SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of autophagic mechanisms in pathological conditions and their therapeutic implications

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The PhD Defense takes place at the Lecture Hall of the Department of Internal Medicine Building ”A”, Faculty of Medicine, University of Debrecen on 2nd of March 2026 at 13:00.

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1. Introduction

Cardiovascular and oncological diseases remain among the leading causes of mortality worldwide, including Europe, the United States, and Hungary. With increasing life expectancy, their prevention, early detection, and treatment represent major clinical and societal challenges. Despite distinct clinical presentations, both share fundamental pathophysiological mechanisms such as chronic inflammation, oxidative stress, obesity, unhealthy lifestyle, and genetic predispositions.

At the cellular level, autophagy and apoptosis are central regulators. Autophagy preserves cellular homeostasis by removing damaged proteins and organelles, supporting cardiac function, while its dysregulation contributes to pathology. In cancer, autophagy may act as a tumor suppressor or, under stress, promote survival and therapeutic resistance.

Despite advances in prevention, diagnosis, and treatment, these diseases still account for the highest global mortality. In Hungary, mortality rates have shown an upward trend. Modern healthcare must therefore balance effective therapy with cost-efficient prevention, which may reduce expenditure by up to 100-fold compared with long-term management of advanced chronic illness.

Cardiovascular and oncological pathologies are interconnected through overlapping risk factors and treatment-related toxicities, giving rise to cardio-oncology, a field addressing therapy-induced cardiotoxicity through integrated clinical and research efforts.

Cutaneous melanoma is among the most aggressive skin cancers, with high metastatic potential and poor survival. Surgery, radiotherapy, and chemotherapy remain standard, but targeted therapies have advanced systemic management. Nevertheless, prognosis in advanced melanoma is still poor. Natural bioactive compounds, including flavonoids, saponins, and cannabinoids, are emerging as potential adjunct therapies. Notably, cannabigerol (CBG), a non-psychotropic cannabinoid, has demonstrated anticancer activity in melanoma models. This thesis emphasizes in vitro studies of melanoma cells, focusing on a novel synthetic CBG derivative that may overcome current therapeutic limitations.

In cardiovascular disease, pathological cardiomyocyte hypertrophy is central, associated with myocardial infarction, ischemia-reperfusion injury, and post-infarction dysfunction. The thesis also examines endothelin-1-induced hypertrophy and β-estradiol-modulated heme oxygenase-1 (HO-1) expression in experimental models.

Overall, this work highlights a holistic approach to cancer and cardiovascular disease management, integrating pharmacological innovation with lifestyle and non-pharmacological strategies for effective prevention and therapy.

2. Aims

Cancer and cardiovascular diseases are major civilization-related disorders driven by complex molecular mechanisms. This thesis investigates two seemingly distinct, yet interconnected, pathological processes using in vitro and in vivo models.

The first part focuses on the antitumor activity of a newly synthesized cannabigerol (CBG) derivative, LE-127/2, in human melanoma cell lines. Natural CBG is a nonpsychotropic phytocannabinoid with reported anticancer effects but limited therapeutic potential due to poor solubility and bioavailability. Synthetic derivatives, including LE-127/2, overcome these limitations, enabling improved in vitro evaluation and potentially enhanced efficacy.

This study aimed to assess how LE-127/2 affects melanoma cell proliferation and viability, whether it induces apoptosis or autophagy, and how classical autophagy markers (LC3-I/II, Beclin-1, p62/SQSTM1) are modulated. Mapping the interplay between apoptosis and autophagy provides insights into the molecular mechanisms of CBG derivatives and may identify novel prognostic or therapeutic targets.

The second part addresses ET-1–induced cardiac hypertrophy. ET-1 is a potent vasoconstrictor and pro-inflammatory peptide that contributes to myocardial infarction, ischemia–reperfusion injury, and chronic heart failure. We focused on heme oxygenase-1 (HO-1), a cytoprotective enzyme whose expression decreases under cardiac stress.

