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**INVESTIGATIONS CONCERNING THE MECHANISMS OF ACTION OF FUNCTIONALIZED
TRITERPENOIDS**

ABSTRACT

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1. BACKGROUND

Cancer remains one of the challenges of modern medicine because it is one of the major causes of death worldwide, and the number of us is constantly growing. However, treatment options are still limited and are associated with an increased number of systemic toxic reactions.

Skin cancer is a common cancer in the Caucasian population. This type of cancer has two subtypes, namely, non-melanoma cancer and melanoma cancer. In turn, non-melanoma cancer is classified into basal cell carcinoma and squamous cell carcinoma. The latter type of cancer is associated with an increased ability to metastasize, being associated with an increased lethality.

Lung cancer is a type of cancer known for its potential for invasiveness and metastasis, ranking among the leading causes of death in cancer globally, both among women and men. Currently, lung cancer therapy includes radiation therapy, chemotherapy, targeted therapy and surgery. However, today, modern medicine is facing an increased mortality associated with lung cancer.

The most common type of cancer is breast cancer. This is the second leading cause of death globally. The most prone to this type of cancer are women aged 45-55 years. Classical therapy for breast cancer includes surgical removal of the affected tissue, chemotherapy, radiation therapy and hormone therapy.

Due to the fact that conventional therapy is associated with the number of toxic effects, and the results are not always positive, recent research has focused on the discovery of new therapeutic compounds that have a targeted action. One of the biological targets researched for the antitumor effect of the compounds is mitochondria. Recent studies have also tried to focus on natural compounds that have a selective antitumor action with mitochondria. One of these compounds of natural origin is oleanolic acid. The actions of this compound are documented in the literature and with side effects such as: anti-inflammatory, antioxidant and antitumor. In order to increase its biological activity, oleanolic acid undergoes a number of derivatives.

These derivatizations include conjugation with Rhodamine B which aims to obtain a compound with selective antitumor action that has mitochondria as a site of action and that can be incorporated into the category of compounds named in the literature "MITOCAN".

2. AIMS OF THE RESEARCH

The aim of this paper included the complex characterization and evaluation of the antitumor mechanism of action of a Rhodamine B-conjugated oleanolic acid derivative synthesized in the laboratory of Prof. Dr. Prof. Dr. René Csuk (Halle, Germany) and hereinafter referred to as "RhodOA", on different experimental models *in silico*, *in vitro* and *in vivo*.

The specific objectives were the following:

1. Evaluation of the *in vitro* effects of RhodOA in terms of antioxidant properties and cytotoxic, anti-migratory and antibacterial effects.

2. Detailed evaluation of the mechanism of action by determining cell morphological changes and at the level of cellular organs, as well as the effect of the compound on cellular respiration.

I. IN SILICO EVALUATION OF THE ANTIPROLIFERATIVE MITOCHONDRIAL TARGETED MECHANISM OF ACTION OF SOME PENTACYCLIC TRITERPENE DERIVATIVES

The aim of the first study was to evaluate by molecular docking the mechanism of action in the mitochondria of pentacyclic triterpene derivatives conjugated with Rhodamine B.

The protein structures required for this study were available from Protein Data Bank RCSB. The software used for molecular docking was Autodock Tools 1.5.6. The ligand molecules corresponding to the triterpene derivatives conjugated with Rhodamine B were: betulinic acid, glycyrrhetic acid, maslinic acid, oleanolic acid, platanic acid and ursolic acid. The structures were drawn using Biovia Draw and converted to 3D using OyRx's Open Babel software. Finally, the scores recorded for the anchored molecules were obtained in the form of values of free binding energy (kcal / mol). Ligand protein binding models were analyzed using Accelrys Discovery Studio 4.1 software.

The results obtained showed that the oleanolic acid derivative conjugated with Rhodamine B has remarkable energy values in the case of NDH and SDH proteins which are, in fact, complexes I and II of the electron transport chain. These results suggest that the oleanolic acid derivative has effects on cell viability due to action in the mitochondria.

