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# **PHD THESIS**

**CONTRIBUTIONS TO THE DEVELOPMENT OF  
BIOINSPIRED STRUCTURES AS POTENTIAL NEXT-  
GENERATION CHEMOTHERAPEUTICS**

## **A B S T R A C T**

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# ABSTRACT

## 1. INTRODUCTION

Cancer, defined as a group of diseases characterized by anomalous cell division and ability to invade neighboring tissues, stands as one of the most pressing causes of death worldwide. According to recent statistics, the incidence of cancer might face unprecedented increases in the future, reaching over 20 million of diagnosed cases in 2030.

Arising from the malignant switch of pigment-sinthesising melanocytes in the basal epidermal layer of the skin, cutaneous melanoma (CM) stands as one of the deadliest neoplasms, accounting for the majority of deaths (approximately 90%) caused by skin cancer each year. CM was ranked the 15<sup>th</sup> most frequent cancers worldwide, presenting an annual incidence that is increasing at a more rapid rate compared to other neoplasms. Currently, there is a vast array of therapeutic options that could be applied in CM treatment, including surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy, nonetheless, they face two main disadvantages that limit their applications, severe adverse reactions and resistance.

Lung cancer (LC) is another major causes of tumor-related deaths worldwide, presenting the highest mortality rate compared to other neoplasms. Non-small cell lung carcinoma (NSCLC), accounts for the majority of LC diagnoses (80–85%) (56), and being further divided into three histologic subtypes—adenocarcinoma (40%), squamous cell carcinoma (25–30%), and large cell carcinoma (10–15%).

Despite the recent advances in the field of chemically synthesized pharmaceutical agents, nature remains the main supplier of bioactive molecules, their therapeutic relevance being well-recognized in oncology as potential alternatives conventional chemotherapeutics.

## 2. AIM AND CONTRIBUTIONS

In the light of these considerations, the present thesis was elaborated to provide an exhaustive investigation of some bioinspired products with diverse chemical structure for their potential application in the next-generation cancer treatment. The personal contributions consist in the portrayal of the safety and therapeutic profile of bioinspired polymeric (MEL-NPs) and lipid (RUT-O and RUT-L) structures as potential novel chemotherapeutics for the treatment of CM and NSCLC, respectively. The original findings

described herein confer a mechanistic overview on their therapeutic effects, by demonstrating that: (i) MEL-NPs present a strong anti-melanoma effect by triggering apoptosis-related cytotoxicity and blocking cell migration by targeting the epithelial-to-mesenchymal transition (EMT); and (ii) only RUT-L exerts antineoplastic effects in vitro against NSCLC by causing a non-apoptotic cell death that is similar to paraptosis due to vacuole formation and oxidative stress generation, while the anticancer properties of RUT-O are absent. Additional to these novelties, the study also contributes to further research regarding the applications in cancer treatment of nature-inspired products by outlining their biocompatibility.

### 3. RESULTS

#### 3.1. SAFETY PROFILE OF MEL-NPs

The cytotoxicity of MEL-NPs in healthy skin-derived cells (HEMa, HaCaT, JB6 CI 41-5a) and melanotic CM cells (SH-4, B164A5) was assessed by evaluating their impact on cell viability after a 24 h treatment. As shown in Figure 1, MEL-NPs significantly lowered the viability of HEMa, HaCaT, and JB6 CI 41-5a cells only at the highest concentration of 100  $\mu\text{g/mL}$  (to 58.89%, 68.43% and 58.14%, respectively). At 10  $\mu\text{g/mL}$ , MEL-NPs exerted a slight stimulatory effect on the cells' viability which increased to 111.14% (HEMa), 105.74% (HaCaT) and 102.53% (JB6 CI 41-5a), while at 25, 50, and 75  $\mu\text{g/mL}$ , it was maintained over 80% in all healthy cell lines.

