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Synthesis and *in vitro* antiproliferative evaluation of PEGylated triterpene acids

Marta Medina-O'Donnell[a], Francisco Rivas[a,*], Fernando J. Reyes-Zurita[b,*], Antonio Martinez[a], Francisco Galisteo-González[c], Jose A. Lupiañez[b], Andres Parra[a,*]

[a] Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain
[b] Departamento de Bioquímica y Biología Molecular I, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain
[c] Departamento de Física Aplicada, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain

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ABSTRACT

A set of PEGylated derivatives of oleanolic and maslinic acids has been semi-synthesised, attaching ethylene glycol, diethylene glycol, triethylene glycol or tetraethylene glycol to the C-28 carboxyl group of these natural triterpenes and some derivatives. Another set of PEGylated derivatives has been semi-synthesised by connecting the same four ethylene glycols to the hydroxyl groups of the A ring of these triterpenic acids, through a carbonate linker, by reaction with trichloromethyl chloroformate. The aqueous solubility of some of these PEGylated derivatives has been compared with that of maslinic acid. The cytotoxic effects of 28 triterpenic PEGylated derivatives in three cancer-cell lines (B16-F10, HT29, and Hep G2) have been assayed. The best results have been achieved with the HT29 cell line, and specifically with the oleanolic acid derivatives having ethylene glycol or tetraethylene glycol attached to the C-28 carboxyl group, which are approximately 27-fold more effective than their natural precursor. Eight PEGylated derivatives have been selected to compare the cytotoxicity results in the HT29 cancer-cell line with those of a non-tumour cell line of the same tissue (IEC-18), four of which were less cytotoxic in the non-tumour cell line. These compounds showed apoptotic effects on treated cells, with percentages of total apoptosis between 20% and 53%, relative to control, at 72 h and IC_{50} concentration, and between 29% to 62%, relative to control, for the same time and IC_{80} concentration. We have also found that with the treatment of these compounds in HT29 cancer cells, cell-cycle arrest occurred in the G0/G1 phase. Finally, we have also studied changes in mitochondrial membrane potential during apoptosis of HT29 cancer cells, and the results suggest an activation of the extrinsic apoptotic pathway for these compounds.

1. Introduction

Natural products have been central to traditional medicine for many years [1,2]. Nature has been a source of drugs since the beginning of history in many cultures, and about half of the drugs available today are related to natural compounds [3,4]. On the other hand, the traditional Mediterranean diet is characterized by the intake of vegetal foods such as grapes and olives, and their corresponding liquid extracts, wine and oil. Consumption of this type of food has often been associated with a low incidence of the different types of cancer [5–8]. The olive-oil industry produces a large amount of waste, solids, and liquids, which represent a serious environmental problem in many producing regions [9,10]. However, these wastes contain a set of remarkable chemical substrates and therefore constitute a potentially valuable resource [11,12].

Pentacyclic triterpene acids, such as oleanolic acid (3β-hydroxyolean-12-en-28-oic acid, OA, **I**) [13] and maslinic acid (2α,3β-dihydroxyolean-12-en-28-oic acid, MA, **II**) [14] are two natural products present in abundance in industrial olive-oil waste [15]. These triterpene acids have some promising biological properties as anti-tumour [16–25], antioxidant [26,27], antimicrobial [28,29], antimalarial [30], anti-inflammatory [31] agents. Certain structural modifications of these triterpenoids can have a strong impact on their biological activities [32–34]. Some of these structural modifications involve the formation of simple derivatives at several functional positions of their skeleton. In recent years, our research group has reported the semi-syntheses of various triterpene derivatives from the corresponding natural products and has evaluated its cytotoxicity and apoptotic capacity in several cancer-cell lines, showing a significant enhancement of these activities [35,36].

Polyethylene glycol (PEG) is a synthetic polymer which, for its non-toxic, non-immunogenic, non-antigenic, and non-amphiphilic proper-

* Corresponding authors.
E-mail addresses: frivas@ugr.es (F. Rivas), ferjes@ugr.es (F.J. Reyes-Zurita), aparra@ugr.es (A. Parra).

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ties, has a key function in drug delivery [37,38]. The strategy called PEGylation is the incorporation of polyethylene glycol reagents of different chain lengths into proteins, peptides or small molecules. Currently, PEGylation technologies are widely used in the preparation of drugs and there are a large number of commercially available PEGylation reagents [39,40]. Recently, our research group has reported the semi-synthesis and antiproliferative evaluation of several PEGylated derivatives of oleanolic and maslinic acids [41].