Key objectives included determining the effects of ET-1 on cell size, viability, and HO-1 levels, assessing whether β-estradiol (β-E) can counteract hypertrophic changes via HO-1 modulation, and exploring the therapeutic potential of targeted HO-1 induction. This approach may provide a novel strategy for managing cardiac hypertrophy and other chronic inflammatory conditions.

Overall, the study aims to elucidate the cellular mechanisms underlying cancer and cardiovascular disease pathogenesis. Investigating LE-127/2's antitumor effects and HO-1 regulation highlights promising avenues for identifying new therapeutic targets and advancing treatment strategies for civilization-related diseases.

3. Materials and Methods I.

3.1.Chemicals

Cannabigerol (CBG; CBDepot s.r.o., Teplice, Czech Republic) was used as the parent compound. LE-127/2, a bis-N-butyl-dihydro-1,3-oxazine derivative of CBG, was synthesized via a Mannich-type reaction of CBG with n-butylamine and formaldehyde.

Vemurafenib (PLX4032, Zelboraf; B-Raf inhibitor, >98% purity) was purchased from Merck (Darmstadt, Germany). Stock solutions were prepared in DMSO (Sigma-Aldrich, St. Louis, USA) and stored at -20 °C. For experiments, stocks were diluted in culture medium to final concentrations of 1.25-80 μM.

3.2.Cell Cultures

Human melanoma cell lines WM35, A2058 (CRL-3601), and WM3000 were used. Nonmalignant HaCaT keratinocytes were used for cytotoxicity assays. Cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere. A2058 and WM35 carry BRAF mutations, while WM3000 harbors N-RAS Q61R mutation.

3.3.LDH Cytotoxicity Assay

HaCaT cells were seeded at 6 × 10^3 cells/well in 96-well plates and treated with LE-127/2 or CBG (1.25-80 μM) for 24-72 h. LDH release into the medium was measured using the CyQUANT™ LDH Assay Kit (Invitrogen) at 490 nm.

3.4.Cell Proliferation Assay

Melanoma cells were seeded at 6 × 10^3 cells/well, treated with LE-127/2, CBG, or vemurafenib (2.5-80 μM), and proliferation was measured using the CellTiter-Blue® assay (Promega). Fluorescence was read at 560/590 nm (excitation/emission) using a Fluostar OPTIMA reader.

3.5.Colony Formation Assay (Clonogenic cell survival assay)

Cells were seeded at 6×10^3 cells/well in 6-well plates and treated with LE-127/2 (5–80 μM). Media was refreshed every third day. After 14 days, colonies were fixed in methanol:acetic acid (3:1), stained with 0.1% crystal violet, and quantified in 2% SDS at 570 nm.

3.6. Protein Expression Analysis

3.6.1. Protein Isolation

Cells were lysed in M-PER buffer with protease/phosphatase inhibitors on ice for 30 min, centrifuged (20,000 rpm, 4 °C, 20 min), and protein concentration determined by BCA assay (Thermo Fisher).

3.6.2. Autophagy Array

Autophagy-related proteins were analyzed using Human Autophagy Array (Assaygene). Cells were treated with 20 μM LE-127/2 for 48 h, proteins isolated, and experiments using arrays were performed according to manufacturer's protocol. Signals were detected using ChemiDoc Imaging System (Bio-Rad).

3.6.3. Western Blot

Proteins (40 μg) were separated on 12% SDS-PAGE, transferred to PVDF membranes, blocked in 5% milk/TBST, and incubated overnight with primary antibodies against LC3I/II, p62, Beclin-1, Atg12, caspase-3, Tom20, HO-1, p53, PDCD-4, Bax, Bcl-2, PARP (1:1000). HPRT was used as loading control. HRP-conjugated secondary antibodies were applied and signals detected by Clarity ECL (Bio-Rad). Band intensity was quantified and normalized to HPRT. All experiments were performed in triplicate.

