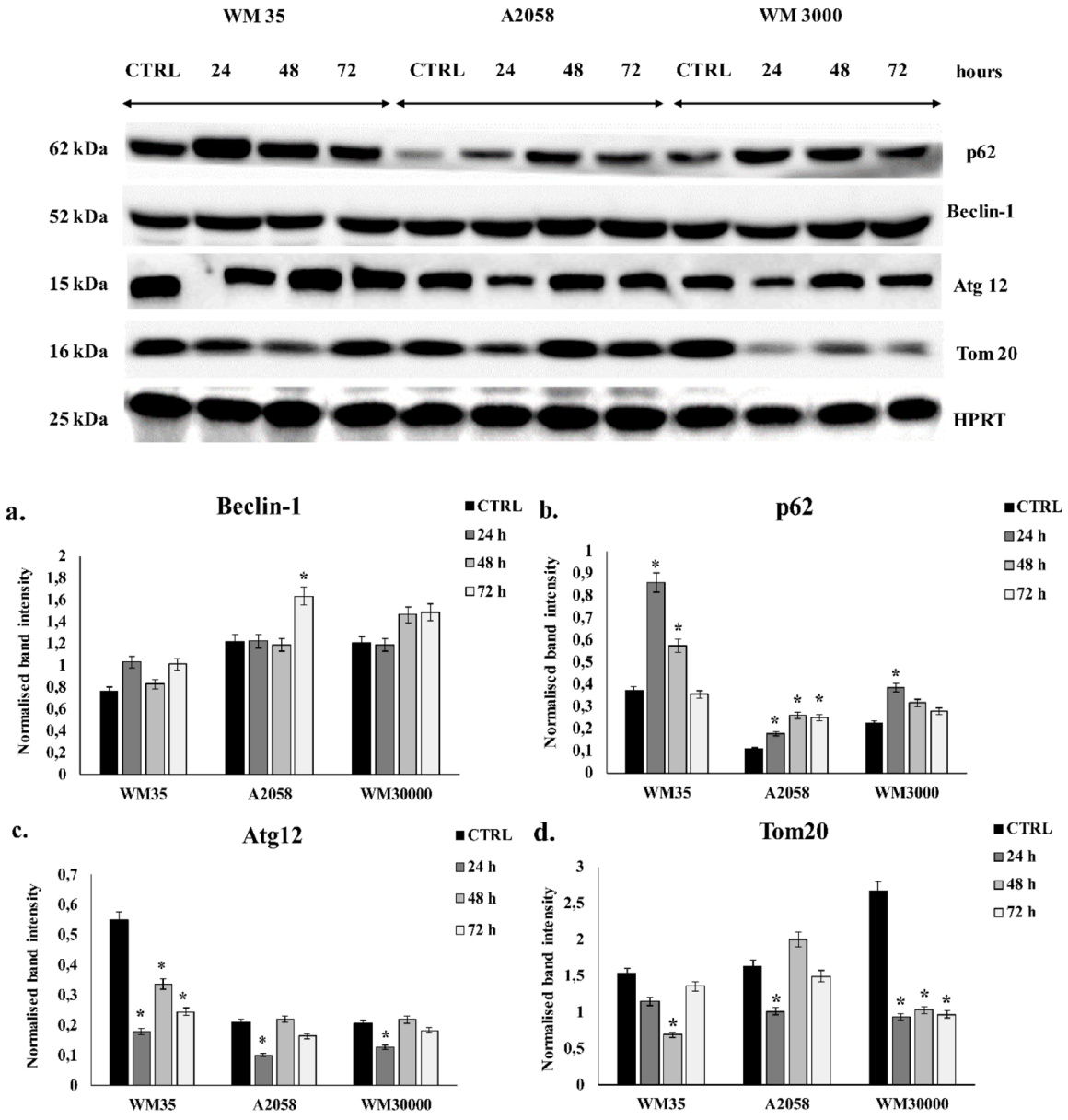

Fig. 8. Western blot analysis of autophagy related protein expression in melanoma cells with 20 μM of LE-127/2 treatment. Bar graphs (a-d) show the levels of proteins (p62, Beclin-1, Atg12 and Tom20) expression normalized to HPRT. Data were calculated from three independent experiments and expressed as the mean ± *S*.D. Significant differences were calculated with two-way ANOVA analysis (* $p < 0.05$).