II. ASSESSMENT OF THE ANTIOXIDANT CAPACITY OF OLEANOLIC ACID DERIVATIVE CONJUGATED WITH RHODAMINE B (AOA)

The aim of the study was to evaluate the antioxidant effect of both the oleanolic acid derivative and pure, unconjugated Rhodamine B. Numerous studies in the field have focused on the antioxidant effect on oleanolic acid, and recent discoveries have shown that it has an antioxidant effect both by direct reaction with reactive oxygen species and by increasing the expression of antioxidant enzymes such as catalase and thioredoxin peroxidase.

The DPPH method was used to evaluate the antioxidant capacity of RhodOA and Rhod. Five concentrations of the oleanolic acid derivative (20, 40, 60, 80 and 100 nM) as well as five concentrations of unconjugated Rhodamine B (20, 40, 60, 80 and 100 nM) were tested. Ascorbic acid was used as a standard for antioxidant activity.

The results obtained showed that both RhodOA and Rhod showed an antioxidant activity similar to ascorbic acid when DPPH was used in a concentration of 0.1 mM. Both compounds showed an antioxidant activity of about 80%, compared to the activity of ascorbic acid of about 94%. Regarding the antioxidant activity of RhodOA and Rhod when DPPH with a concentration of 1 mM was used, it was observed that they have a lower antioxidant activity, but nevertheless, the values obtained for the antioxidant activity were over 50%.

In conclusion, RhodOA has a strong antioxidant activity, similar to that of ascorbic acid, when DPPH is used in a concentration of 0.1 mM. However, when DPPH is used at a concentration of 1 mM, the antioxidant activity of the compounds is lower, but it is over 50%.

III. CYTOTOXIC AND ANTIPROLIFERATIVE ACTIVITY OF RHODOA COMPARED WITH OLEANOLIC ACID

The aim of this study was to evaluate the cytotoxic and antimigrating effect of RhodOA compared to that of pure, non-derivatized oleanolic acid on the healthy human keratinocyte cell line - HaCaT and on human melanoma tumor cell lines - A375, pulmonary adenocarcinoma and A549 - MDA-MB-231. The decreasing effect of cell viability produced by RhodOA and Rhod was determined by MTT assay, and the ability to inhibit cell proliferation was assessed using the scratch technique. To evaluate the cytotoxic effect, cells were stimulated for 24, 48 and 72 hours with five concentrations of RhodOA (20, 40, 60, 80 and 100 nM), five concentrations of unconjugated Rhodamine B (20, 40, 60, 80 and 100 nM) and five concentrations of pure oleanolic acid (20, 40, 60, 80 and 100 nM).

The results obtained showed that RhodOA has a cytotoxic effect depending on the time and dose used. The decreasing effect of cell viability produced by RhodOA was observed only in the case of tumor cell lines, while the healthy cell line of human keratinocytes was not affected. At the same time, unconjugated Rhodamine B did not show visible effects on cell viability. Compared to the effect of the RhodOA derivative, oleanolic acid, did not show a major effect on cell viability. The value of cell viability was around 90-100% even after a stimulation of 72 hours.

In conclusion, by derivatizing oleanolic acid and conjugating with Rhodamine B, a compound with increased antitumor activity is obtained. An important aspect is that the oleanolic acid derivative does not show toxic effects on healthy human keratinocytes, this indicating the selectivity towards tumor cells.

IV. IMMUNOFLUORESCENCE ASSAY

The aim of this study was to evaluate the changes produced by RhodOA on the nucleus, mitochondria and actin fibers, thus providing a more complex picture of the mechanism of action of the oleanolic acid derivative.

The immunofluorescence technique was performed on the human melanoma tumor cell line - A375 and on the human keratinocyte cell line - HaCaT. The tumor cell line was chosen based on the results obtained by the MTT technique, A375 being the most affected tumor cell line. RhodOA was tested in four different concentrations, three of which were then used to evaluate the effect on cellular respiration (10, 20 and 30 nM) and the highest concentration used in the cell viability study (100 nM). DAPI staining was used to visualize the nucleus, Alexa Fluor® 555 Phalloidin antibody was used for actin fiber visualization, and Anti-COX IV antibody Mitochondrial marker was used for mitochondrial visualization.