3.6.4. Immunofluorescence staining

Melanoma cells on coverslips were treated with 20 μM LE-127/2 for 48 h, fixed in methanol, permeabilized with 0.1% Triton X-100, incubated with LC3 primary antibody (1:100) overnight, and Alexa-488 secondary antibody (1:1000) for 1 h. Nuclei were stained with 0.5 μg/mL DAPI and cells visualized under an inverted fluorescence microscope (Carl Zeiss).

3.7. Statistical Analysis

All experiments were performed in triplicate. Data were analyzed using unpaired Student's t-test in GraphPad Prism 5.01. Significance was set at $*p<0.05$.

4. Materials and Methods II.

4.1.Cell Culture of H9c2 cells

Rat H9c2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. For passaging, remaining medium was removed, cells were washed with PBS (pH 7.4), and detached with trypsin-EDTA for 15 min. Cells were collected in suspension, centrifuged at 1100 rpm for 6 min at 26 °C, and resuspended in fresh medium with serum. Cells were plated at approximately 100,000 cells/mL for further experiments.

4.2.In Vitro Hypertrophy Induction

To induce hypertrophy, H9c2 cells were treated with endothelin-1 (ET-1, E7764, Sigma). The potential protective effect of β-estradiol (β-E, Sigma) was evaluated by pre-treating cells prior to ET-1 exposure. Cells were switched from serum-containing medium to serum-free DMEM with 1% antibiotics and incubated for 24 h at 37 °C, 5% CO_2. ET-1 was then added at concentrations of 100 nM, 1000 nM, and 10,000 nM. For the 1000 nM ET-1 group, cells were pretreated with 200 nM of β-estradiol, 6 h prior to ET-1 addition. Treatments were repeated for two consecutive days, after which RNA and protein were isolated.

4.3.Cell Morphology Analysis

For morphological analysis, H9c2 cells were seeded onto 24-well plates at ~8,000 cells/well. Cells were treated as described, then fixed with 4% formalin for 15 min and permeabilized with 0.1% Triton-X for 15 min. The cytoskeleton was stained with FITC-conjugated phalloidin, and nuclei were stained with DAPI. Images were captured using a Zeiss Axio Scope.A1 fluorescence microscope, and cell area was measured using ZEN 2012 software, analyzing 200 cells per group, reported in μm^2.

4.4.Cell Viability Assay (MTT)

Cell viability was assessed after ET-1 and/or β-E treatment using the MTT assay. H9c2 cells were plated in 96-well plates at ~2,500 cells/well in serum-free medium, treated with ET-1 and selected groups with β-E. After 48 h, MTT solution (5 mg/mL in PBS) was added and incubated for 2 h at 37 °C. Formazan crystals formed by viable cells were dissolved in isopropanol for 30 min at 37 °C, and absorbance was measured at 570 nm and 690 nm using a FLUOSTAR Optima spectrophotometer.

4.5.Statistical Analysis

Results are presented as mean ± SEM. Statistical analysis was performed using Student's t-test and two-way ANOVA in GraphPad Prism 5. Differences were considered statistically significant at *$p<0.05$.

5. Results I.

5.1.Investigation of the cytotoxicity of LE-127/2 and the parent compound CBG using LDH-based assay

LDH is the most commonly applied marker for testing the cytotoxic effects of novel potential drug candidates. Plasma membrane damage leads to the release of LDH into the cell culture medium. Accordingly, extracellular LDH content can be quantified through a coupled enzymatic reaction, in which LDH converts lactate to pyruvate while reducing NAD^+ to NADH.

