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EXPERIMENTAL RESEARCH REGARDING THE *IN VITRO* AND *IN VIVO* EFFECTS OF A MASLINIC ACID DERIVATIVE

ABSTRACT

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1. BACKGROUND

Cancer represents a leading cause of death globally and it is expected that the number of cases to increase in the following years.

Skin cancer represents one of the most common cancers diagnosed in Caucasian population. Skin cancers are divided into non-melanoma skin cancers (NMSC) and melanoma skin cancers (MSC). The two most common cutaneous malignancies are squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), followed by melanoma, the latter being also associated with a high mortality rate. Exposure to ultraviolet B (UVB) radiation is one of the most important risk factor in the development of this type of cancer.

The standard treatment for pacients with cancer (surgery, chemo- and radiotherapy) is constantly associated with side effects that can variably affect their life quality. Phytotherapy represents nowadays a highly investigated supportive therapeutic approach, view the comprehensive characterization of the cellular and subcellular effects of the phytochemical components.

Among these, olive oil and its derivatives have been systematically studied as a rich source of phytochemicals with numerous therapeutic effects. Recently, there has been a growing interest in the characterization of maslinic acid, a naturally occurring pentacyclic triterpene, mainly found in *Olea europaea* L.

Maslinic acid is known to possess various therapeutic effects, namely anti-inflammatory, anti-diabetic, anti-oxidant, anti-bacterial, anti-parasitic, and anti-HIV, as well as cardio- and neuroprotective properties. Furthermore, the compound exerts an important antitumor activity via complex, partially characterized mechanisms.

The antitumor effect of maslinic acid was reported in several types of cancers, including colorectal, urinary bladder, pancreatic, liver, prostate, esophagus, stomach, lung and salivary gland astrocytoma, as well as several cell lines of melanoma. The chemopreventive and antitumor potential of maslinic acid involves at least 3 proven activities, namely, pro-apoptotic, anti-proliferative, and anti-angiogenic.

Recently, the scientific community's interest shifted towards the maslinic acid derivatives since many of them displayed antitumor properties that were superior to the ones of the parent compound. Derivatization is a technique aimed at obtaining novel compounds with improved activity.

2. AIMS OF THE RESEARCH

The present research work was purported to comprehensively characterize and assess the different properties of a benzylamide derivative of maslinic acid (Benzyl (2α, 3β) 2,3-diacetoxy-olean-12-en-28-amide) synthesized and kindly provided by Prof. Dr. René Csuk (Halle, Germany), and further referred to as “EM2”, on *in vitro* and *in vivo* experimental models.

The specific objectives were as follows:

1. Characterization of morphology and ultrastructure of the EM2 compound (Benzyl (2α, 3β) 2,3-diacetoxy-olean-12-en-28-amide) and assessment of its *in vitro* effects (antioxidant, antimicrobial activity, cytotoxicity and proliferation studies).
2. *In vivo* evaluation of EM2 effects in experimental models of acute inflammation and carcinogenesis.

I. PHYSICO-CHEMICAL CHARACTERIZATION OF A BENZYLAMIDE DERIVATIVE OF MASLINIC ACID

The aim of the first study was to characterize the benzylamide derivative of maslinic acid "EM2" (Benzyl (2α, 3β) 2,3-diacetoxy-olean-12-en-28-amide), kindly provided by Prof. Dr. René Csuk.

The morphology of the compound was assessed by transmission electron microscopy (TEM) whereas scanning electron microscopy and energy dispersive X-ray analysis (SEM-EDAX) were performed in order to establish the ultrastructure of the sample. Thermogravimetry (TG) and differential scanning calorimetry (DSC) analysis showed the thermal behaviour of the compound.

Fourier transform infrared spectroscopy (FTIR) and Fourier transform-Raman spectroscopy (FT-RAMAN) analysis were further performed to display the bands characteristic for EM2.

