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SIMU SEBASTIAN CLAUDIU



PHD THESIS

**THE IMPACT OF FEMALE SEX HORMONES ON SKIN AND
BREAST CANCER DEVELOPMENT. EXPERIMENTAL
MODELS**

ABSTRACT

PhD Supervisors

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Introduction

Cancer refers to several diseases whose main characteristics are abnormal cell growth and multiplication, that persists after the removal of the original trigger, forming a mass called a tumor. These tumors can be either benign (that are unable to spread from their original location) or malign (that can invade other tissues). Malign tumors are referred to as cancers and based on the type of cells they originated from, can be split into several categories: carcinomas, sarcomas, blastomas, germ cell tumors, lymphomas and leukemias.

Skin is one of the components of the cutaneous organ, together with the cutaneous appendages. Skin is the largest organ in the body, covering its external surface, and serves a number of functions, including physical, chemical and mechanical barrier, sensory organ for a number of stimuli, secretion, vitamin D synthesis and thermoregulation. The skin is a complex organ, composed of three layers, with different structures and roles.

The incidence of skin cancers has increased in recent years. Their main cause is exposure to ultraviolet radiation, although hereditary factors play an important role in increasing the carcinogenic risk. Skin cancers can be classified as non-melanoma skin cancers or malignant melanomas, each main category having a number of subtypes.

Breast cancer represents the most frequent form of cancer diagnosed in women, and the second leading cause of cancer related mortality in women. Breast cancers can be split into in situ and invasive carcinomas, which can be further split into several subcategories, based on their original type of tissue.

Estradiol and progesterone are the two most significant steroid hormones in adult women. Estrogen's effects at the tissular level depends the receptors they bind to. There are two types of estrogen receptors, ER α and ER β , with different genetic profiles but equal affinities for estradiol but the effects of their activation is different and depends on the signaling ratio between the two subtypes.

Estrogen has many physiological functions, but elevated hormonal levels have been linked to the with increased incidence of some cancers, especially breast and endometrial cancers.

Ethinyl estradiol, a semi-synthetic exogenous estrogen, is widely used in oral contraceptives. In addition to the contraceptive action, it lowers testosterone levels and can

delay the progression of prostate cancer and has shown a protective effect against osteoporosis and breast cancer.

Progesterone is an endogenous sex hormone that plays an important role in the regulation of the menstrual cycle, fertility and embryogenesis.

Levonorgestrel is a synthetic progestogen used in combined oral contraceptives or as a single agent in emergency contraception.

Oral contraceptives represent the most common and safe form of reversible contraception and can be used to treat acne, control dysmenorrhea and reduce the risk of ovarian, colon and endometrial cancers. Despite their many benefits, oral contraceptives have a number of serious side effects, such as myocardial infarction and thromboembolisms.

Motivation of the study

The overall aim of my PhD thesis was to assess the influence of female sexual hormones on the evolution of hormone-dependent cancers.

To this end, I used synthetic variants of the two hormones that are commonly found in oral contraceptives, Ethinylestradiol, Levonorgestrel and a combination of the two on human and murine melanoma, and breast cancer cell lines. In addition, I assessed the thermal stability of Ethinylestradiol found in oral contraceptives compared its binding affinity to estrogen receptors through molecular docking, using the PyRx software.

Study 1. Thermal Degradation Process of Ethinylestradiol – Kinetic Study

Objective:

The main objective of this study was to perform an isoconversional kinetic study for Ethinylestradiol, as the literature data offered no information regarding the processes of heterogeneous decomposition for this compound.

Materials and methods:

The study was performed using Ethinylestradiol purchased from Sigma-Aldrich (St. Louise, MO, USA), without further purification.

FT-IR studies were performed using a Shimadzu Prestige-21 spectrometer (Duisburg, Germany), at 24 °C. The operating parameters set were: resolution of 4 cm⁻¹ within the spectral range of 400–4000 cm⁻¹, using KBr pellets.