We performed three independent experiments, with each LE-127/2 and CBG concentration tested in triplicates. Notably, CBG already significantly reduced HaCaT cell growth at a concentration of 10 μM, indicating that CBG exerts toxic effects on normal cells (keratinocytes) even at relatively low concentrations. The drastic decrease in cell growth was observed within 72 hours after CBG treatment. In contrast, even after treatment with 80 μM LE-127/2, no significant cell damage was detected in HaCaT cells, and microscopic morphological observations also did not reveal major alterations in the non-tumorigenic HaCaT cells treated with LE-127/2. The cytotoxic effect of LE-127/2 was also tested in other nonmalignant cell types (H9c2 and NIH-3T3 fibroblast cells). In H9c2 cells, significant cell death was observed only after 40 μM LE-127/2 treatment, 48-72 hours following exposure, whereas CBG exhibited toxicity already at 20 μM in these cells. Interestingly, fibroblast cells showed the lowest sensitivity to LE-127/2 treatment. Similar to HaCaT cells, fibroblasts only exhibited sensitivity after treatment with 80 μM LE-127/2, and no significant cytotoxic effect was detected up to 48 hours. In contrast, CBG was toxic to fibroblasts already at 20 μM concentration.

5.2.Comparison of the cytotoxic effects of LE-127/2, CBG, and Vemurafenib on melanoma cell lines

To examine the cytotoxic effects of LE-127/2, three different human cutaneous melanoma cell lines (WM35, A2058, and WM3000) were used. The effects of LE-127/2 were compared with those of its parent compound CBG, and vemurafenib, used in as an active clinical therapeutic agent. For all experiments, the compounds were administered to melanoma cells in

increasing concentrations (2.5 μM to 80 μM). Cell proliferation activity was monitored for 24 to 72 hours using the CellTiter-Blue Assay.

Proliferative activity was determined based on fluorescence values, and the results were normalized to the untreated control (DMSO). According to the results, vemurafenib inhibited the proliferation of melanoma cells more effectively than either LE-127/2 or CBG, with a significant reduction already observed at 2.5 μM. The highest efficacy was detected in WM35 cells, where vemurafenib significantly inhibited proliferation within 24 hours, resulting in the death of nearly 90% of the cells.

The mother compound of CBG also proved to be more effective than its newly synthesized derivative, LE-127/2. It exerted similar inhibitory effects on the proliferation of all tested cell lines. At 5 μM, CBG significantly reduced cell proliferation, while in the WM3000 cell line, already 2.5 μM concentration exhibited significant inhibitory activity.

Compared to vemurafenib and CBG, LE-127/2 demonstrated weaker antiproliferative activity in all melanoma cell lines tested. Significant inhibition by LE-127/2 was only measurable at 20 μM across all three lines, although some effects were already detectable at 24 and 48 hours. However, statistically significant differences *($p<0.05$) were only observed after 72 hours. An exception was the A2058 cell line, which appeared more sensitive to LE-127/2 than the other two lines. Accordingly, the effect of LE-127/2 on melanoma cell lines was found to be both dose- and time-dependent manners.

When comparing the efficacy of vemurafenib, CBG, and LE-127/2, it was established that at 20 μM all three compounds significantly inhibited proliferation in all melanoma cell lines tested. Importantly, while the effects of LE-127/2 only became detectable at higher concentrations compared to vemurafenib and CBG, cytotoxic effects on nonmalignant HaCaT cells were only observed at 80 μM, suggesting that healthy, non-cancerous cells are considerably less sensitive to LE-127/2 at lower concentrations.

5.3.Inhibition of colony formation

Clonogenic survival assays were used to investigate the effect of LE-127/2 on the colony forming capacity of human cutaneous melanoma cell lines (WM35, A2058, and WM3000). Based on the results of proliferation assays, cells were treated with increasing concentrations of LE-127/2 (5-80 μM) for two weeks, incubated at 37 °C under 5% CO_2 and 95% air, and subsequently subjected to colony formation assays.

WM3000 and A2058 cells displayed similar colony-forming abilities, and in both cells, LE-127/2 significantly reduced clonogenic capacity already at 10-20 μM. However, LE-127/2

inhibited colony formation more effectively in A2058 and WM3000 cells at 10 μM compared to WM35 cells. The number of colonies in all tested lines was reduced approximately 2-3-fold upon treatment with 20 μM LE-127/2 compared to untreated controls. The results of the clonogenic assays are consistent with proliferation data, showing that LE-127/2 exerted a comparable, intense inhibitory effect on both cell growth and colony formation at 20 μM, with dose- and time-dependent activity against human melanoma cells.