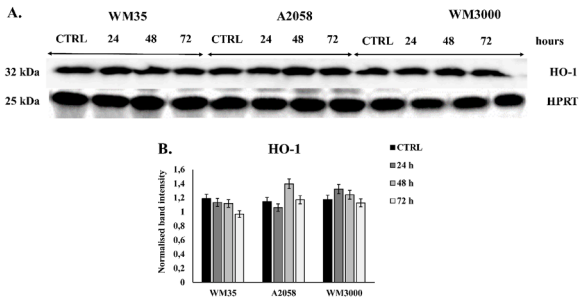

Fig. 9. Protein expression level of HO-1 protein in WM35, A2058 and WM3000 cells. Detection of HO-1 expression in drug-free control melanoma cells and LE-127/2 treated cells (A). Bar graphs show the level of proteins normalized to HPRT (B), data were calculated from three independent experiments and were expressed as the mean ± *S*.D. Significant differences were calculated with two-way ANOVA analysis (* $p < 0.05$).

kinase, inducing apoptosis by blocking the mutation-driven constitutive MAPK pathway signaling, thus, improve overall survival of patients (Mandel et al., 2022). Also, the inhibition of BRAF in combination with MEK inhibitors in melanoma therapy, proved to be impressively successful although, yet the acquired resistance to such drugs as vemurafenib, often arised in most of the patients, the mechanism of which is poorly understood. Moreover, the strong metastatic nature of melanoma makes long-term resistive treatment difficult (Tóvári et al., 2023). The resistance developed against the drug during the long-term treatment of metastatic patients is one of the disadvantages of vemurafenib, and another disadvantage is its toxicity to the intact human cells, which manifested in a few adverse reactions, such as skin reactions, photosensitivity, headache, and arthralgia (Gouravan et al., 2018; Shahriari et al., 2019). To overcome these limitations, several studies are currently addressed to explore naturally based compounds holding potential in the treatment of melanoma.