Thermo-Analytical Investigations were performed to assess the stability of the samples, using a Netzsch STA 449 C instrument (Netzsch-Gerätebau GmbH, Selb, Germany), in the range of 20–500 °C, air atmosphere, at 2, 4, 6, 8, and 10 °C/min heating rates. Each sample

was weighed in aluminum crucibles and the analysis was performed under artificial air at a flow rate of 20 mL/min.

The kinetic study on the main decomposition process of Ethinylestradiol was carried out using the AKTS—Thermokinetics Software (AKTS AG TechnoArk, Siders, Switzerland) for the Friedman and Flynn-Wall-Ozawa methods, while the classical Kissinger method was employed using a template file created by our research team.

Results:

An exploratory method for characterizing EE was FTIR spectroscopy.

In the spectral region $3650\text{--}3100\text{ cm}^{-1}$, the stretching vibrations $\nu(\text{O-H})$ for both OH moieties from the EE structure are seen as a broad band, suggesting the strong H-bonding between the molecules in solid state, overlapped with the sharp band of superficially adsorbed water.

The bands at 2972.31 , 2935.66 , and 2866.22 cm^{-1} reflect the symmetric and asymmetric stretching vibrations for different C-H bonds, specifically (C-H), including the ones from CH_3 moiety and CH_2 ones.

The FTIR spectra of compounds containing -CC- moieties, such as the ethinyl moiety of the compound, exhibits characteristic bands that are highly individualized at 2357.01 and 2322.29 cm^{-1} . These bands are crisp and weak and are well defined. The bands from 1614.42 , 1585.49 , 1500.62 , 1471.69 , 1446.61 , and 1435.04 cm^{-1} , as well as for $\text{as}(\text{CH}_3)$ and $\text{as}(\text{C}=\text{C})$, are caused by symmetric C-C stretching (CH_2). The bands at 1373.32 , 1357.89 , 1298.09 , and 1286.52 cm^{-1} result from symmetric methyl bending, with the first two bands corresponding to the hydroxyls, (COH). The bands at 1255.66 and 1056.99 cm^{-1} are likely caused by (C-O) vibration, while the other bands from the fingerprint are caused by skeletal vibrations and by different combination bands.

EE is thermally stable up to a temperature of $71\text{ }^\circ\text{C}$, at which point a mass loss process starts and lasts until a temperature of $101\text{ }^\circ\text{C}$. The first step of this process is initiated by the elimination of the sample's superficially adsorbed water.

Anhydrous EE is stable up to a temperature of $177\text{ }^\circ\text{C}$, at which point a decomposition process starts and overlaps the melting of the compound. This suggests the presence of polymorphic form II rather than polymorphic form I, which melts at $146\text{ }^\circ\text{C}$. The primary decomposition of EE occurs between 187 and $324\text{ }^\circ\text{C}$, which is accompanied by an exothermic effect on the DSC curve with a peak at $287\text{ }^\circ\text{C}$. As the temperature rises, the overlapping thermal degradation process occurs, further complicating the thermoanalytical profile.

The kinetic analysis was carried out over processed DTG, obtained in dynamic air atmosphere for the following five heating rates β : 2, 4, 6, 8, and 10 °C/min. The main decomposition process of EE that was subjected to kinetic analysis is the one that takes place after the formation of anhydrous form. A preliminary kinetic study was realized using the Kissinger method, which assumes that the degree of conversion is a constant and non-dependent of the heating rate at the DTG peak.

For the main stage of EE degradation, the value for E_a (activation energy) is 107.91 kJ/mol.

The differential method of Friedman (Fr) and the integral method of Flynn–Wall–Ozawa (FWO), two isoconversional methods, were used to evaluate the values of E_a of the decomposition vs. conversion degree α . The decomposition process is shifted to higher temperatures due to thermal inertia of the sample, as the heating rate increases.

By comparing the results from the Kissinger kinetic method and the two isoconversional ones, the obtained values for the decomposition activation energies are in good agreement. The fitting of T_{max} points from the Kissinger method suggests that the degradative mechanism of EE under thermal stress is independent of the heating rate.