5.4. Protein expression analysis

The three main types of programmed cell death are apoptosis, autophagy, and necrosis. The interplay and transitions between these processes are closely linked to the pathomechanisms of numerous human diseases. Both autophagy and apoptosis play pivotal roles in melanoma development and metastasis formation. Therefore, our aim was to explore the effects of LE-127/2 on the expression of key proteins associated with autophagy and apoptosis.

5.4.1. Evaluation of the Human Autophagy Array results

To monitor changes in protein expression following LE-127/2 treatment, we used a Human Autophagy Array with protein samples isolated from cells treated with 20 μM LE-127/2 for 48 hours (for details, see the "Materials and Methods" section). The specific antigens on the array were incubated with the proteins extracted from cells, and subsequent steps followed the manufacturer's protocol.

The spot intensities corresponding to antigen-specific antibodies were proportional to the relative expression levels of the given antigen in the tested samples. Accordingly, by comparing arrays of treated and untreated cells, we identified relative changes in protein expression. LC3A showed primarily perinuclear and nuclear localization, while LC3B was distributed throughout the cytoplasm and also appeared in nucleolar regions.

Array analysis revealed that expression of LC-3 (LC3A and LC3B), Beclin-1, and Sequestosome 1/SQSTM1/p62 (p62) protein were increased in all tested melanoma cell lines following LE-127/2 treatment compared to untreated controls. Notably, Beclin-1 expression was particularly elevated in A2058 cells, exceeding the levels observed in the other two cell lines.

5.4.2. Confirmation of LC3, p62, Beclin-1, and Atg12 expression by Western blot

Results clearly confirm that LC-3 expression levels were modified by treatment. Expression of LC-3II was elevated in WM35 and A2058 melanoma cells after 24 hours, while in the WM3000 line, this change appeared only after 48 hours of treatment. This suggests that

the cytosolic form of LC-3 (LC-3I) conjugates with phosphatidylethanolamine to form LC-3II, whose increased levels serve as a hallmark of autophagy activation. These findings imply that LE-127/2 can induce autophagy in melanoma cell lines within 24-48 hours. The increase in LC-3 expression detected by Western blot after 48 hours of LE-127/2 treatment was consistent with array results showing elevated LC-3II levels in treated cells.

Immunofluorescent labeling further confirmed these findings, as cells treated with LE-127/2 exhibited more intense green fluorescence in the cytoplasm than untreated controls. This is likely due to the appearance of "LC-3 puncta" (punctate structures), characteristic of autophagy activation.

5.4.3. Detection of other autophagy-related proteins following LE-127/2 treatment

p62 expression markedly increased within 24 hours of LE-127/2 treatment in all tested cell lines, then gradually decreased in WM35 and WM3000 cells at 48 and 72 hours, respectively. In contrast, A2058 cells exhibited a continuous increase in p62 levels throughout the 72-hour period.

Beclin-1 expression showed only mild, non-significant increases during treatment, except in the A2058 line, where expression rose significantly after 72 hours, consistent with array results. Atg12 expression decreased 24 hours after LE-127/2 treatment, followed by gradual increases at 48-72 hours, matching array data.

We also examined Tom20 expression, known to play a key role in melanoma cell death. Tom20 mediates mitochondrial signaling of reactive oxygen species (ROS) and is central to mitophagy. LE-127/2 treatment significantly reduced Tom20 expression after 24 hours, but levels returned to baseline after 48 and 72 hours in WM35 and A2058 cells. In WM3000, however, Tom20 expression decreased in a time-dependent manner, remaining significantly lower than control levels.

It is noteworthy that across the 72-hour treatment period, expression of autophagy proteins did not show a clear trend but instead fluctuated, which pattern was reflected in both array and Western blot analyses. Protein Array data also revealed changes in additional autophagy-related proteins beyond those confirmed by Western blot, though detailed analysis exceeds the scope of this study.