C. sativa (Cannabis sativa L., Cannabaceae) produces over 500 bioactive compounds; among these are cannabinoids: Δ9-

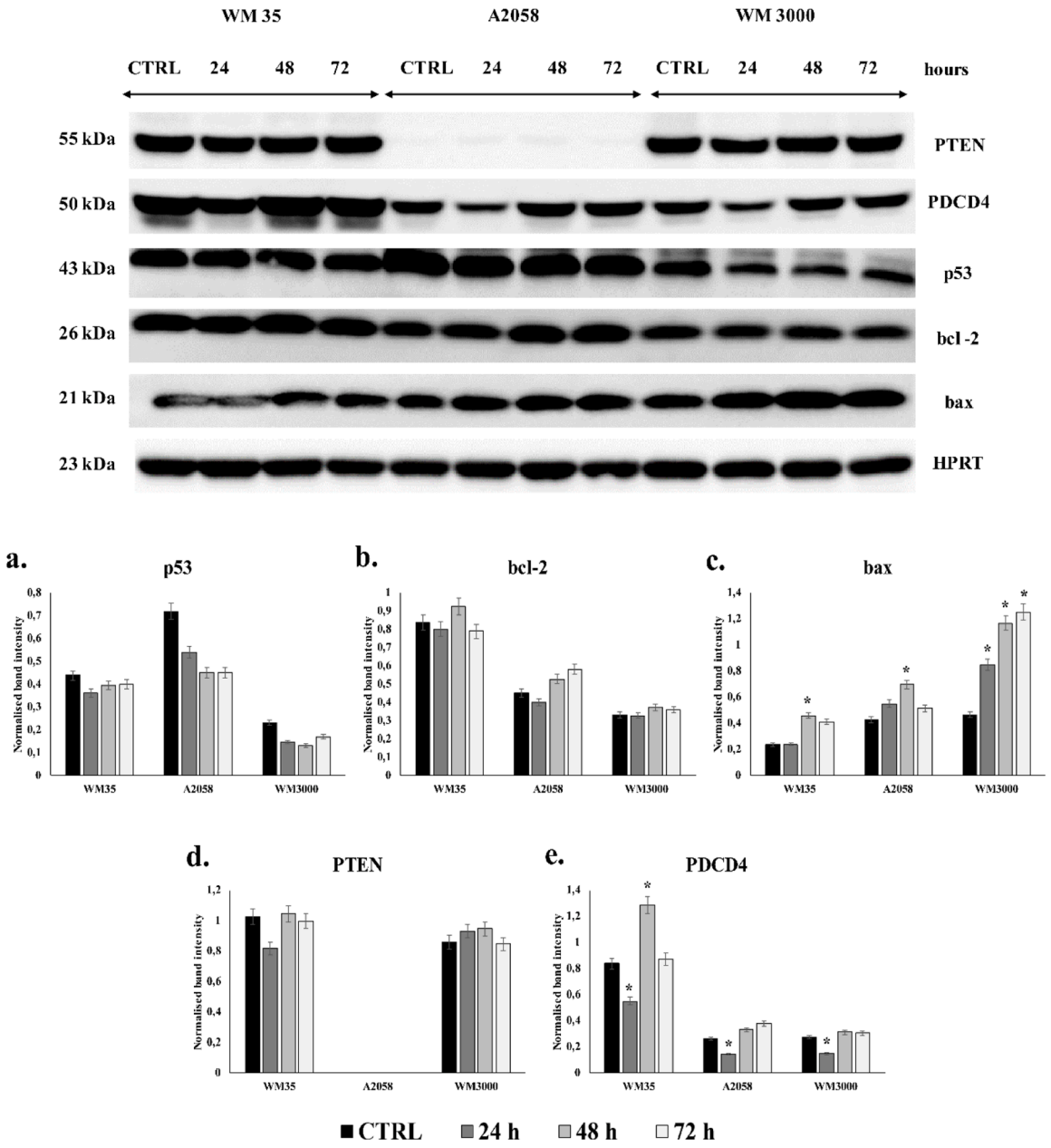

Fig. 10. Detection of apoptosis related proteins in LE-127/2 treated melanoma cells. PTEN, PDCD4, p53, bcl-2 and bax proteins were detected at appropriate molecular weights (upper panel). Bar graphs show the level of proteins normalized to HPRT (a-e). Data were calculated from three independent experiments and expressed as the mean ± *S*.D. Significant differences were calculated with two-way ANOVA analysis (*$p < 0.05$).

tetrahydrocannabinol (THC) and cannabidiol are intensely investigated for their anti-cancer functions (Ahmed et al., 2019). However, less is known about the effect of another constituent of *C. sativa*, the cannabigerol (CBG), although it has been shown to exhibit anti-tumor properties in some kind of tumors including melanomas (Ligresti et al., 2006). CBG has been described to show significant antiproliferative effect in other aggressive tumors than melanoma (Lah et al., 2021), thus, CBG modulates the differentiation of myeloid cancer, and glioblastoma as well (Lah et al., 2022; Wyrobnik et al., 2023). Despite of beneficial biological properties of CBG (Vacek et al., 2021), its low water solubility and moderate bioavailability makes limit to clinical use, however these properties can be improved by chemical modification. Recently, E. Lőrincz et al., synthesized a series of a new nitrogen-containing derivatives of CBG, and showed the biological properties of these compounds (Lőrincz et al., 2023). The nitrogen content of the derivatives can improve the binding to different receptors, enzymes or other proteins, and gives the opportunity for salt formation and thereby increasing water solubility.

These properties can help increase the bioavailability, which probably result in much less of the compound being used in patients than the parent compound itself. Moreover, Lőrincz et al., showed that salt formation does not alter the biological effects (Lőrincz et al., 2023).