The isoconversional method of Friedman reveals a variation outside the $\pm 10\%$ limit, outside the medium value of E_a for EE solely at conversions superior to 85%. This variation is not observed in the case of the integral method of Flynn–Wall–Ozawa, even if the individual E_a values have the tendency to decrease with the advance of the reaction.

Study 2. Insights into the Behavior of Triple-Negative MDA-MB-231 Breast Carcinoma Cells Following the Treatment with 17 β -Ethinylestradiol and Levonorgestrel

Objective:

The objective of this study was to assess in vitro the impact of 17 β -Ethinylestradiol and Levonorgestrel on the proliferation and metastatic behavior of the highly aggressive and invasive triple-negative breast cancer cell line MDA-MB-231.

Materials and methods:

17 β -Ethinylestradiol, Levonorgestrel, PBS, trypsin-EDTA solution, DMSO, FBS, penicillin/ streptomycin mixture, and MTT reagent were purchased from Sigma Aldrich, Merck KgaA. The cell culture medium, DMEM–ATCC® 30-2002™, was bought from ATCC . All reagents used in the present study were of analytical grade purity and for cell culture use.

Our study was performed using the MDA-MB-231 breast cancer cell line, which was acquired from ATCC, then cultured in their specific growth medium—DMEM supplemented with 10% FCS and 1% antibiotic mixture.

The cell viability assessment was performed by the means of the MTT assay, the absorbance values were measured at 570 and 630 nm using Cytation 5.

The potential impact of the hormonal treatment on MDA-MB-231 cells' morphology and confluence was evaluated using an Olympus IX73 inverted microscope. The analysis of the photos was performed using the cellSens Dimensions v.1.8. Software.

The Hoechst 33342 staining assay was performed to analyze the effects of EE, LNG, and EE-LNG at MDA-MB-231 cells' nuclear level after 24 h of treatment. The experimental protocol was applied according to manufacturer's recommendations.

The influence of oral contraceptives on the migratory character of the MDA-MB-231 cells was evaluated by the means of a scratch assay using the AutoScratch™ Wound Making Tool provided by BioTek® Instruments Inc., Winooski, VT, USA. The protocol was performed as recommended by the manufacturer.

In order to quantify the effect of the contraceptives in terms of cell migration, the difference between the initial and after 24 h wound widths was determined. The migration rate (%) was calculated according to a formula described previously by Felice et al.

To evaluate the impact of OCs on gene expression, a reverse-transcription polymerase chain reaction (RT-PCR) was performed.

Results:

In the present study, the MDA-MB-231 breast cancer cell line was exogenously treated with EE, LNG, and EE-LNG solutions in DMSO. Increasing concentrations (0.05-10 μ M) were tested for three-time intervals: 24, 48, and 72 h.

Although the 24 h results indicate a cytotoxic effect, a stimulatory effect has been noticed after the 48 h treatment. The effect was dose dependent. The cell viability percentages assessed after the 72 h treatment with EE-LNG were similar to control. However, a slight decrease was induced by EE-LNG 10 μ M.

The pictures of the MDA-MB-231 cells and nuclei taken after 24h of treatment suggest a loss of cell confluence after the treatment with EE, LNG, and EE-LNG solutions, as compared to the control (STP and TRX) and DMSO. The roundness and non-adherence of the cells noticed after the stimulation with OCs suggest cell death and are more prominent in the case of EE 10 μ M, LNG 0.05 μ M, and EE-LNG 0.05 μ M. As opposed to STP and TRX, the OCs induced no visible changes in the morphological aspect of the viable adherent cells.

After the stimulation with EE, LNG, and EE-LNG at 0.05 μ M and 10 μ M we noticed relevant changes to the aspect of the nuclei, such as nuclear fragmentation, membrane blebbing, and apoptotic bodies. No signs of necrosis were noticed after the cells' treatment with OCs.