In this study, other PEGylated derivatives of oleanolic and maslinic acids have been semi-synthesised to test for their biological activities. Firstly, we prepared a set of PEGylated derivatives, attaching ethylene glycol, diethylene glycol, triethylene glycol or tetraethylene glycol to the C-28 carboxyl group of the natural triterpenes (OA and MA) and some derivatives. Secondly, another set of PEGylated derivatives was semi-synthesised by connecting the same four ethylene glycols to the hydroxyl groups of the A ring of the triterpenic acids (OA and MA), through a carbonate linker, by reaction with trichloromethyl chloroformate. We have also compared the aqueous solubility of some PEGylated derivatives of MA with that of MA. Finally, we also tested the cytotoxic effects of these 28 PEGylated derivatives on three cancer-cell lines (B16-F10, HT29 and Hep G2). We have selected eight of these derivatives to compare the cytotoxicity results in the HT29 cancer-cell line with those of a non-tumour cell line from the same tissue (IEC-18). Four of these compounds were then chosen to perform various cytometric assays. All of the cytotoxic compounds tested were active in the apoptotic process. In addition, we established the percentage of cells in the different cell-cycle phases. Finally, we studied the changes in the mitochondrial membrane potential (MMP) to formulate hypotheses on the plausible apoptotic mechanisms activated by the different compounds tested.

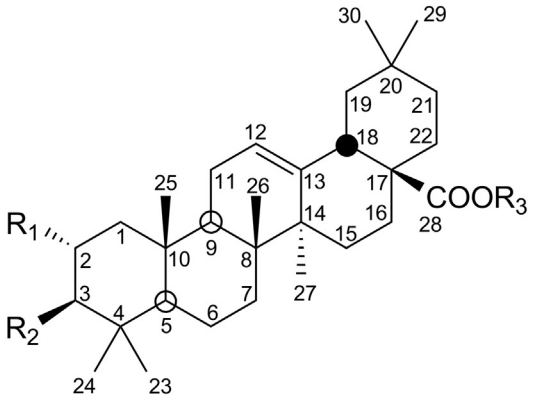
2. Results and discussion

2.1. Semi-synthesis of PEGylated derivatives on the C-28 carboxylic group

Oleanolic acid (**I**, 3β-hydroxyolean-12-en-28-oic acid, OA) and maslinic acid (**II**, 2α,3β-dihydroxyolean-12-en-28-oic acid, MA) are natural pentacyclic triterpenes with an oleanane skeleton (Fig. 1). These compounds (OA and MA), widely present in the plant kingdom, were obtained from wastes from the olive-oil industry [42]. These triterpene acids were acetylated to protect the hydroxyl group/s of the A ring, yielding the corresponding 3β-acetylated (**III**, OA-Ac) or 2α,3β-diacetylated (**IV**, MA-Ac) derivatives [43,44]. Similarly, to protect the C-28 carboxyl group, these triterpene acids (OA and MA) were benzylated, producing the corresponding benzyl derivatives (**V**, OA-Bn, and **VI**, MA-Bn) [45,46].

The starting compounds **I–IV** were used to get a set of PEGylated triterpene derivatives by forming an ester bond between the C-28 carboxylic group and several polyethylene glycol reagents with different chain lengths. Four PEG reagents (mono-, di-, tri-, or tetra-ethylene glycol) were chosen to analyse how much influence the length of this glycol chain has on the chemical and biological properties of these PEGylated derivatives. The semi-syntheses of these PEGylated derivatives were performed in two consecutive reactions. The first consisted of activating the C-28 carboxyl group of each triterpene acid by forming the corresponding TBTU-derivative. Thus, each starting product (**I-IV**) was treated with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumtetrafluoroborate (TBTU), in the presence of *N*,*N*-diisopropylethylamine (DIEA), in DMF [41]. In the second step, each of these four activated C-28 intermediates were now treated with the corresponding PEG reagent (mono-, di-, tri-, or tetra-ethylene glycol), in the presence of K_2CO_3, in dioxane, yielding 16 PEGylated derivatives (**1–16**) (Scheme 1).

The NMR spectra of these compounds (**1–16**) were similar to their corresponding precursors (**I–IV**), the main differences being the proton signals of the PEG group of each compound. Thus, the ^{1}H NMR spectra showed a signal at about δ_H 4.20 due to the methylene group attached directly to the C-28 carboxyl group of the corresponding triterpene, and between δ_H 3.80–3.50, other signals due to the other methylene groups of the corresponding PEG group. The ^{13}C NMR spectra of these compounds (**1–16**) differed from those of their corresponding precursors (**I–IV**), in the number of signals due to the PEG group of these derivatives (2, 4, 6, or 8 signals, respectively), which were situated between 60 and 70 ppm.



I: $R_1 = R_3 = H$, $R_2 = OH$ (oleanolic acid, **OA**)

II: $R_1 = R_2 = OH$, $R_3 = H$ (maslinic acid, **MA**)

III: $R_1 = R_3 = H$, $R_2 = OAc$ (3β-acetyl oleanolic acid, **OA-Ac**)

IV: $R_1 = R_2 = OAc$, $R_3 = H$ (2α,3β-diacetyl maslinic acid, **MA-Ac**)

V: $R_1 = H$, $R_2 = OH$, $R_3 = Bn$ (28-benzyl oleanolate, **OA-Bn**)

VI: $R_1 = R_2 = OH$, $R_3 = Bn$ (28-benzyl maslinate, **MA-Bn**)

Fig. 1. Structures of precursors (**I–VI**).