5.4.4. Effect of LE-127/2 treatment on HO-1 expression levels

HO-1 overexpression promotes chemoresistance and activates protective autophagy mechanisms. Interestingly, while HO-1 upregulation in melanoma cells supports tumor

progression, it may also reduce tumor growth and metastatic potential. Due to this dual role, we aimed to investigate how treatment with 20 μM LE-127/2 affects HO-1 expression in WM35, A2058, and WM3000 melanoma cell lines. Western blot analysis revealed no significant differences between LE-127/2-treated and untreated controls. Only a slight increase in HO-1 expression was observed after 72 hours of the treatment.

5.4.5. Expression of p53, Bax, Bcl-2, PDCD4, and PTEN

The tumor suppressor protein p53 plays a central role in the molecular network regulating cell death. Its activation halts tumor cell growth through both p53-dependent and p53-independent apoptotic pathways. Quantification of p53 protein levels by Western blot revealed differential expression among the three melanoma lines. Following LE-127/2 treatment, p53 expression decreased, but this was not statistically significant.

The expression of bax and bcl-2 proteins increased compared to controls, but a statistically significant rise was observed only for bax protein, particularly after 48 hours of treatment.

We also examined PTEN protein expression, noting that the A2058 line does not express PTEN. The lack of which likely contributes to the enhanced proliferative capacity and more aggressive phenotype of these cells compared to WM35 and WM3000.

Since PDCD4 is a tumor suppressor known to influence survival in metastatic melanoma patients, we also analyzed its expression under LE-127/2 treatment. After 24 hours, PDCD4 levels significantly decreased, but returned to baseline after 48-72 hours. Interestingly, in the WM35 cells PDCD4 expression increased nearly 1.5-fold compared to controls after 48 hours.

5.4.6. PARP activation induced by LE-127/2

In all three melanoma cell lines treated with LE-127/2, increased expression of cleaved PARP was observed compared to controls. LE-127/2 treatment induced cleavage of full-length PARP (116 kDa) into its cleaved form (89 kDa), with levels rising significantly in a time-dependent manner. Moreover, cell line-specific differences in PARP activation were observed, with the earliest and strongest activation detected in WM35 cells.

Caspase-3 expression showed significant changes 24-48 hours after the treatment with LE-127/2 in WM35 and A2058 cells, whereas in WM3000 cells no significant changes were detected.

6. Results II.

6.1. Changes in cell surface upon treatment

Our results demonstrate that application of endothelin (ET-1) in the dose range of 100 nM to 10,000 nM caused a significant increase in cell surface area, indicative of edema formation in this cell group. Pretreatment with β-estradiol (β-E) at 200 nM prevented the ET-1 (1000 nM) -induced increase in cell surface. Ethanol (E) was used as a solvent in all experimental groups, except for the control group.

6.2. Cell survival (%) in H9c2 cardiomyoblasts

No reduction in cell viability (%) was observed in H9c2 cells compared to controls when ethanol (E, 0.01%) was used as a solvent for the compounds and in combination with β-estradiol (β-E) treatment. Ethanol and β-estradiol alone, at the applied concentrations, did not alter cell viability. In contrast, increasing concentrations of endothelin-1 (ET-1, 100 nM to 10,000 nM) resulted in a significant decrease in cell survival. However, in the experiment where 200 nM β-E was co-administered with 1000 nM ET-1, cell survival increased significantly. Thus, β-E counteracted the cytotoxic (cell-damaging, lethal) effect of 1000 nM ET-1.

6.3. In vitro analysis – Heme oxygenase-1 (HO-1) protein and Western blot in H9c2 rat cardiomyoblasts

The results showed that co-treatment with 1000 nM ET-1 and 0.01% ethanol (E) significantly decreased HO-1 levels ($p<0.05$). In contrast, cotreatment with 200 nM β-estradiol (β-E) and 0.01% ethanol (E) did not cause a significant decrease in HO-1 levels. In experiments where 200 nM β-estradiol (β-E), 1000 nM ET-1, and 0.01% ethanol (E) were applied together in the culture medium, a significant increase in HO-1 levels was observed ($+p<0.05$) compared to the group without β-E. These results indicate that 200 nM β-estradiol treatment prevented the ET-1-induced reduction in HO-1 levels, contributing to enhanced cell survival.