To determine the impact of contraceptives on the migratory ability of MDA-MB-231 breast cancer cells, the scratch assay was applied, using two concentrations (0.05 μ M and 1 μ M) for every sample. Untreated cells (control) presented the highest migration rate (85.97%), followed by the cells treated with LNG 0.05 μ M (85.01%) and EE-LNG 1 μ M (81.67%). The most potent anti-migratory effect was noticed in the case of EE 0.05 μ M and 1 μ M with wound healing rates of 46.38% and 13.91%, respectively.

Significant inhibition of the wound healing rate was observed in the cells treated with LNG 1 μ M (70.73%) and EE-LNG 0.05 μ M (53.04%), respectively.

The changes in the expression of vimentin were analyzed via the RT-qPCR technique after a 48 h stimulation period with EE, LNG, and EE-LNG solution, using the lowest and the highest concentrations tested. OCs induced relevant changes in the expression of vimentin as follows: EE and LNG up-regulated vimentin mRNA expression, significant changes being noticed for EE 0.05 μ M and LNG 10 μ M. Although the differences between control and EE-LNG groups were not statistically significant, the association of EE with LNG decreased the gene expression at both concentrations.

Study 3. Binding Affinity of 17- β -ethinylestradiol to Normal and Mutant Types of Estrogen Receptors

Objective:

The aim this study was to compare the binding mode and affinity of EE2 to the ER α , some of its important mutant isoforms, and ER β , as this information could assist in understanding specific aspects regarding the connection between EE2 biological activity and certain ERs signaling correlated pathologies.

Materials and methods:

The protein structures corresponding to ER α and ER β were obtained from the RCSB Protein Data Bank. They were prepared as suitable targets for molecular docking using Autodock Tools 1.5.6.

Molecular docking was carried out with the PyRx software (Version 0.8) using Vina's scoring function. Molecules were docked in the estrogen ligand binding domain of each protein structure, using default docking parameters. Recorded scores for docked molecules were given as free binding energy values (kcal/mol). Ligand protein binding particularities were analyzed using Accelrys Discovery Studio 4.1 (Dassault Systemes Biovia).

Results:

The calculated binding energies (ΔG) of the two docked compounds are below the -6 kcal/mol threshold value suggested by Shityakov et al., which correlates with their estrogenic activity.

EE2 accommodations in both ER α and ER β binding domains are extremely similar to that of E2. In both cases the phenolic -OH is responsible for hydrogen bond (HB) formation with residues Glu353, Arg394; however, in the case of E2, an additional HB is formed between the alcoholic -OH and His524.

When superimposing the two docked molecules a topological similarity can be observed. Both molecules are oriented in a similar manner and the coplanarity of the two structures is relatively high.

The same features and similarities are present when the two molecules were docked in ER β . In both cases the phenolic -OH is responsible for HB formation with residues Glu305, Arg346 but, in the case of E2, an additional HB is formed between the alcoholic -OH and His475. The structure orientation similarity, between E2 and EE2 is present here as well.

Our results indicated a decrease in binding affinity (higher ΔG values) related to the mutated forms of ER α compared to the non-mutant structure, but the decrease is not significant enough to conclude that there is a drop in EE2 activity involving mutated ER α mutant types.

In mutant forms that adopt the specific antagonist binding conformation the usual binding mode of EE2 is significantly affected.

Study 4. Ethinylestradiol and Levonorgestrel as Active Agents in Normal Skin, and Pathological Conditions Induced by UVB Exposure: In Vitro and In Ovo Assessments
Objective:

In this study, we focused on the evaluation of the cytotoxic profile of EE, LNG, and their association (EE + LNG), with and without UVB irradiation, on healthy cell lines and tumor cell lines by in vitro and in vivo techniques.

Materials and methods:

EE and LNG were acquired from Sigma Aldrich (Munich, Germany).

The healthy cell lines we used were: HaCaT—immortalized human keratinocytes (ATCC, LGC Standards GmbH, Wesel, Germany), 1BR3—human skin fibroblast (90011801, ECACC General Collection, Salisbury, UK), HEMa—primary human epidermal melanocytes (ATCC, LGC Standards GmbH), JB6Cl41-5a—newborn mice epidermis (CRL-2010™, ATCC, LGC Standards GmbH).