In the present study we examined the antiproliferative effect of a recently synthesized CBG derivative, LE-127/2, in three different melanoma cell lines and compared its effect with vemurafenib (Lőrincz et al., 2023). A2058 and WM35 cell lines carry a BRAF mutation, while WM3000 carries mutation of the N-RAS gene, thus, not surprising that WM35 and A2058 cell proliferation activity was inhibited already by a low concentration of BRAF mutant selective vemurafenib (as low as 2.5 μM) and proved to be more effective in these two cell lines than in WM3000. LE-127/2 showed less impressive inhibition on cell proliferation of the examined melanoma cell lines than vemurafenib, yet at 20

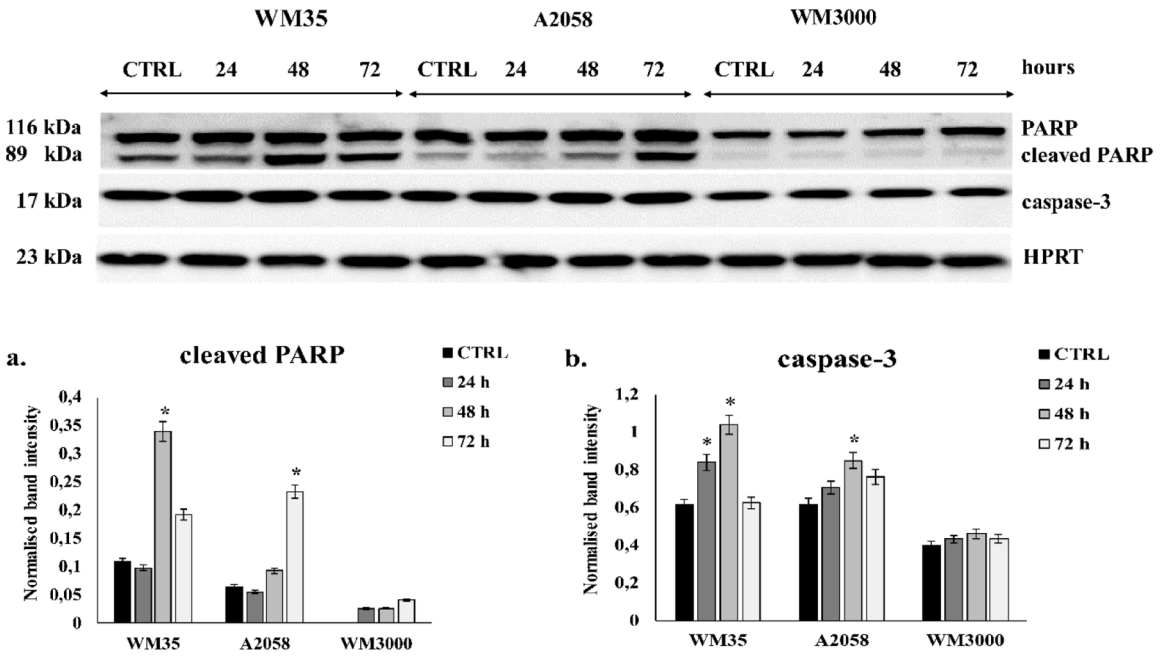

Fig. 11. Detection of PARP expression in LE-127/2 treated melanoma cells by Western blot. Expression level of PARP/cleaved PARP affected by LE127/2 treatment in all examined cell lines (upper panel and lower panel (a) and (b). Bar graphs show the quantification of proteins normalized to HPRT. Data were calculated from three independent experiments and expressed as the mean ± *S*.D. Significant differences were calculated with two-way ANOVA analysis (*$p < 0.05$).

μM of LE-127/2 exerted approximately 50% suppression on the growth of all examined cells regardless of the mutation type. These properties of LE-127/2 emphasize the advantages of this new compound over vemurafenib, since it is non-selective and can exert similar anti-proliferative effect regardless of mutation type. Thus, we assumed that the new CBG presumably might be useful in the treatment of melanomas with different molecular background in comparison with vemurafenib.