Our results indicated that EM2 has irregular shape with spherical forms and can form agglomeration. The sample size is of the micro order; this can also be due to formed agglomerations in the sample. It consists of 3 elements, namely C, O and N. Furthermore, the compound underwent an exothermic effect at 414.4°C accompanied by an important mass loss of 91.8% on TG curve, effect that can be assigned to the oxidative degradation of EM2.

Due to FTIR analysis, we can observed the C=O group, specify to a strong band located at 1741.72 cm-1 and the NH group, specify for stretching vibration at 3425.58 cm-1, respectively.

II. ASSESSMENT OF THE ANTIOXIDANT CAPACITY OF EM2 AND ZINC

This study was aimed to investigate the antioxidant activity (AOA) of EM2 alone, and in co-administration with $ZnCl_2$, respectively. Zinc is an essential microelement which plays an important role in human cutaneous biology, with anti-inflammatory and antimicrobial properties and an important role in wound healing. Upon UVB exposure, intracellular zinc release is induced. Accordingly, we hypothesized that the antioxidant activity of EM2 could increase in the presence of zinc.

In order to evaluate the antioxidant capacity of EM2 and $ZnCl_2$ DPPH radical scavenging assay was used. Nine ethanolic solutions of maslinic acid derivative were prepared, with the following concentrations (200μM, 150μM, 100μM, 50μM, 25μM, 12.5μM, 10μM, 5μM, 1μM), while the concentrations for $ZnCl_2$ were 300μM, 200μM and 100μM, respectively. Ascorbic acid was used as standard to compare the antioxidant activity.

Our results indicated that all the nine concentrations of EM2 displayed a low antioxidant activity (approx. 25%) as compared to the ascorbic acid one (95%). When $ZnCl_2$ was co-administrated, the antioxidant activity of the samples showed a decrease with the increase of $ZnCl_2$ concentration. A minor increase in the antioxidant effect was recorded when the lowest concentration of $ZnCl_2$ (100μM) was used.

In conclusion, contrary to our initial hypothesis, in the presence of $ZnCl_2$, there is a concentration-dependent decrease in the antioxidant activity of EM2.

III. EVALUATION OF THE ANTIMICROBIAL AND ANTIFUNGAL ACTIVITY OF EM2 AND ZINC

The aim of the present study was to evaluate the antimicrobial and antifungal activity of EM2 (10 mM) ± $ZnCl_2$ (100 μM and 10 mM) on several bacterial strains (*B. cereus; E. faecalis;*

E. coli; S. aureus; K. pneumoniae; P. mirabilis; P.aeruginosa; S. pneumoniae; S. pyogenes; Y. enterocolitica; S. flexneri; S. enterica) and fungi (*C. albicans; C. parapsilosis*).

The antimicrobial and antifungal activity was determined by means of disk diffusion method. Gentamycin (10 µg) was used as a control for bacilli and staphylococci, gentamycin (120 µg) for streptococci and enterococci and fluconazole (25 µg) for Candida. The zone of inhibition for the antibiotics and the antifungal agent was reported in millimeters and was between 16-19 mm.

The results showed that EM2 elicited a strong antibacterial activity against *Streptococcus pyogenes* (inhibition zone 20 ± 0.26 mm) and a mild one against *Staphylococcus aureus* with an inhibition zone of 13 ± 0.19 mm.

Co-administration of EM2 + $ZnCl_2$ at the lowest concentration tested (100 µM) increased the inhibition zone for *Enterococus faecalis* (14.05 ± 0.39 mm). Increasing $ZnCl_2$ concentration (10 mM) induced the reduction of the antimicrobial effect on this bacterial strain (inhibition zone 11 ± 0.15 mm). Similar effects were obtained on *E. faecalis* when $ZnCl_2$ was administered alone (inhibition zone 14.01 ± 0.29 mm for 100 µM and 11.02 ± 0.39 mm for 10 mM, respectively).

No antimicrobial effect of either compound could be detected on other bacterial strains. Furthermore, neither EM2 alone nor in co-administration with $ZnCl_2$ showed any antifungal properties.