The tumor cell lines were: A375—human melanoma (CRL-1619™, ATCC, LGC Standards GmbH) and B164A5—murine melanoma (94042254; Sigma-Aldrich Chemie GmbH, Munich, Germany).

The specific reagents for cell culture (DMEM, EMEM, Dermal Cell Basal Medium, and Adult Melanocyte Growth Kit) were purchased from ATCC (LGC Standards GmbH); non-essential amino acids, FBS, antibiotics mixture (penicillin/streptomycin), PBS, trypsin/EDTA and Trypan blue were acquired from Sigma-Aldrich (Munich, Germany).

UVB exposure was performed at 312 nm, at a dose of 40 mJ/cm² by means of Biospectra system (Vilber Lourmat, France). Immediately after irradiation, PBS was replaced with culture medium ± test compounds. The stimulation with test compounds (LNG, EE, and EE + LNG) was performed after UVB irradiation.

The Alamar blue assay was used to test cell viability.

The migratory character of the cells used in the present study was evaluated by means of a scratch assay. Representative images were recorded by using an Olympus IX73 inverted microscope equipped with DP74 camera (Olympus, Tokyo, Japan) and the wound widths were measured with CellSense Dimension 1.17 (Olympus, Tokyo, Japan).

Flow cytometry analysis was performed to test the impact of our test compounds on cell apoptosis, using an annexin V-FITC apoptosis detection kit (eBioscience, Vienna, Austria).

Cells were analyzed by flow cytometry (FACSCalibur; Becton Dickson, Franklin Lakes, NJ, USA) and unstimulated cells were used as controls. The results were processed using Flowing Software Version 2.5.1 (developed by Perttu Terho, Cell Imaging Core, Turku Centre for Biotechnology, Turun Yliopisto, Finland).

The evaluation of hormones biocompatibility and toxicity was assessed in ovo by the Hen's Egg Chorioallantoic Membrane Test (HET-CAM), which was carried out following ICCVAM recommendations and adapted to our conditions.

Results:

The effect induced by test compounds (EE, LNG and EE + LNG) on healthy human and murine skin (HaCaT, 1BR3, HEMa and JB6 Cl 41-5a) cells, and melanoma (A375 and B164A5) cell viability in the presence/absence of UVB irradiation, was assessed using the Alamar blue assay. Irradiation of HaCaT, 1BR3, HEMa and JB6 Cl 41-5a cells with UVB (40 mJ/cm²) resulted in a significant reduction of cells viability (66.30% viable HaCaT, 74.75% viable 1BR3, 58.25% viable HEMa, and 60.85% viable JB6 Cl 41-5a, respectively) as compared to control cells.

Stimulation of healthy cells with EE (1 and 10 μ M) for 24 h led to the following results: HaCaT cells—a slight decrease of viability in a dose-dependent manner (92.90% at 1 μ M and 82.01% at 10 μ M), 1BR3 cells—88.04% viable cells at 10 μ M, HEMa cells—82.25% viable cells at 10 μ M, JB6 Cl 41-5a cells—the viability was not affected as compared to control cells.

LNG had no influence on HaCaT, 1BR3, and JB6 Cl 41-5a cell viability after 24 h stimulation at the lowest concentration tested—1 μ M, whereas at 10 μ M it was recorded a decrease for HEMa.

The lowest viability rates were observed in the groups of cells that were irradiated with UVB and stimulated with the combination of hormones—EE + LNG (at 10 μ M) but these viability percentages were higher than the ones recorded for the cells that were only UVB-exposed (HaCaT: 78.55% vs. 69.30%; 1BR3: 83.31% vs. 74.75%, HEMa: 82.46% vs. 58.25%, and JB6 Cl 41-5a: 79.83% vs. 60.85%), which might indicate a recovery effect induced by EE+ LNG.

Similar experimental conditions were applied for A375 and B164A5 melanoma cells to evaluate the effects induced by test compounds \pm UVB irradiation on cells viability in a 24 h frame. UVB irradiation of human and murine melanoma cells determined a significant decrease of cell viability (around 75%) as compared to control cells.