The obtained results showed that the oleanolic acid derivative causes changes in all the organs studied in this study, in the case of the human melanoma cell line. Thus, a condensation of chromatin characteristic of the process of cell death by apoptosis was observed in the nucleus. At the level of actin fibers, it was observed that the highest concentration tested by RhodOA, 100 nM, causes a condensation of actin fibers. Finally, at the level of the mitochondria, an increase of the number of mitochondria located predominantly around the nucleus was observed. All of these changes were not observed, however, in the case of healthy human keratinocytes, suggesting that the derivative has a selective effect on melanoma cells.

In conclusion, the oleanolic acid derivative is able to produce changes in the morphology of the nucleus, mitochondria and actin fibers. It is important to note that these changes were observed exclusively in the

case of the tumor cell line. For this reason, we can deduce that by derivatizing oleanolic acid a compound with an increased and selective biological activity is obtained.

V. CELL RESPIROMETRY STUDY

The aim of this study was to highlight the effects produced by the oleanolic acid derivative on cellular respiration, both in the case of the human melanoma tumor cell line and in the case of the healthy human keratinocyte cell line. To determine the effect on cellular respiration, three different concentrations of RhodOA (10, 20 and 30 nM) were tested, and cellular respiration was determined by high-resolution respirometry studies (Oxygraph-2k Oroboros Ltd.).

Regarding the effects of the oleanolic acid derivative on the human keratinocyte line, the results obtained showed that RhodOA causes an increase in all respiratory rates. On the other hand, on the human melanoma line, RhodOA, causes in a dose-dependent manner, a decrease in respiratory rates. For this reason, it can be stated that RhodOA causes a decrease in oxygen consumption. In addition, RhodOA has been shown to inhibit active respiration and decrease the maximum respiratory capacity of the electron transfer system. In addition to these changes, RhodOA also causes a dose-dependent decrease in the basal respiration dose.

By determining the effect of RhodOA on cellular respiration, we can conclude that it causes a decrease in all respiratory rates in the case of the human melanoma cell line, while in the case of the healthy human keratinocyte cell line, it has no toxic effect. Correlating the antitumor effect observed in viability and immunofluorescence studies with the effect on mitochondrial function, we can conclude that the new derivative of oleanolic acid conjugated with Rhodamine B can be classified as "MITOCAN".

VI. CHORIOALLANTOIC MEMBRANE (CAM) ASSAY

The aim of this study was to observe the effect of the compound RhodOA on blood vessels in terms of its antiangiogenic effect, but also to observe the potential irritant and anti-irritant effect of the derivative on the chorioallantoic membrane.

To determine the effect of RhodOA on angiogenesis, five derivative concentrations (20, 40, 60, 80 and 100 nM) were tested and eggs were observed for five days. Regarding the irritative and anti-irritant effect of the oleanolic acid derivative, it was tested in two concentrations, 80 and 100 nM, and the changes in the blood vessels of the chorioallantoic membrane were observed for five minutes.

The obtained results showed that after three days of application of the derivative on the chorioallantoic membrane, it causes a decrease in the number of newly formed capillaries. The strongest anti-angiogenic effect was observed at 100 nM. Regarding the potential irritating effect of the derivative, it was not observed even in the case of the highest concentration tested. After applying the 100 nM concentration, no changes in the blood vessels were observed. In contrast, when RhodOA is applied before the application of sodium dodecyl sulfonate (SDS) known to be an irritant to blood vessels, RhodOA causes a decrease in the irritating effect of SDS.

In conclusion, RhodOA has an increased biological activity, having a pronounced effect on angiogenesis. Regarding the irritating effect, it was not observed on the chorioallantoic membrane, even at

the highest concentration tested. RhodOA also did not cause major changes in blood vessels and did not affect the viability of chicken embryos.

VII. EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF RHODOA

Scopul studiului a fost evaluarea activității antimicrobiene a derivatului de acid oleanolic asupra mai multor specii bacteriene (*Streptococcus mutans*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*).

