ORIGINAL ARTICLE

ASSESSMENT OF THE ANTIOXIDANT EFFECT OF A MASLINIC ACID DERIVATIVE IN AN EXPERIMENTAL MODEL OF ACUTE INFLAMMATION

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Manuscript received: September 2016

Abstract

Maslinic acid (MA) is a pentacyclic triterpenoid recognized for multiple therapeutic effects including antidiabetic, anti-inflammatory, antitumor, antioxidant and antiviral properties. Structural changes of MA were performed in order to obtain new derivatives with enhanced properties. The aim of the study was to evaluate the effect of a benzylamide derivative of maslinic acid ("EM2") (Benzyl (2α, 3β) 2,3-diacetoxy-olean-12-en-28-amide) on acute inflammation-induced oxidative stress. The effect of EM2 on oxidative stress was assessed in an experimental model of turpentine oil-induced acute inflammation in rats 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). The compound was administered prior to and after the induction of the inflammation. In the experimental model of acute inflammation only prophylactic administration elicited an antioxidant effect through a decrease of MDA, the effect being comparable to that of diclofenac ($p < 0.01$).

Rezumat

Acidul maslinic (AM) este o triterpenă pentaciclică ce posedă numeroase efecte terapeutice precum: antidiabetic, anti-inflamator, antitumoral, antioxidant şi antiviral. Modificări structurale ale AM au fost efectuate pentru obţinerea unor derivaţi ce prezintă efecte terapeutice îmbunătăţite. Scopul prezentului studiu constă în evaluarea efectului unui derivat de acid maslinic ("EM2") (Benzil (2α, 3β) 2,3-diacetoxi-olean-12-en-28-amida) la nivelul stresului oxidativ indus de inflamaţia acută. Efectul compusului EM2 a fost studiat pe un model experimental de inflamaţie acută indusă de uleiul de terebentină prin determinarea nitriţilor şi nitraţilor totali (NOx), statusului oxidativ total (TOS), răspunsul antioxidant total (TAR), indexul stresului oxidativ (OSI), tiolilor (SH) şi malondialdehidei (MDA). Compusul a fost administrat anterior şi respectiv, după inducerea inflamaţiei. În cazul modelului experimental de inflamaţie acută doar administrarea profilactică a compusului a prezentat un efect antioxidant prin scăderea nivelului MDA, efectul fiind comparabil cu cel al diclofenacului ($p < 0,01$).

Keywords: benzylamide derivative of maslinic acid, inflammation, oxidative stress, antioxidant

Introduction

Maslinic acid is a pentacyclic triterpenoid mainly found in the olive tree (*Olea europaea* L.) [16] recognized for multiple therapeutic effects, such anti-inflammatory [8, 11], antioxidant [17] and anti-tumour [14, 20].

The antitumoural activity of maslinic acid was reported to occur in several types of cancer, including: pancreatic [14], colon [23] and melanoma [20]. Similar cytotoxic effects have been recently reported for its derivatives [27]. Derivatization is a technique used in order to obtain compounds with improved therapeutic effects.

Maslinic acid anti-inflammatory effect was evaluated on rat astrocytes cultures stimulated with lipopolysaccharide by a research group. These authors reported a significant anti-inflammatory effect by inhibiting the production of nitric oxide and tumor necrosis factor alpha (TNF-α) [11].

Besides the *in vitro* anti-inflammatory activity of maslinic acid, *in vivo* effects have also been reported. In a mouse model of ear oedema, maslinic acid extracted from *Eriobotrya japonica* inhibited TPA-induced inflammation (ID_{50} = 0.13 mg/ear) [2]. Furthermore, many plants (*Ecballium elaterium* L., *Bryonia alba* L. [12], and marine microalgae

(*Phyllophora pseudoceranoides* [29]) are known to possess anti-inflammatory and antioxidant properties.
The present study aimed to characterize the effect of a single daily dose of EM2 (Benzyl (2α, 3β) 2,3-diacetoxy-olean-12-en-28-amide) (Figure 1) in the setting of oxidative stress induced by acute experimental inflammation.