To sum up, EM2 antimicrobial activity mainly targets infections with cocci bacteria. Administration of $ZnCl_2$ (100 µM) together with EM2 proved to be beneficial particularly on *E. faecalis* species.

IV. CYTOTOXIC AND ANTIPROLIFERATIVE ACTIVITY OF EM2 AND ZINC

This present study was aimed to evaluate the cytotoxic and antimigratory effects of EM2 and $ZnCl_2$ on normal keratinocytes (HaCaT) and on various tumor cell lines (A375, B164A6, B16F0, MDA-MB-231, MCF7, HepG2).The cytotoxic effect was measured by MTT assay and the migratory ability was examined by scratch assay. In order to assess the cytotoxic effect, cells were stimulated for 24, 48 and 72h with different concentrations of EM2 alone (1, 5, 10, 25 and 50 µM), $ZnCl_2$ (100 µM) or with EM2 + $ZnCl_2$ (100 µM).

The data obtained showed that EM2 is a cytotoxic compound on tumor cells, while toxic effects were recorded in healthy human keratinocytes. The strongest effect in tumor cells was observed 72h post-stimulation. The antimigratory capacity of the tumor cells was affected after stimulation with EM2. When tested on normal HaCaT human keratinocytes, EM2 stimulated cells proliferation at all tested concentrations

In summary, our findings indicate that EM2 has a cytotoxic effect on the tested cancer cell lines especially at 72h after stimulation. Even more interesting, the compound showed no cytotoxic activity on normal/healthy keratinocytes, indicating its selectivity towards tumor cells.

V. EFFECTS OF EM2 ON THE CHORIOALLANTOIC MEMBRANE ASSAY

The purpose of the present study was to determine the potential toxicity and to investigate the anticancer and antiangiogenic effects of EM2 (100 µM) on a melanoma cell line using the *in vivo* chorioallantoic membrane (CAM) assay. The biologic material used for the CAM assay consists of fertilized hen (*Gallus gallus domesticus*) eggs.

EM2 effects on the normal developing chorioallantoic membrane was assessed after daily application for 5 days of the compound. EM2 properties were further investigated in an *in vivo* melanoma model using the CAM assay after SK-MEL-2 melanoma cells were inoculated inside a sterile ring previously placed on the CAM.

The results indicated that after 5 doses of EM2 daily applied on the normal developing CAM, no signs of toxicity were registered. EM2 presented good tolerability, having similar survival rates as the control group. Similar to the effect on the normal allantoic vasculature, the administration of EM2 on the developing SK-MEL-2 melanoma induced no toxicity, the viability of specimens being similar to that of the non-treated and the non-tumor samples.

An important observation regarding the effect elicited by EM2 upon the SK-MEL-2 developing melanoma is that no secondary tumors were formed; only a few scattered cells were noticed in the proximity of the application ring on day 5, as compared to the control specimens indicating a possible antimetastatic potential of the EM2.

To conclude, EM2 shows no toxicity when applied *in vivo*, on normal and tumor tissues, which makes it a good candidate as therapeutic agent. It induces the limitation of invasiveness of N-RAS mutated melanoma tumors *in vivo* in CAM assay, by possibly targeting tumor angiogenesis and melanoma microenvironment, but without important effect on the proliferation index of tumor cells.

VI. ASSESSMENT OF EM2 AND ITS METABOLITE IN RAT BLOOD BY LIQUID CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY

This present study was aimed to assess the blood level of EM2 and its metabolite by high-throughput liquid chromatography coupled with tandem mass spectrometry method (LC-MS/MS) after oral and intraperitoneal administration, respectively.

The maslinic acid derivative (50 mg/kg of body weight) was administered via the oral and intraperitoneal route to rats. Blood was automatically collected using the Culex System. The samples were analyzed using the validated analytical method and the blood levels of EM2 and its metabolite were determined. The analytical method that was developed for the simultaneous analysis of the two compounds showed good specificity due to the tandem mass spectrometry (MS/MS) detection method.