Activitatea antimicrobiană a fost determinată prin metoda de difuziune în agar. Ca și control a fost folosită gentamicina pentru bacili și stafilococi și gentamicina pentru streptococi și enterococi.

Rezultatele obținute au sugerat că derivatul de acid oleanolic prezintă o activitate antibacteriană ușor superioară antibioticului control în cazul speciilor *Streptococcus mutans* și *pyogenes*. În ceea ce privește restul speciilor bacteriene, activitatea RhodOA la cea mai mare concentrație testată (100 nM) a fost similară cu cea a antibioticului control. Excepția, în care RhodOA a arătat o activitate antibacteriană mai mică decât cea a antibioticului control a fost observată în cazul *Escherichia coli* și *Klebsiella pneumoniae*.

În concluzie, derivatul acidului oleanolic, RhodOA, este un potențial agent antibacterian în infecția cu *Streptococcus pyogenes* și *Streptococcus mutans* atunci când este utilizat la o concentrație de 100 nM. Cu toate acestea, sunt necesare studii suplimentare pentru a elucida mecanismul acțiunii antibacteriene

FINAL CONCLUSIONS

Characterization of the Rhodamine B conjugated oleanolic acid derivative led to following final conclusions:

1. In silico study performed by applying molecular docking approach revealed that RhodOA, the derivative of oleanolic acid conjugated with Rhodamine B, targets mitochondria by binding to proteins specific for complex I and II of cellular respiration
2. RhodOA showed a strong antioxidant effect comparable to ascorbic acid in the experimental design employed by using the 0.1 mM DPPH radical scavenging assay.
3. RhodOA exerted a pronounced dose- and time-dependent cytotoxic effect on all tumor cell lines tested (A375, A549, and MDA-MB-231), melanoma cell line – A375 being the most susceptible to compound's effect. The most significant cytotoxic effect was observed after a 72h treatment. In addition, the compound showed a safe profile in human keratinocytes, suggesting a selective cytotoxic behavior oriented towards tumor cells.

4. The cytotoxic activity induced by RhodOA in melanoma cells is characterized by the appearance of apoptotic specific signs, as: changes of cells shape (roundish cells) with condensation of actin fibers at the edges and nuclear fragmentation and condensation. No morphological changes were detected in human keratinocytes following RhodOA treatment, highlight the selective cytotoxic behavior.
5. Acute administration of RhodOA on permeabilized human melanoma cells determined a decline of cellular respiration characterized by a decrease of all mitochondrial respiratory parameters rates, including basal respiration, active respiration and maximal respiratory capacity, This effect was noticed only in melanoma cells, in human keratinocytes – HaCaT being described a stimulatory effect.
6. RhodOA proved a moderate antiangiogenic effect on the blood vessels of chick chorioallantoic membrane, a very low irritant effect on the chorioallantoic membrane and a moderate anti-irritant effect.
7. RhodOA showed a significant antibacterial activity only against the strains of *Streptococcus pyogenes* and *Streptococcus pyogenes* when used at a concentration of 100 nM.

ORIGINAL CONTRIBUTIONS

The original contributions can be summarized as follows:

1. Assessment of the mechanism of action of mitochondrial targeting by a series of triterpene derivatives, including the oleanolic acid derivative conjugated with Rhodamine B
2. Assessment of the antioxidant activity of RhodOA compared to Rhod and Vitamin C.
3. Assessment of the cytotoxic activity of RhodOA on several types of tumor cell lines, as well as the ability of RhodOA to inhibit tumor cells migration
4. Assessment of the ability of RhodOA to modify the morphology of the nucleus, mitochondria and actin fibers in order to determine the mechanism of antitumor action
5. Assessment of RhodOA influence on cellular respiration by both in the case of the human melanoma cells and in the case of the healthy human keratinocytes
6. Assessment of the antiangiogenic activity and potential irritant and anti-irritant effect of RhodOA on the chorioallantoic membrane
7. Assessment of the antibacterial activity of RhodOA in several bacterial strains.

Keywords: oleanolic acid derivative, melanoma, lung cancer, breast cancer, cellular respiration, mitochondria, antioxidant activity, antimicrobial activity

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