Materials and Methods

Chemicals and reagents
EM2 with a cytostatic activity comparable to maslinic acid (being more toxic to cancer cells than to primary human fibroblasts) was a kind gift from Prof. Rene Csuk. The compound was obtained as previously mentioned in the literature [27] (Figure 1).

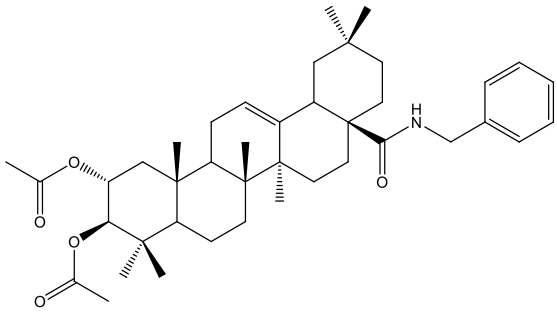

Figure 1.
Chemical structure of EM2

Experimental turpentine induced inflammation
The study protocol was approved by the Institutional Animal Ethical Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca, Romania. The experiments were performed on adult male Wistar-Bratislava albino rats, weighing 200 - 250 g. Inflammation was induced by injection of turpentine oil (6 mL/kg b.w., im) [22]. EM2 was administered orally by gavage (50 mg/kg b.w./day) [26] for 10 days. The animals were randomly assigned to six groups (n = 5): a group was treated orally only with EM2 for 10 days (EM2 group), a group was injected with turpentine oil in day one and then treated for 10 days with EM2 (T/EM2 group), a group was treated for 10 days with EM2 and then injected with turpentine oil in day 10 (EM2/T group), a positive inflammation group was injected with turpentine oil in day one and then treated orally with saline solution (1 mL NaCl 0.9%/day) for 10 days (T group), a group of anti-inflammatory control was injected with turpentine oil in day one and then treated orally for 10 days with diclofenac (T/DIC) (20 mg/kg b.w./day), and a negative control group (CONTROL) was treated orally only with saline (1 mL NaCl 0.9%/day) for 10 days.
Animals were fed *ad libitum* and kept under standard conditions (constant temperature and humidity of 22.5 ± 2°C and 55 + 5%, 12 h light/dark cycle).
At the end of the study rats were anesthetized using a combination of ketamine (50 mg/kg b.w.) and xylazine (20 mg/kg b.w.) [13], blood was withdrawn by retro-orbital puncture, serum separated and stored at -80°C until use. The experiments were performed in triplicate. At the end of the experiments the animals were sacrificed by cervical dislocation.
Assessment of oxidative stress
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 malondi-aldehyde (MDA) in the serum.
First serum samples were passed through 10 kDd filters (Sartorius AG, Goettingen, Germany) and contaminant proteins were removed by extraction with a 3:1 (v:v) solution of methanol/diethyl ether. The sample methanol/diethyl ether ratio was 1:9 (v:v) [9].
The Griess reaction was used to indirectly determine NOx. Briefly, 100 µL of 8 mg/mL VCl3 were added to 100 µL of filtered and extracted serum supernatant in order to reduce nitrate to nitrite, followed by the addition of the Griess reagents, 50 µL of SULF (2%) and 50 µL of NEDD (0.1%). After 30 min incubation at 37°C, the sample absorbance was registered at 540 nm. The concentration of serum NOx was determined using a sodium nitrite-based curve, and expressed as nitrite µmol/L [18].
TOS of serum was measured using a colorimetric assay [6]. This assay measures the oxidation of ferrous ion to ferric ion in the presence of various reactive oxygen species in an acidic medium. The ferric ion was detected by reaction with xylenol orange. Assay measurements were standardized using hydrogen peroxide (H_2O_2) as the oxidative species, and the assay results are expressed in µmol H_2O_2 Equiv/L.
TAR was measured in serum using a colorimetric assay [5]. In this assay the rate of hydroxyl radical production by the Fenton reaction was monitored by following the changes in the absorbance of coloured dianisidyl radicals. Upon addition of a serum sample, the hydroxyl radical initiated oxidative reactions are suppressed by antioxidant the subsequent colour change, thereby effectively measuring the total anti-oxidant capacity of the serum. This assay is calibrated using trolox and results are expressed as mmol trolox Equiv/L.
OSI, an indicator of the degree of oxidative stress [9], was calculated as follows: OSI (Arbitrary Unit) = TOS (mol H_2O_2 Equiv/L)/TAR (mmol trolox Equiv/ L).
SH were estimated by using Ellman's reagent [10]. To a final volume of 4.0 mL, there were added 0.2 mL serum and 0.6 mL of 20 mM tris-HCl buffer pH 8.2, followed by addition of 0.04 mL of 10 mM 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB) in