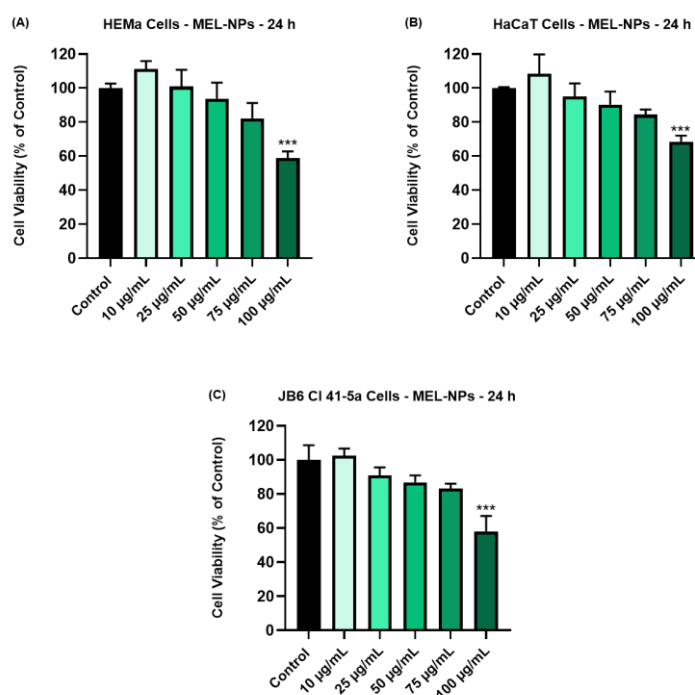


Figure 1. Influence of MEL-NPs (10, 25, 50, 75 and 100  $\mu\text{g/mL}$ ) on the viability of (A) HEMa, (B) HaCaT and (C) JB6 CI 41-5a healthy skin-derived cells following a 24 h treatment. The data were normalized to control (representing

cells without treatment) and expressed as means  $\pm$  SD of three independent experiments conducted in triplicate. The statistical differences between Control and MEL-NPs-treated groups were determined using the one-way ANOVA analysis and the Dunnett's multiple comparisons post-test (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$  versus control).

To investigate the potential irritant effect of the obtained MEL-NPs using a 3D in vitro system, the Skin irritation assay on EpiDerm inserts was performed (Figure 2). A significant impact on the viability of EpiDerm tissues was obtained in the case of SDS 1% used as positive control which reduced it to 9.69% compared to DPBS used as negative control. MEL-NPs (100  $\mu\text{g/mL}$ ) showed no irritant potential in vitro, the viability of the treated tissues being over 50%. Furthermore, MEL-NPs showed a stimulatory effect, increasing the viability of EpiDerm inserts to 111.44%.

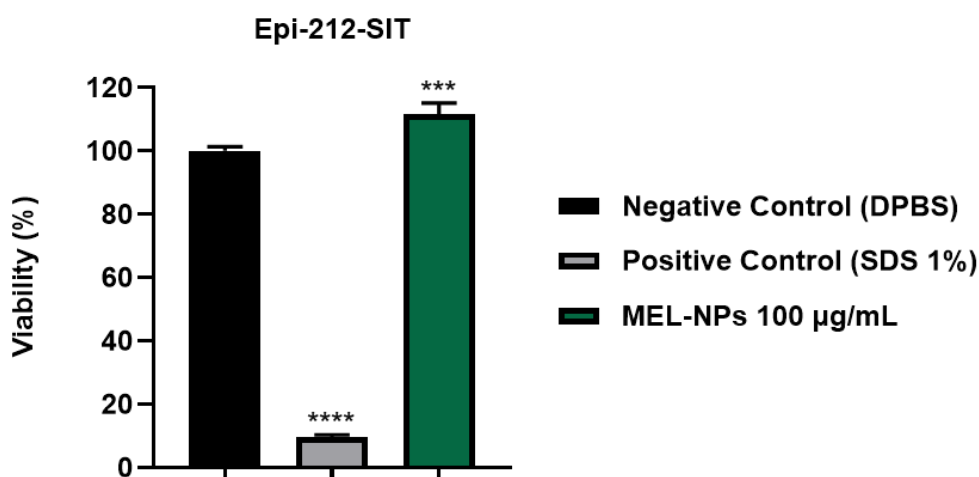
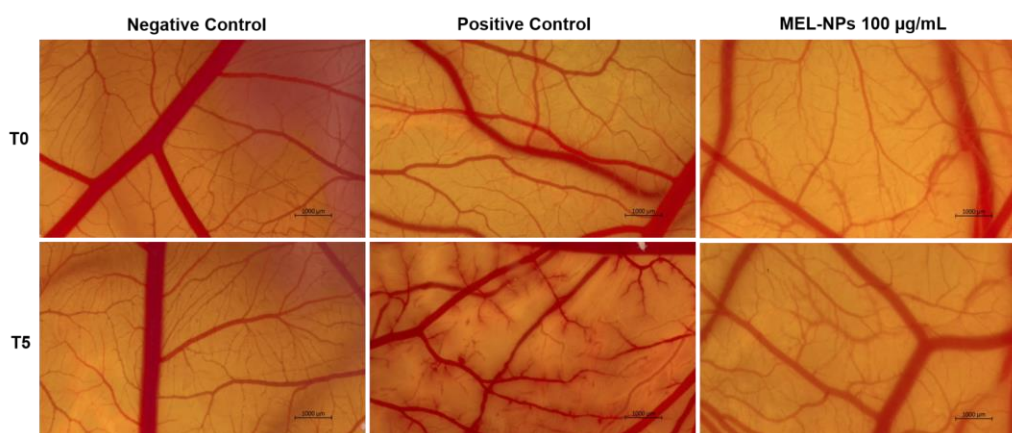