Our results showed that EM2 had a maximal concentration 4 hours after oral administration, and 5 hours after i.p. administration, respectively. The compound showed a lower bioavailability after oral vs. intraperitoneal administration.

In summary, the LC-MS/MS method developed and validated in this paper has a short runtime, a simple and inexpensive sample preparation technique, and is robust, all important features for high-throughput methods used in routine bioavailability studies.

VII. ASSESSMENT OF THE EFFECTS OF EM2 IN AN EXPERIMENTAL MODEL OF ACUTE SYSTEMIC INFLAMMATION

The aim of this present study was to characterize the effect of a single daily dose of EM2 in the setting of oxidative stress induced by acute experimental inflammation.

Inflammation was induced by the injection of turpentine oil (6 mL/kg b.w., im) to Wistar-Bratislava albino rats. EM2 was given orally by gavage (50 mg/kg b.w./day) for 10 days (EM2 was administered pre and post-turpentine-induced inflammation and also to rats that were not subjected to inflammation). Diclofenac was used as a control for the anti-inflammatory effect and was given orally for 10 days (20 mg/kg b.w./day).

Oxidative stress was evaluated by measuring total nitrites and nitrates (NOx), total oxidative status (TOS), total antioxidant response (TAR), oxidative stress index (OSI), total thiols (SH) and malondialdehyde (MDA) in the serum.

To sum up, the most important results of this study indicated that EM2 induced a decrease in the parameters of oxidative stress but only when adminitered to healthy rats.

Administration before and after turpentine oil-induced inflammation had a low effect on the oxidative stress. Only the prophylactic administration of the compound showed an inhibitory effect on MDA formation that was comparable to the effect of diclofenac.

VIII. EVALUATION OF THE EFFECTS OF EM2 AND ZINC IN AN EXPERIMENTAL MODEL OF ACUTE LOCAL INFLAMMATION

The present study was purposed to characterise the effects of various topically applied formulations (EM2 1% hydrogel, zinc (1% and 5%) hydrogel and EM2 1% plus zinc (1% and 5%)) in an animal model of 12-o-tetradecanoylphorbol-13-acetate (TPA) induced ear inflammation.

The TPA-induced inflammation model is easily obtained and offers rapidly data regarding the beneficial or toxic activity of newly obtained compounds. In order to overcome to poor solubility of EM2 and to improve drug delivery, the compound was incorporated in a hydrogel formulation either alone or in combination with $ZnCl_2$.

The results obtained indicated that topical application of EM2 reduced the TPA-induced inflammation compared to mice treated with hydrogel blank. The histopathological analysis revealed that the group treated with EM2 1% hydrogel presented blood vessels with mild hyperemia + and some of them showed leukocytes margination. The interstitial edema was moderate ++. In the groups treated with $ZnCl_2$ the dermis was hyalinized and the epidermis showed acanthosis. The edema was lower in the group treated with the higher concentration of $ZnCl_2$ (5%). The groups treated with EM2 + $ZnCl_2$ showed edema similar to that observed in the EM2 group, while the dermis hyalinization was similar to that observed in the $ZnCl_2$ group.

In conclusion, the groups treated with EM2 ± $ZnCl_2$ presented an anti-inflammatory effect demonstrated by the mitigation in local edema and induction of favourable changes in the dermis.

IX. ASSESSMENT OF THE EFFECTS OF EM2 IN MURINE MODELS OF CHEMICAL/PHOTOCHEMICAL INDUCED SKIN CARCINOMA AND IN ISOLATED MOUSE LIVER MITOCHONDRIA

The aim of the present study was to analyse the effects of EM2 in two different SKH1 mice models of skin carcinoma induced by i) exposure to chemical agents 7,12-dimetilbenzantracen (DMBA) and 12-o-tetradecanoylphorbol-13-acetate (TPA); and ii) exposure to chemical agents (DMBA and TPA) and ultraviolet B radiations (UVB) followed by investigating the effects on mitochondrial respiration parameters and H_2O_2 production in isolated liver mitochondria.