absolute methanol and 3.16 mL of absolute methanol. After 15 min at room temperature the tubes were centrifuged at 3,000 xg for 20 min, supernatant was collected and the absorbance was registered at 412 nm.

MDA was measured as an index of lipids per-oxidation using thiobarbituric acid (TBA) reagent [19]. To 0.2 mL serum was added 0.2 mL, 8.1% (w/v), sodium dodecylsulphate, 1.5 mL 20% (v/v) acetic acid and 1.1 mL of 0.8% (w/v) TBA to make up the volume to 3 mL. The tubes were heated in a water bath at 95°C for 1 h and cooled immediately under running tap water. To each tube, 1.0 mL water and 5.0 mL solution of butanol and pyridine (15:1 v/v) were added, vortexed and centrifuged at 800 xg for 20 min. The upper layer was aspirated out and colour intensity measured at 532 nm. 1,1,3,3-tetra ethoxy propane was used as the reference.

All of the spectroscopic measurements were performed using a Jasco V-530 UV-Vis spectrophotometer (Jasco International Co., Ltd., Tokyo, Japan).

Statistical analysis

All results were expressed as the mean ± SD for normally distributed data. Otherwise, the median and first quartile (Q1) and third quartile (Q3) were reported. One way ANOVA test was performed to determine the variability among groups. Significant differences among groups were calculated by post-hoc Bonferroni multiple comparison tests. Statistical significance was set at $p < 0.05$. Analyses were performed using GraphPad Prism 5.

Results and Discussion

Assessment of Oxidative Stress

In order to determine EM2 effect in an acute experimental inflammation, markers of oxidative stress, including TOS, TAC, OSI, NOx, SH and MDA, were measured in the serum from turpentine oil-treated rats. Oral administration is one of the most common used treatment methods. Therefore, in order to evaluate the anti-inflammatory effect of EM2, the compound was orally administrated to Wistar rats. The dose used (50 mg/kg b.w.) was previously described in the literature for maslinic acid [15, 24, 25].

Assessment of Serum Nitrites and Nitrates Levels (NO). NO production can be assessed indirectly by measuring serum nitrites and nitrates levels [1].

Turpentine oil is a non-antigenic inflammatory stimulus [4] that activates phagocytes. The process includes reactive oxygen species (ROS) and NO production. At low NO fluxes, NO reacts with ROS to generate reactive nitrogen species (RNS) and amplify the oxidative stress, whereas at higher levels of NO nitrosation reactions would predominate [7].

Yang *et al*. evaluated the effect of two maslinic acid derivatives on nitric oxide production and radical-scavenging activity [30]. The two compounds, 2-O-trans-p-coumaroyl maslinic acid (1) and 2-O-caffeoyl-maslinic acid (2) were extracted from *Hippophae rhamnoides* L. Compound 2 showed a significant inhibition of NO production in RAW 264.7 cells, while compound 1 presented only a small suppression of NO probably due to the caffeoyl group present in the second compound. Compound 2 also indicated antioxidant activity, effect that can be linked with the higher number of hydroxyl groups [30].