The effect of parent CBG on the cell proliferation was stronger than the effect of LE-127/2, which showed approximately 50% inhibition on cell growth only at 20 μM of the compound. However, we would like to highlight the positive feature of LE-127/2, it has low cytotoxic effect measured on HaCaT (normal keratinocytes) cells by LDH assay, compared to the effective concentration (20 μM). It was toxic only at 80 μM. Treatment with LE-127/2 resulted in neither a significant cell number reduction of HaCaT cells nor morphological changes. Whereas, studying the parent CBG on HCaT cells, it proved to be toxic despite its impressive inhibitory effect on the proliferation of melanoma cells. Earlier studies also proved that CBG is cytotoxic in fibroblasts and keratinocytes (HaCaT) (Lah et al., 2022). It is important to emphasize although the new cannabigerol derivative, LE-127/2 effective only at higher concentrations than its parent CBG, the lower efficacy does not reduce its advantages over vemurafenib or CBG. The low toxicity presumably suggests that this drug candidate could be a possible application in the therapy of melanoma alone or in combination with approved drugs without causing damage in surrounding normal non-malignant cells. Our further cytotoxicity studies on H9c2 cardiomyocytes and fibroblasts also confirmed that LE-127/2 is less toxic to these cells than the parent CBG, which further emphasizes this compound's benefits in clinical application as it is believed to have fewer untoward effects than CBG. Therefore, the research on LE-127/2 deserves to be deepened to explore whether our results are translatable in the clinical application and whether LE-127/2 could be used alone or in combination with other therapeutics as a novel approach in cancer treatment including melanomas. However, preclinical studies are needed to demonstrate that LE-127/2 is a potential drug candidate for melanoma therapy (Wang et al., 2020; Riera et al., 2022).

Clonogenic assays used to investigate survival of treated cells after chemotherapy or irradiation resulted in a significant inhibition on colony formation at 20 μM, suggesting that this compound might be useful against metastases or in the treatment of metastatic melanomas. Based on the obtained results, the combination of conventional therapies with cannabinoids are suggested to exert its antitumor effect in addition to the palliative effect that is a primarily reason of cannabinoid applications among patients (Viereckl et al., 2022).

Resistance developed in melanoma to chemotherapies are often associated with induction or inhibition of autophagy, therefore, the effects of LE-127/2 were studied on autophagic related proteins. Elevated levels of LC-3, p62 and Atg12 as basic markers of autophagy were detected upon the treatment with LE-127/2. Interestingly, the major marker of autophagy, LC-3II showed a significant increase during the 24–48 h treatment and it was dropped to the level of untreated controls at 72 h after the application of LE-127/2. Beclin-1 and LC-3 are involved in the nucleation phase, while p62 as a cargo receptor plays a role in the removal of aggregated molecules and organelles deliver to autophagosomes following interaction with LC-3II, thus most likely this is the reason why increased level of p62 was detected at 72 h in all LE- 127/ 2 treated cells (Lee et al., 2021). We assume, regarding the action mechanism of LE-127/2, that autophagy can be primarily an intracellular degradation pathway that occurs before apoptosis and may lead to the programmed cell death prior to apoptosis (Viereckl et al., 2022). According to obtained results related to LC-3II, we suggest that initiation of autophagy occurs between 24 and 48 h after the application of LE-127/2, and later, to complete the cells death possibly partly apoptosis and necrosis takes the driving over the initially induced autophagy. Based on the published data, we suggest the LE-127/2 inhibits cell viability of cancer cells through several types of cannabinoid receptors, the activation of which may stimulate the synthesis and accumulation of ceramides, and then triggers the induction of p8 and p38, which followed by inhibition of Akt-mTORC1 axis and initiation of the autophagy process, which is upstream of apoptotic processes (Peeri and Koltai, 2022). It is needed to study the expression of different cannabinoid receptors (which are not examined in the present study) in melanoma cells to clarify the clear mechanism, however according to what has been published inhibition of Akt via possibly other involved molecules leads to apoptosis via the intrinsic mitochondrial pathway (Peeri and Koltai, 2022).

Interestingly, the examined Tom20 protein, which just recently showing importance in melanomas, initially decreased upon the treatment with LE-127/2, however between 48 and 72 h its level increased back to the level of untreated control. It was detected in BRAF mutant melanoma cell lines (WM35 and A2058), while it was decreased in WM3000 cell lines carries N-RAS mutation. It may be suggested that LE-127/2 inducing Tom20-Bax-caspase-3 pathway and downstream results in release of cytochrome-c, consequently activating caspcase-3. Once, caspase-3 is initiated, it activates downstream other caspases (6 and 7) to activate the apoptotic pathways in melanoma cells leading to activation of apoptosis (Zhou et al., 2018). Thus, the substrate of activated caspases (3 and 7) will lead to PARP cleavage, and accordingly as a result of LE-127/2 treatment remarkably increased the expression of cleaved PARP in the studied melanoma cells, the activation of which perhaps together with cell death induced by autophagy, causes eventually a death of cancer cells exposed to LE-127/2.