Exposure to UVB radiation followed by stimulation with EE, LNG, or EE + LNG led to a significant dose-dependent decrease of A375 cell viability percentage, compared to the effects induced by each test compound/UVB alone, (EE vs. EE + UVB: 66.54% vs. 58.72%; LNG vs. LNG + UVB: 69.78% vs. 67.59%; EE + LNG vs. EE + LNG + UVB: 56% vs. 49.69%).

In the case of B164A5 cells, UVB irradiation followed by stimulation with test compounds produced an increase of the cells' viability compared to the values obtained for each test compound (EE vs. EE + UVB: 56.84% vs. 74.46%; LNG vs. LNG + UVB: 59.27% vs. 78.06%; EE + LNG vs. EE + LNG + UVB: 47.23% vs. 80.59%). A similar effect was observed in the case of pigmented human melanoma cells—RPMI-7951

A dose-dependent apoptotic activity was noticed in the case of both cell lines. As compared to control cells, the strongest apoptotic effect on non UVB-irradiated A375 human melanoma cells was induced by EE and EE + LNG at the highest concentrations tested—10 μ M.

LNG exerted a lower pro-apoptotic activity at the same concentration. UVB exposure of A375 cells, followed by addition of 1 μ M of test compounds led to a significantly increased percentage of early apoptotic cells: 22.62% for LNG; 31% for EE and 27% for EE + LNG.

UVB irradiation combined with the highest concentration of test compounds triggered percentages of the early apoptotic population like the ones recorded for the test compounds in non UVB-exposed cell population.

HaCaT cells showed no significant morphological changes after stimulation with EE, LNG, and EE + LNG (1 μ M). After UVB irradiation, HaCaT cells drastically changed their morphological aspect. The most affected cells were the ones stimulated with EE + LNG.

The results for 1BR3 cells were similar to those obtained for HaCaT.

Stimulation of HEMA cells with EE and LNG (1 μ M) had no effects on cells morphology. EE + LNG induced a slight modification of HEMA cells morphology. UVB irradiation influenced the melanocytes' shape and their confluence, and the association with EE + LNG seemed to be the most noxious.

24 h post-exposure to UVB, HEMA cells stimulated with EE and LNG partially recovered their initial form. Murine epidermis JB6 Cl 41-5a cells showed a good confluence in the absence of UVB radiation, and the test compounds did not perturb the shape of the cells; whereas after UVB exposure, the cells stimulated with test compounds seemed to be protected by UVB deleterious effects.

The control cells exposed to UVB were most affected, displaying a low level of confluence and major changes of their morphological aspects, characteristics that were partially recovered after 24 h post-irradiation.

In the case of A375, the control cells displayed a normal epithelial morphology, after 24 h. A decrease of A375 control cells confluence was recorded after UVB radiation and some detached and floating cells were noticed. The EE and LNG stimulation of cells exposed to UVB led to some changes in cells' shape, mainly after EE + LNG treatment indicating the process of apoptosis, the results agreeing with the reported cell viability data. In the case of pigmented human melanoma cells—RPMI-7951, the test compounds had no impact on cells morphology, but after UVB irradiation, significant changes were observed in all groups, effects that were almost completely reversed after 24 h and test compound stimulation.

In the case of murine melanoma cells—B164A5, the cells exposed to UVB irradiation seemed to be the most greatly affected in terms of cell morphology, showing a round shape with dendrites and shrinkage. Changes in B164A5 melanoma cells shape were also observed after stimulation with test compounds, in non-UVB irradiated cells. On the other hand, B164A5 cells exposed to UVB followed by stimulation with test compounds revealed a confluence increment and minor changes in cells morphology

LNG stimulation did not interfere with the migration of human and murine healthy skin cells, the wound widths at 24 h being like the ones measured for control cells.

After EE stimulation, a stimulatory trend in all cell lines could be mentioned as compared to control cells; however, the most significant stimulation was seen with 1BR3 cells (52.37% vs. 40.09% on 1BR3 cells).