In the present study, after 10 days from turpentine oil administration serum NOx was not significantly elevated in the inflammation group ($p > 0.05$) as compared to the control rats (Figure 2). Compared to group T, diclofenac decreased NO production ($p < 0.05$). Interestingly, EM2 administrated alone elicited an important NO lowering effect when compared to control and T animals ($p < 0.01$). Treatment with EM2, either prior or after inflammation induction, did not interfere with NO synthesis ($p > 0.05$). Perhaps NO slight increase due to the inflammation was compensated by EM2 inhibitory activity.

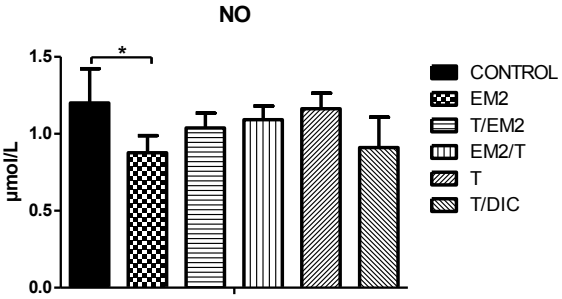

Figure 2.
EM2 effects on serum NO concentration

Assessment of Serum Total Oxidant Status (TOS). The measurement of TOS reflects the additive oxidative effect of different molecules [6]. Turpentine-induced inflammation increased TOS ($p < 0.001$) and treatment with diclofenac significantly reduced it ($p < 0.01$). Treatment with EM2 before and after inflammation induction had no important effect on TOS ($p > 0.05$). EM2 alone had no important effect ($p > 0.05$) when compared to the control group (Figure 3).

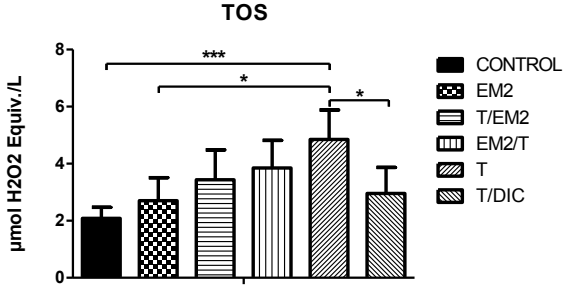

Figure 3.
The effect of EM2 administration on TOS

Assessment of Serum Total Antioxidant Response (TAR) and Total Thiols (SH). TAR has been developed since *in vivo* antioxidant systems work together and not isolated [5]. It is known TAR to be inversely proportional to the oxidative stress [1]. In Figure 4 it is represented that treatments with EM2 alone, before or after inflammation induction showed no significant change of TAR when compared to the inflammation group ($p < 0.05$). The same was found in diclofenac group.

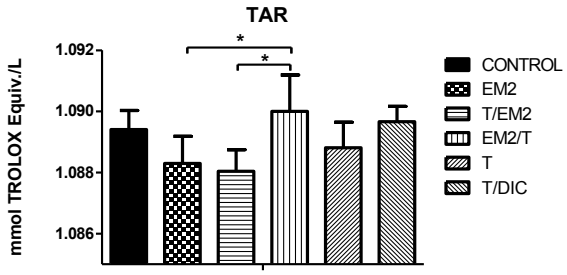

Figure 4.
The effect of EM2 administration on TAR

Free thiol groups of proteins are mainly responsible for their antioxidant effect. During the study total thiols (SH) level was not significantly influenced by the EM2 treatments too ($p > 0.05$) (Figure 5).