EM2 was topically applied as a 1% hydrogel, twice per week, after the appearance of papillomas. Our results indicate a decrease in tumor volume following treatment with EM2 in both chemical and photochemical skin carcinoma models.

Another objective of this study was to investigate the effect of EM2 on isolated liver mitochondria from mice from the two experimental animal models. Liver mitochondria were isolated from SKH1 mice by differential centrifugations technique at 4ºC. The study of mitochondrial respiratory rates was performed by measuring the oxygen consumption rates using the high-resolution respirometry method in the presence of complex I (glutamate/malate) and complex II (succinate, plus rotenone to inhibit complex I) substrates.

Our results indicated that in female mice with chemical induced skin carcinoma, the group treated with EM2 1% hydrogel elicited in complex I (CI) an increase in all parameters, State 2 (basal respiration), OXPHOS (ADP-stimulated respiration) and ETS - maximal

respiration (titration with the classical uncoupler FCCP), while in complex II (CII) supported respiration the basal respiration was decreased in the treated group and OXPHOS and ETS presented a slightly increase. On the other hand male mice with chemical induced skin damage displayed on both CI and CII a decrease in all respiratory rates following treatment with EM2 1% hydrogel.

Female mice exposed to photochemical damage exhibited on CI a decrease in basal and active respiration and an increase in ETS parameter in mice with topical application of EM2 hydrogel. Furthermore, on CII, all respiratory rates were increased after EM2 treatment.

Reactive oxigen species (ROS) production was appraised in isolated liver mitochondria using the Amplex Red assay. The results obtained revealed that in mice with chemical induced skin carcinoma, EM2 treatment induced a significant decrease of H_2O_2 production following complex I activation, an effect that could not be observed in the case of complex II activation. Furthermore, mice with photochemical induced lesions did not display a significant effect on mitochondrial H_2O_2 production following EM2 treatment.

To sum up, our data denoted that topical application of EM2 as a 1% hydrogel elicited a beneficial effect on the chemical and photochemically-induced skin lesions. However, heterogeneous results were obtained with respect to mitochondrial respiration and ROS production, that showed sex-, and substrate-depedency and also varied according to the skin cancer model. The compound mitigated the oxidative stress in the model of chemical cancer, an effect that was not evident anymore in the more severe form of photochemical skin cancer.

FINAL CONCLUSIONS

1. The physico-chemical characterization of the benzylamide derivative of maslinic acid, EM2, revealed that the compound has irregular shape with spherical forms and can form agglomeration. The sample size is of the micro order and it consists of 3 elements, namely C, O and N.
2. EM2 revealed a mild antioxidant activity, which was concentration-independent in the range of 1-200 μM. The antioxidant activity of EM2 was slightly enhanced by the association with $ZnCl_2$ (at the concentration of 100 μM).
3. The antibacterial activity of EM2 was significant against infections with cocci bacteria. Administration of $ZnCl_2$ (100 μM) together with EM2 proved to be beneficial against *E. faecalis.*
4. EM2 has a cytotoxic effect on tumor cells (melanoma, breast carcinoma, hepatocellular carcinoma) especially at 72 h post-stimulation. The cytotoxic effect was more potent on human melanoma cells than on murine melanoma cells. The compound did not show cytotoxic activity on healthy keratinocytes indicating its selectivity towards tumor cells.
5. EM2 induces the limitation of invasiveness of N-RAS mutated melanoma tumors *in vivo* in CAM assay, by possibly targeting tumor angiogenesis and melanoma microenvironment, but without important effect on the proliferation index of tumor cells. Following EM2 application, no secondary tumors were formed upon the SK-MEL-2 developing melanoma, indicating a possible antimetastatic potential of the EM2.
6. The compound showed a lower bioavailability after oral vs. intraperitoneal administration.
7. EM2 was associated with a mild antioxidant effect in experimental model of acute systemic inflammation. In particular, the prophylactic administration of the compound showed an inhibitory effect on MDA that was comparable to the effect of diclofenac.
8. EM2 elicited a mild anti-inflammatory effect in an experimental model of local, TPA-induced acute ear inflammation.