Among several other proteins connected to apoptosis, such as p53, bcl-2, bax and PDCD4, analyzed by Western blot only the bax protein was expressed significantly at 48 h after the LE-127/2 treatment compared to the drug-free control cells. Expression level of p53 protein was not changed significantly in none of the cell lines upon the treatment, presumably, the p53-dependent pathway is not activated significantly during the LE-127/2 induced apoptosis, most likely it is related to the fact that the PI3K/Akt pathway could be inhibited in BRAF or N-RAS mutant melanomas.

Recently another important protein, PDCD4, which functions as a tumor suppressor has been frequently studied and found a relationship between p53 and PDCD4, which seems to be involved in tumor progression also in melanoma (Yang et al., 2023). PDCD4 is associated with p53 mRNA and suppresses its translation. As a result of LE-127/2 treatment we found that at the beginning of the treatment, at 24 h, the level of PDCD4 dropped down, however, it was elevated at 48–72 h, particularly markedly increased in WM35 cells. Therefore, it is conceivable that PDCD4 activation by LE-127/2 suppresses the p53 dependent apoptosis in melanoma cells.

We also may consider that the increased level of PDCD4 contribute to the blockage of the PI3K/Akt apoptotic signaling pathway and with this effect, it gives even more space to the autophagy inducing effect of the compound LE 127 / 2 (Ferris, 2022).

Finally, the activity of HO-1 induces cytoprotection which can elicit different responses in cancer cells, and it is known some *C.sativa* derivatives induce the synthesis of HO-1, promoting autophagy and cell death (Cardile et al., 2023). According to our data, HO-1 was not affected by LE-127/2, therefore, cell death in examined melanoma cells was probably not mediated by HO-1 induction.

We could emphasize the fact that autophagy has been described to play crucial role in the combating resistances toward targeted therapies in melanoma. For instance, Verykiou et al., published that autophagy has a tumor-suppressor function, therefore, activation of autophagy by cannabinoids has been shown to induce cytotoxicity of melanoma, which may potentially overcome the resistance of melanoma to BRAF/MEK inhibition (Verykiou et al., 2019). In summary, based on the literature and our results, it can be assumed that CBG, and its synthetic derivatives, including LE-127/2 may function as autophagy modulatory drug, potentially contributing to overcome drug resistance in melanoma (Verykiou et al., 2019). In addition, among other derivatives of cannabis, CBG contributed to inhibition of cancer stem cells (CSC) self-renewal, thus overcoming drug resistance, presumably, LE-127/2 may have a similar effect on the development of resistance (Al Hmada et al., 2024).

The precise mechanism of LE-127/2 most probably could be revealed only in specific microenvironment. Compound LE-127/2 may activate different receptors at the same time, however, to clarify this it is necessary to carry out studies using agonists and antagonists, which we will attempt in the near future.

5. Conclusions

The results of the present study show that the investigated new compound, LE-127/2, is effectively reduced proliferation of all three examined melanoma cell lines at a concentration of 20 micromoles.

Low cytotoxicity of LE-127/2 on HaCaT cells raises that LE-127/2 probably might be a potential drug candidate with possibly less untoward effects and toxicity, therefore, the application of LE-127/2 may offer a new approach to overcoming drug resistance in melanoma cells, however, further in vitro and in vivo studies are required to explore the properties and the action mechanism of this new drug candidate.

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CRediT authorship contribution statement

Ágnes Tósaki: Writing – original draft, Software, Methodology, Data curation. **Zsuzsanna Szabó:** Writing – original draft, Methodology, Conceptualization. **József Király:** Visualization, Software, Methodology, Investigation, Data curation. **Eszter Boglárka Lőrincz:** Data curation. **Virág Vass:** Software, Data curation. **Bence Tánczos:** Software, Formal analysis. **Ilona Bereczki:** Funding acquisition, Data curation, Conceptualization. **Pál Herczegh:** Supervision, Methodology. **Éva Remenyik:** Writing – original draft, Supervision. **Árpád Tósaki:** Writing – original draft, Supervision, Funding acquisition. **Erzsébet Szabó:** Writing – original draft, Supervision, Methodology, Data curation, Conceptualization.

Declaration of competing interest

All authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106920.

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