6.4. In vivo analysis – Plasma HO-1 levels and Western blot in rat cardiomyoblast-derived heart tissue

Based on the in vitro findings, the effects of 1000 ng/kg ET-1 and 200 ng/kg β-estradiol (β-E) were investigated in in vivo rat heart experiments. Results showed that 1000 ng/kg ET-1 significantly decreased plasma HO-1 protein levels compared to the control group, whereas β-E treatment alone did not produce significant changes. Co-administration of 200 ng/kg β-E prevented the ET-1-induced reduction in plasma HO-1 levels. Similar results were observed using Western blot analysis of HO-1 expression in heart tissue. Therefore, the elevated HO-1

levels appear to express protection in cardiomyoblasts, consistent with the in vitro observations in H9c2 cells. Taken together, these findings suggest that the protective effects are likely mediated through modulation of the HO-1 signaling pathway.

7. New findings

Effect of LE-127/2, a CBG derivative, on human cutaneous melanoma cell lines:

- Exhibited antiproliferative effects on melanoma cell lines independent of mutation status.
- Low cytotoxicity was observed in normal keratinocytes and cardiomyoblasts, indicating a favorable side effect profile for potential clinical application.
- Effectively inhibited colony formation, suggesting anti-metastatic potential.

New results regarding the presumed mechanism of action of LE-127/2:

- Induction of autophagy (increased levels of LC-3II, p62, Atg12).
- Activation of apoptosis involving caspase-3/7–PARP activation, with the PI3K/Akt pathway playing a dominant role.
- Time-dependent increase in PDCD4 expression in response to LE-127/2.
- HO-1 levels did not change significantly, remaining nearly unchanged, consistently not involved in the cellular response and not induced by LE-127/2.

Findings from cardiovascular research:

- ET-1 decreases HO-1 activity, resulting in cell damage and hypertrophy.
- β-estradiol pretreatment contributes to HO-1 elevation, protects cells, and mitigates ET-1– induced damage.
- β-E pretreatment significantly inhibited cell surface enlargement in ET-1 treated cell groups.
- ET-1– induced hypertrophy was reduced by β-estradiol treatment, confirmed in vivo.
- ET-1–induced cell damage leading to hypertrophy and cell death can potentially be prevented by β-estradiol (β-E) treatment, in which the HO-1 enzyme system plays a crucial role.

- HO-1 has a dual role: protective in the cardiovascular system, but may support tumor cell survival in cancers.
- It was confirmed that changes in HO-1 expression levels depend on the applied compound.

8. Summary

Cardiovascular and oncological diseases, classified among civilization-related disorders, represent the leading causes of mortality worldwide. The development of these two major and prevalent disease groups involves several overlapping molecular mechanisms, the recognition of which may facilitate the identification of more effective therapeutic strategies and thereby improve clinical outcomes.

In the management of cutaneous melanoma, surgical excision remains the primary approach; however, immunotherapies and targeted therapies have emerged as dominant treatment modalities. The BRAF V600E mutation, detected in more than half of melanoma patients, led to the introduction of vemurafenib, a selective BRAF inhibitor, as a first-line targeted therapy. Vemurafenib effectively suppresses the MAPK signaling pathway and prolongs survival; nevertheless, its clinical utility is limited by the rapid onset of resistance and severe toxic side effects. These limitations underscore the importance of exploring alternative agents, including naturally derived compounds such as phytocannabinoids and their synthetic analogues. Cannabigerol (CBG), although less well studied than other cannabinoids, has demonstrated antineoplastic properties; however, its poor aqueous solubility and limited bioavailability restrict therapeutic application. To overcome these barriers, we investigated the effects of a synthetic nitrogen-containing CBG derivative, LE-127/2, in human cutaneous melanoma cells. Owing to its improved solubility and bioavailability, LE-127/2 may represent a promising candidate for therapeutic development.