Figure 2. Viability percentage of EpiDerm skin model inserts (EPI-212-SIT) at 18 h post-treatment with MEL-NPs 100  $\mu\text{g/mL}$ . One-way analysis of variance (ANOVA) followed by Dunnett's post-test was employed to determine the statistical differences between sample-treated inserts and negative control-treated inserts ( $p < 0.0001$  indicated by \*\*\*\*). Positive control is represented by SDS 1%, and negative control is represented by DPBS.

The irritant potential of MEL-NPs 100  $\mu\text{g/mL}$  was evaluated in ovo by applying the HET-CAM test (Figure 3). Distilled water ( $\text{H}_2\text{O}$ ) used as negative control induced no alteration on the vascular structure, while SLS 1% (positive control) caused haemorrhage, lysis, and coagulation shortly after its application on the CAM. No severe vascular impairments were observed after the exposure of the CAM to MEL-NPs, except for slight signs of lysis (at 100  $\mu\text{g/mL}$ ) at the end of the treatment.



**Figure 3.** Stereomicroscopic images taken before the application of the evaluated samples (Negative Control – distilled water, Positive Control - Sodium Lauryl Sulfate, and MEL-NPs 100 µg/mL on the chorioallantoic membrane (T0) and 5 minutes after their application (T5). The scale bars represent 1000 µm.

The calculated IS for the tested samples is presented in Table 1. The highest IS value was obtained for SLS 1%. In the case of MEL-NPs 100 µg/mL, the IS slightly increased to 0.49. MEL-NPs were classified as non-irritant on the CAM at these concentration, lacking vascular toxicity.

**Table 3.** Irritation score values for negative control (H<sub>2</sub>O), positive control (SLS 1%), and MEL-NPs (10, 50, and 100 µg/mL).

Sample	Irritation Score (IS)	Irritant Potential
Negative Control (H <sub>2</sub> O)	0.07	Non-irritant
Positive Control (SLS 1%)	19.88	Strong irritant
MEL-NPs 100 µg/mL	0.49	Non-irritant