The combination of the two hormones—EE + LNG induced an inhibitory effect on HaCaT cells migration, showing a wound closure rate of 58.18%, whereas in the case of 1BR3 and JB6 Cl 41-5a, the effect was stimulatory. The very low wound healing rate (40.08%) of 1BR3 control cells was due to their low proliferation ability in specific culture conditions per day.

A stimulatory effect on HEMa cells migration was observed after EE and LNG stimulation (the gap was almost covered—mainly after EE) as compared with control cells. Moreover, the combination EE + LNG also augmented the migratory capacity of HEMa cells.

The in vitro wound healing assay revealed that the melanoma cells' (A375—human melanoma, B164A5—murine melanoma) migratory capacity was not inhibited by EE and EE + LNG stimulation (1 μ M), moreover, a stimulatory effect could be mentioned; still, the fact that the wound was also covered with some detached cells must be considered.

For EE, the wound closure rate was 82.81% on human melanoma cells and 85.29% on the murine melanoma cell line. In contrast, the same concentration of LNG (1 μ M) showed a wound healing rate of only 63.98% in the case of human melanoma cells, and 53.94% in the case of the murine melanoma cell line. Similar results were obtained for human pigmented melanoma cells—RPMI-7951.

The potential toxicity of the test compounds (EE, LNG, and EE + LNG) was also assessed in vivo, using the in ovo chick chorioallantoic membrane as a biological environment.

The effects induced by the test compounds, along with the positive and negative controls were registered as photographs representing the upper surface of the chorioallantoic membranes before and after 5 min of contact with the solutions. Prior to the determination of the irritation score, the results recorded for irritation severity were considered.

PBS, DMSO 1%, LNG (1 and 10 μ M), and the lowest concentration of EE (1 μ M) showed none of the three endpoints (hemorrhage, coagulation and lysis). For these samples, we registered a viability of more than 24 h.

EE (10 μ M) showed late and limited signs of hemorrhage or coagulation, and early, though limited signs of vasodilatation. EE + LNG (1 and 10 μ M) application induced slight and limited coagulation, in a dose-dependent manner. The highest mean irritation score was

recorded for the positive control, SDS. For the samples that induced a weak irritant effect the death was registered within the first 24 h.

SDS induced major vascular damage on the chorioallantoic membrane. All three endpoints: hemorrhage, coagulation, and lysis, were reported only for SDS. The death of the specimen was registered within 60 min.

General Conclusions

Anhydrous EE has a good thermal stability (up to 177 °C), with the main decomposition process taking place in the 175–375 °C range in a single-step process, invariable with the modification of heating rate of the sample.

The potential influence of EE, LNG, and their association on the behavior of the highly aggressive MDA-MB- 231 hormone-independent breast cancer cells highly dependent on the exposure time, test compound, and concentration. At 24 h, EE presented the highest cytotoxicity, LNG showed a stimulatory trend regarding cell survival, while EE + LNG behaved similarly to EE. At 48h, all samples increased the cell viability and number. EE and LNG upregulated the expression of vimentin, suggesting a stimulation of cell migration, while their combination was associated with an anti-migratory effect.

Regarding the binding affinity of EE, by means of molecular docking against normal and mutant type ER. even though mutant variants of ER suffer conformational change due to amino acid changes, obtained binding energies of E2 did not decrease significantly. It is probable that the conformational changes don't affect agonist binding, only when the amino acid changes directly affect the ligand binding domain.

EE, LNG, and their combination (EE + LNG) did not interfere with human and murine healthy skin cell viability at the lowest concentration tested but induced a significant cytotoxic effect on melanoma cells. Higher concentrations of hormones and adding UVB irradiation increased the cytotoxic effect in both healthy and A375 human melanoma cells.

In the case of murine melanoma cells—B164A5, the association of hormones and UVB stress led to an increase of viable cell percentage and a decrease of early apoptotic cells, which suggests a possible role of melanin in the protection of melanoma cells against hormonal treatment.

In ovo experiments confirmed the harmless activity of the hormones at low doses, but at higher concentrations they produced a weak irritant effect.