Unlike the mutation-specific action of vemurafenib, LE-127/2 exerted moderate but mutation-independent antiproliferative effects across melanoma cell lines, while displaying minimal toxicity toward non-malignant cells. Mechanistically, LE-127/2 induced autophagy, as evidenced by the upregulation of LC-3II, p62, and Atg12, followed by the activation of apoptotic pathways, including caspase-3/7 activation, PARP cleavage, and Bax upregulation. Notably, p53 expression remained unchanged, suggesting that the PI3K/Akt pathway may serve as a central mediator of LE-127/2 activity.

Clonogenic survival assays revealed that LE-127/2 significantly impaired colony formation, indicating potential efficacy against metastatic progression. Furthermore, LE-127/2 treatment did not alter HO-1 expression, implying that its mechanism of action is independent of this enzyme. Collectively, these findings identify LE-127/2 as a potential therapeutic candidate for overcoming drug resistance and for application in combination therapies, although further in vitro and in vivo investigations are required to fully elucidate its mechanism of action.

Our previous studies addressed the cytoprotective role of the heme oxygenase-1 (HO-1) enzyme. HO-1 possesses antioxidant, anti-inflammatory, and cytoprotective properties, which are of particular importance within the cardiovascular system. Endothelin-1 (ET-1), a 21-amino acid potent vasoconstrictor peptide, contributes to the development of hypertrophy, oxidative stress, and cell death. Experimental data demonstrated that ET-1 downregulates HO-1 activity and expression, thereby exacerbating cellular damage.

Pretreatment with β-estradiol (β-E) was shown to attenuate ET-1–induced cytotoxic effects, preventing cellular swelling, improving survival, and maintaining HO-1 levels. This hormone-mediated protective effect is, at least in part, attributable to the activation of HO-1–dependent defense mechanisms. Combined treatment with ET-1 and β-E prevented the decline of HO-1 expression, thereby alleviating pathological alterations. In vivo, β-E administration reduced ET-1-induced cardiac hypertrophy, an effect likely related to modulation of mTOR activity as well as regulation of oxidative stress and autophagy.

Cumulative evidence indicates that both autophagy and apoptosis play critical roles in the pathophysiology of cardiovascular diseases and cancer. HO-1 demonstrates a dual function: while its upregulation exerts protective effects in the cardiovascular system, in malignancies it may enhance tumor cell survival. A deeper understanding of these context-dependent mechanisms is essential for the rational design and selection of therapeutic strategies, ultimately determining the success of clinical interventions.

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Candidate: Ágnes Tósaki
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List of publications related to the dissertation

1. **Tósaki, Á.**, Szabó, Z., Király, J., Lőrincz, E. B., Vass, V., Tánczos, B., Bereczki, I., Herczegh, P., Remenyik, É., Tósaki, Á., Szabó, E.: A new cannabigerol derivative, LE-127/2, induces autophagy mediated cell death in human cutaneous melanoma cells.
Eur. J. Pharm. Sci. 203, 1-15, 2024.
DOI: http://dx.doi.org/10.1016/j.ejps.2024.106920
IF: 4.7

2. Barta, T., **Tósaki, Á.**, Haines, D. D., Balla, G., Lekli, I., Tósaki, Á.: Endothelin-1-induced hypertrophic alterations and heme oxygenase-1 expression in cardiomyoblasts are counteracted by beta estradiol: in vitro and in vivo studies.
Naunyn-Schmiedebergs Arch. Pharmacol. 391 (4), 371-383, 2018.
DOI: http://dx.doi.org/10.1007/s00210-018-1462-z
IF: 2.058

List of other publications

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Total IF of journals (all publications): 17,589
Total IF of journals (publications related to the dissertation): 6,758

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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