### 3.2. ANTI-TUMOR EFFECT OF MEL-NPs AGAINST CM

The 24 h treatment of melanotic CM cells with MEL-NPs caused a concentration-dependent decline in cell viability that reached statistical significance at all concentrations for A375 cells and starting with the concentration of 25 µg/mL for SH-4 and B164A5 cells (Figure 4). The viability of SH-4 cells was gradually reduced by MEL-NPs from 87.27% (at 10 µg/mL) to 73.98% (at 25 µg/mL), 61.12% (at 50 µg/mL), 53.98% (at 75 µg/mL), and 46.81% (at 100 µg/mL), respectively. The percentage of viable B164A5 cells was also lowered by the 24 h exposure to MEL-NPs to 86.95% (at 10 µg/mL), 72.66% (at 25 µg/mL), 67.30% (at 50 µg/mL), 61.23% (at 75 µg/mL), and 55.24% (at 100 µg/mL),

respectively. The viability of A375 cells after the 24 h exposure to MEL-NPs was 83.86% (at 10  $\mu\text{g/mL}$ ), 63.82% (at 25  $\mu\text{g/mL}$ ), 53.72% (at 50  $\mu\text{g/mL}$ ), 43.95% (at 75  $\mu\text{g/mL}$ ), and 37.41% (at 100  $\mu\text{g/mL}$ ).

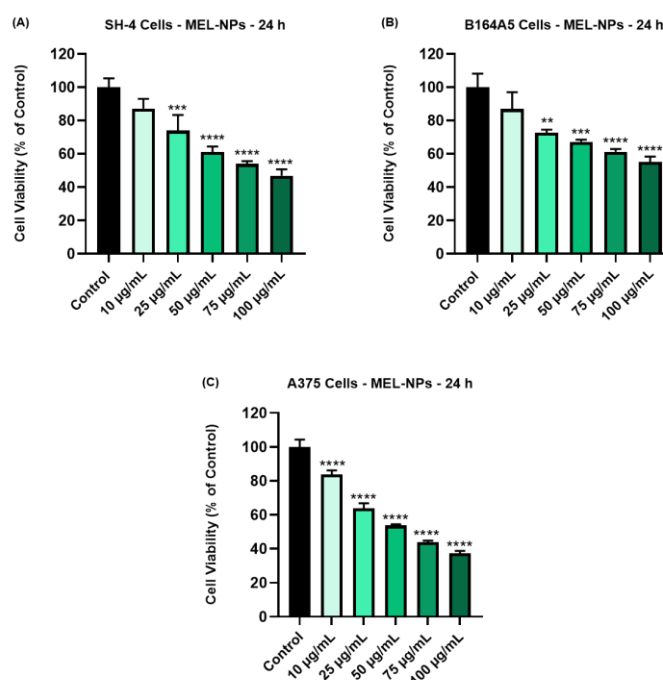


Figure 4. Influence of MEL-NPs (10, 25, 50, 75 and 100  $\mu\text{g/mL}$ ) on the viability of (A) SH-4 and (B) B154A5, and (C) A375 cutaneous melanoma (CM) cells following a 24 h treatment. The data were normalized to control (representing cells without treatment) and expressed as means  $\pm$  SD of three independent experiments conducted in triplicate. The statistical differences between Control and MEL-NPs-treated groups were determined using the one-way ANOVA analysis and the Dunnett's multiple comparisons post-test (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$  versus control).

### 3.3. SAFETY PROFILE OF RUT-O AND RUT-L

To investigate the potential cytotoxicity of the obtained derivatives and their parent compounds at the pulmonary level, the impact of RUT-O and RUT-L (at concentrations ranging between 25 and 125  $\mu\text{M}$ ) on the viability of EpiAirway<sup>TM</sup> 3D human tissues was assessed following a 24 h incubation. The obtained results, graphically represented in Figure 5, indicate that the tested compounds caused no significant impairment in the EpiAirway<sup>TM</sup> tissues' viability. At the lowest concentrations (25 and 50  $\mu\text{M}$ ), both derivatives slightly elevated the viability percentages that reached values over 100%. At 100  $\mu\text{M}$ , the viability of the inserts remained similar to the control for both RUT-O and RUT-L, while at 125  $\mu\text{M}$ , the compounds reduced their viability to 98% and 94%, respectively.