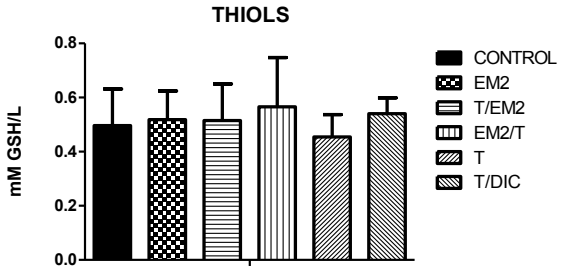

Figure 5.
The effect of EM2 administration on total thiols (SH)

Assessment of Malondialdehyde Levels (MDA) and Oxidative Stress Index (OSI). MDA is a product of lipid peroxidation that can induce alteration at mitochondrial level and can affect the body response against oxidative stress [3]. In a mouse model of diabetic retinopathy, intravitreal administration of ursolic acid, another pentacyclic triterpene, induced a significant decrease of MDA [28]. As showed in Figure 6 MDA level was increased in the inflammation group ($p < 0.001$) and diclofenac had an important inhibitory effect ($p < 0.01$). Only EM2 alone ($p < 0.001$) and the prophylactic EM2 treatments reduced MDA synthesis. In EM2/T group the effect was comparable to that of diclofenac ($p < 0.01$).

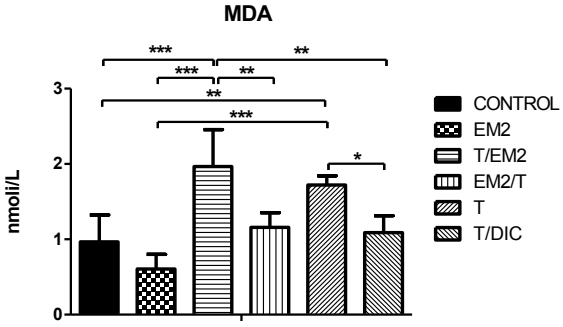

Figure 6.
The effect of EM2 administration on MDA

OSI is calculated as the ratio between TOS and TAR and is a parameter of the degree of oxidative stress [9]. In the inflammation group OSI was significantly elevated ($p < 0.001$) compared to the control group, and diclofenac treatment decreased it ($p < 0.01$). T/EM2 and EM2/T did not induce a significant decline in OSI ($p > 0.05$) (Figure 7).

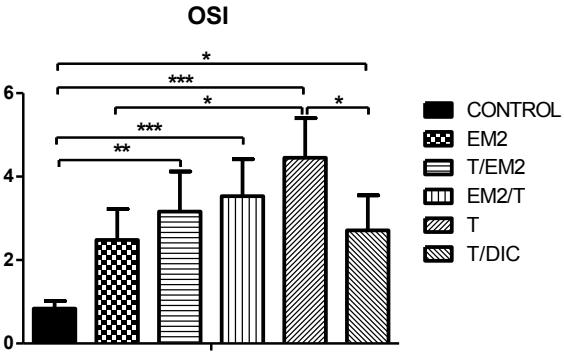

Figure 7.
The effect of EM2 administration on OSI

Our previous results showed that EM2 possesses antitumour and antimicrobial properties. We recently demonstrated that 72 h incubation with EM2 induced a cytotoxic effect on A375 human melanoma cell line and B164A5 murine melanoma cells in a dose dependent manner [21]. Furthermore, the derivative was tested for its antimicrobial activity on several bacterial strains. The compound elicited at a concentration of 10 mM a significant antibacterial activity for *Streptococcus pyogenes* and *Staphylococcus aureus* [21].

Conclusions

When administrated in healthy rats, EM2 decreased the parameters of oxidative stress, NOx, TOS, OSI and MDA. However, 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.

Further studies are required to assess the pharmacokinetics and pharmacodynamics of this compound in the setting of experimental models of chronic, non-communicable diseases, including cancer.

Acknowledgement

The work was supported by the university grant for young researchers PII-C4-TC-2016-16441-10 (I.Z.P.).

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