9. In murine models of skin carcinoma, topical application of EM2 elicited a beneficial effect on the chemical and photochemically-induced skin lesions.
10. In isolated liver mitochondria, gender- and substrate- dependent changes in respiratory rates were described; these changes also varied according to the experimental model of carcinogenesis.
11. EM2 induced a significant decrease in H_2O_2 production in the presence of complex I dependent substrates in the case of chemical (but not photochemical) skin carcinoma model.

ORIGINAL CONTRIBUTIONS

The original contributions can be summarized as follows:

1. Assessment of the antioxidant effects of the benzylamide derivative of maslinic acid effects alone or in combination with $ZnCl_2$.
2. Assessment of the antimicrobial and antifungal properties of EM2 ± ZnC_{l2} in several bacterial strains.
3. Evaluation of the cytotoxic and antiproliferative properties of EM2 in several tumor cell lines and normal human keratinocytes.
4. Assessment of the EM2 toxicity, the effects on the developing SK-MEL-2 melanoma model, as well as the implications on the normal and melanoma angiogenesis in the CAM model.
5. Validation of the LC-MS/MS method for the quantification of the benzylamide derivative of maslinic acid (EM2) and its metabolite.
6. Assessment of the EM2 effects in two experimental models of systemic and local acute inflammation.
7. Assessment of EM2 effects topically applied as hydrogel in two experimental models of carcinogenesis.
8. Assessment of the changes in respiratory function and ROS production in isolated liver mitochondria elicited by the malignant process in the presence vs. the absence of topical treatments.

Key words: maslinic acid derivative, skin cancer, inflammation, mitochondria, antioxidant activity, antimicrobial activity

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LIST OF PUBLISHED PAPERS

1. **Ioana Zinuca Pavel**, Corina Danciu, Camelia Oprean, Cristina Adriana Dehelean, Delia Muntean, René Csuk, Danina Mirela Muntean. *In Vitro Evaluation of the Antimicrobial Ability and Cytotoxicity on Two Melanoma Cell Lines of a Benzylamide Derivative of Maslinic Acid*. **Anal Cell Pathol (Amst). 2016**; 2016: 2787623. doi: 10.1155/2016/2787623.

(ISI journal, IF: 1.078)

2. **Ioana Zinuca Pavel**, Cristina Adriana Dehelean, Lenard Farczadi, Dana Maria Muntean, Laurian Vlase, Corina Danciu, René Csuk, Florin Birsasteanu, Danina Mirela Muntean. *Assessment of a Maslinic Acid Derivative and its Metabolite in Rat Blood by Liquid Chromatography Coupled with Mass Spectrometry*. **Rev Chem (Bucharest). 2017**; 68(5), 1089-1094.
   (ISI journal, IF: 1.232)

3. **Ioana Zinuca Pavel**, Alina Elena Pârvu, Cristina Adriana Dehelean, Laurian Vlase, René Csuk, Danina Mirela Muntean. *Assessment of the Antioxidant Effect of a Maslinic Acid Derivative in an Experimental Model of Acute Inflammation*. **Farmacia, 2017**; 65(3), 390-395.
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4. **Ioana Zinuca Pavel**, Oana Andrada Iftode, Iulia Pinzaru, Dorina Coricovac, Alina Moaca, Claudia Farcas, Sebastian Claudiu Simu, Codruta Soica, Cristina Dehelean, Andrei Motoc. *Skin Specific Cells and UVB Damage – An experimental assessment*. **Rev Chem (Bucharest). 2017**; 68(6), 1227-1331.
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