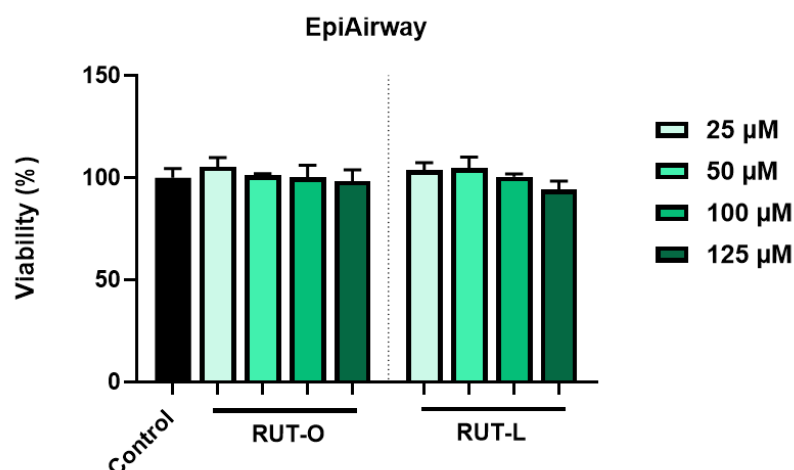


Figure 5. Viability of EpiAirway™ 3D human tissues after 24 h of exposure to rutin oleate (RUT-O) and rutin linoleate (RUT-L) at 25, 50, 100, and 125  $\mu$ M. The results were presented as viability percentages (%) normalized to control (non-treated cells) and expressed as means  $\pm$  standard deviation of three experiments performed in triplicate. The statistical differences between the control and the treated groups were analyzed using the one-way ANOVA and Dunnett's multiple comparisons tests.

### 3.4. ANTI-TUMOR EFFECT OF RUT-O AND RUT-L AGAINST NSCLC

The potential impact of RUT-O and RUT-L (25, 50, 100, and 125  $\mu$ M) on the viability of NCI-H23 cells was explored at the end of a 24 h treatment. As revealed in Figure 6, a clear distinction between the cytotoxic profiles of RUT-O and RUT-L was obtained at this stimulation interval, with the cell viability percentages varying depending on the tested compound and concentration. Thus, RUT-O exerted no anti-tumor activity on this NSCLC cell line, on the contrary, a stimulatory effect was noticed at all concentrations, with the viability values rating between 117% (at the lowest concentration – 25  $\mu$ M), and 113% (at the highest concentration – 125  $\mu$ M). RUT-L, on the other hand, induced a concentration-dependent reduction in the viability of NCI-H23 cells, the lowest and most significant values being obtained at the highest tested concentrations (100  $\mu$ M – 63%, and 125  $\mu$ M – 47%).



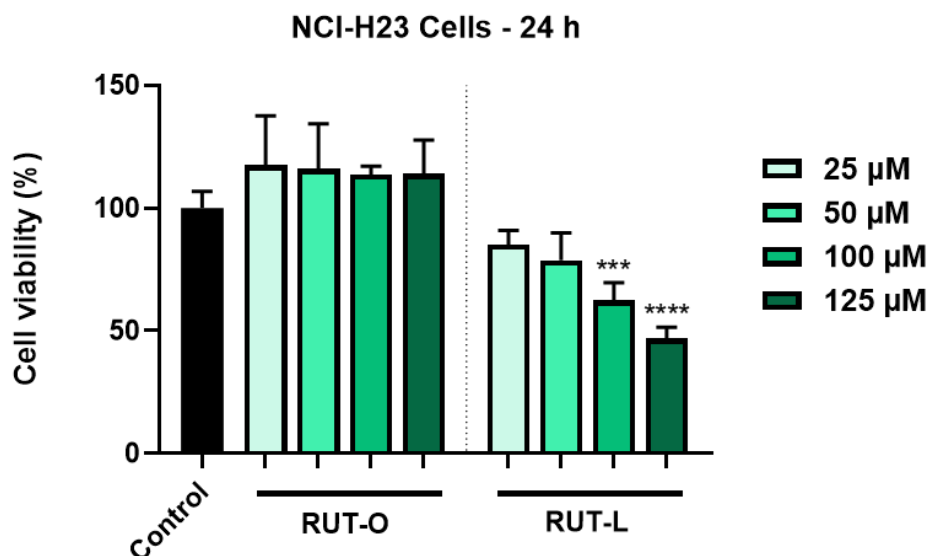


Figure 6. Graphical representation of the percentage of viable NCI-H23 cells after a 24 h treatment with rutin oleate (RUT-O) and rutin linoleate (RUT-L) at 25, 50, 100, and 125  $\mu\text{M}$ . The results were presented as viability percentages (%) normalized to control (non-treated cells) and expressed as means  $\pm$  standard deviation of three experiments performed in triplicate. The statistical differences between the Control and the treated groups were analyzed using the one-way ANOVA and Dunnett's multiple comparisons tests (\*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ ).

To assess whether the morphology of NCI-H23 cells undergoes any changes following their treatment with RUT-O and RUT-L, a microscopic evaluation was performed (Figure 7). RUT-O showed no significant changes in NCI-H23 cells' morphology compared to control at the tested concentrations. RUT-L triggered a dose-dependent decrease in cell confluence starting from the concentration of 25  $\mu\text{M}$ , which was more prominent at 100 and 125  $\mu\text{M}$  and accompanied by several cytotoxic signs (i.e., cell shrinkage, rounding, and detachment). An interesting change in the cells' appearance was observed after their 24 h treatment with RUT-L at high concentrations (100 and 125  $\mu\text{M}$ ), namely cytoplasmic vacuolation (white arrows), evidenced by the formation of numerous irregular vesicles in the cytoplasm that covered the perinuclear space. At the highest tested concentration (125  $\mu\text{M}$ ), the cytoplasmic vacuolation was also associated with massive cell shrinkage.

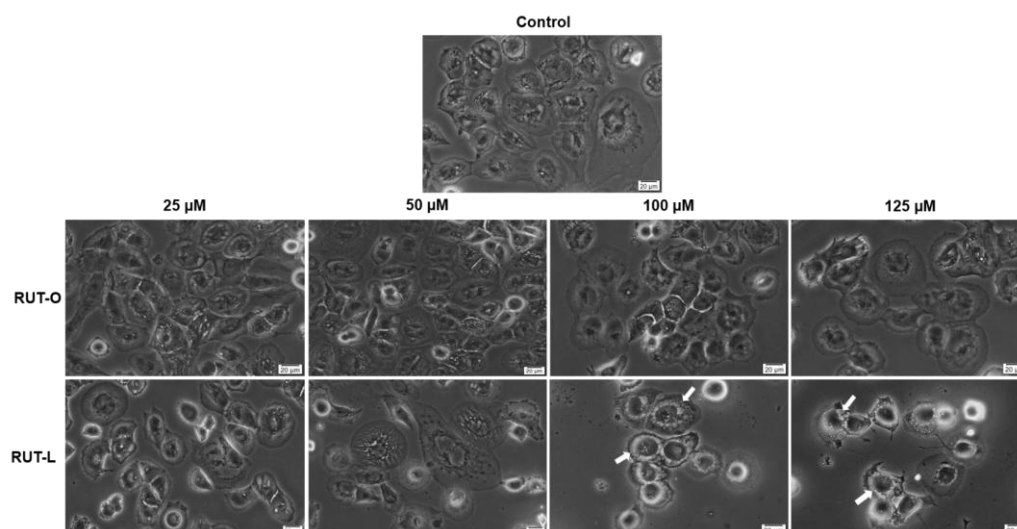


Figure 7. Representative images illustrating the morphology and confluence of NCI-H23 cells post-treatment with rutin oleate (RUT-O) and rutin linoleate (RUT-L) at 25, 50, 100, and 125  $\mu\text{M}$  for an interval of 24 h. The white arrows indicate cells presenting cytoplasmic vacuolation. The scale bars indicate 20  $\mu\text{m}$ .

#### 4. CONCLUSIONS

The findings related herein show that MEL-NPs selectively target CM cells, while also presenting a proper, but concentration-dependent cytocompatibility in healthy cutaneous cells, and lacking irritant effect in 3D EpiDerm™ tissues. These results also describe the lack of toxicity in EpiAirway™ 3D reconstructed tissues as well as the differential therapeutic properties retained by RUT lipophilic derivatives and the potential implication of RUT-L in non-apoptotic cell death pathways, such as paraptosis.