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Research paper

Diamine and PEGylated-diamine conjugates of triterpenic acids as potential anticancer agents

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ABSTRACT

A set of 18 amide derivatives of oleanolic or maslinic acid has been semi-synthesised. Twelve were diamine conjugates at C-28 of these triterpenic acids and the other six were PEGylated-diamine derivatives. The cytotoxic effects of these 18 triterpenic derivatives in three cancer-cell lines (B16-F10, HT29, and Hep G2) have been assayed, and have been compared to three non-tumour cell lines of the same or a similar tissue (HPF, IEC-18, and WRL68). The cell viability percentages for the non-tumour HPF line for almost all diamine conjugates of the tested triterpenic acids ranged from 81% to 94%. The best cytotoxic results were achieved with the diamine conjugates of oleanolic or maslinic acid with the shortest and the longest diamine chain (IC_{50} values from 0.76 μM to 1.76 μM), on the B16-F10 cell line, being between 140- and 20-fold more effective than their corresponding precursors. Four diamine conjugates of these triterpenic acids showed apoptotic effects on treated cells of the B16-F10 line, with total apoptosis rates, relative to control, of between 73% and 90%. The DNA-histogram analysis revealed that all compounds tested produced cell-cycle arrest in B16-F10 cells, increasing the number of these cells in the S phase. All the compounds analysed, except one, did not cause changes in mitochondrial-membrane potential during apoptosis of the B16-F10 cancer cells, suggesting an activation of the extrinsic apoptotic pathway for these compounds.

1. Introduction

Chemotherapy is a longstanding treatment against cancer, despite that it usually causes significant side effects [1,2]. A number of new anti-cancer drugs have been developed from natural sources or by structural modification of natural products [3,4]. The search for better cytotoxic agents remains key in the discovery of drugs with anticancer activity. The semi-synthesis of new products through the structural modification of the functional groups of natural compounds can produce derivatives with greater biological activity and with fewer side effects [5,6].

Triterpenoids, natural compounds present in many plants from nature, have been used as anti-carcinogenic, anti-inflammatory, anti-oxidant, antiviral, anti-bacterial, antifungal and anti-diabetic agents and which exhibit hepato-, cardio-, and neuroprotective properties [7]. Particular attention has been paid to the study of the anticancer ability of these triterpenic compounds because they show relevant cytotoxic properties against tumour cells while maintaining low activity against normal cells [8–13].

Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid, OA, **I**) [14] and maslinic acid (2α,3β-dihydroxyolean-12-en-28-oic acid, MA, **II**) [15] are two naturally occurring pentacyclic triterpenic compounds which are abundant in industrial olive-oil waste [16]. These triterpenic acids have promising pharmacological properties [17,18]. Certain structural modifications of these triterpenoids, which in many cases imply the formation of simple derivatives in the functional groups of the molecules, can have a strong impact on their biological properties [19–21]. In recent years, our research group has reported the semi-synthesis of a large number of derivatives of these natural triterpenic acids, which inhibit the proliferation and induce apoptosis in several cancer-cell lines [22–25].

A relatively high percentage of drugs have an amide group in

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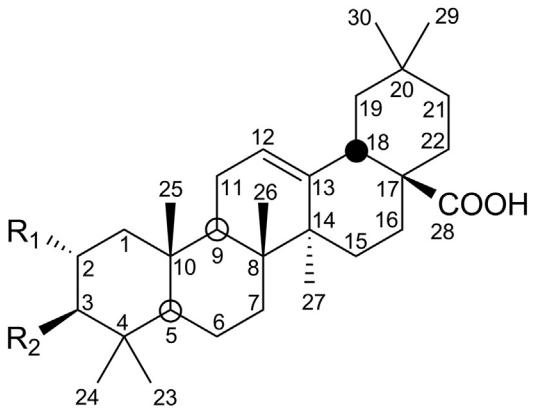
their structure. Furthermore, several studies have recently been published on the increase in the biological activities of the C-28 amino conjugates of various pentacyclic triterpenic acids with respect to their natural precursors [26–31]. Also, several diamine conjugate dimers of oleanolic acid have been designed and synthesised for various biological applications [32,33]. These studies support the contention that the synthesis of triterpenic amide derivatives can be effective in the search for novel cytotoxic agents to treat cancer.

In this study, several C-28 amide derivatives of oleanolic or maslinic acids have been semi-synthesised to test for their biological properties. Firstly, a set of 12 derivatives was prepared, attaching propane-1,3-diamine, hexane-1,6-diamine, or decane-1,10-diamine to one or two units of oleanolic or maslinic acid, through the carboxyl group of C-28 of these natural triterpenes. Secondly, another set of six PEGylated derivatives was semi-synthesised by connecting each of the six C-28 amide derivatives of OA or MA, with a free amino group, to a PEG-acid reagent (3,6,9-trioxadecanoic acid). Finally, the cytotoxic effects of these 18 amide derivatives on three cancer-cell lines (B16-F10, HT29, and Hep G2) were tested, and the results of 12 of them, the most active, were compared to three non-tumour cell lines of the same or a similar tissue (HPF, IEC-18, and WRL68). Percentages of live non-tumour cells were calculated using the IC_{50} values of the triterpenic compounds on the corresponding cancer cells. Eight of these compounds were selected to perform various cytometric assays. All tested cytotoxic compounds were active in the apoptotic process. In addition, we established the percentage of cells in the different cell-cycle phases, and we also 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. Chemistry

Oleanolic acid (**I**, 3β-hydroxyolean-12-en-28-oic acid, OA) and maslinic acid (**II**, 2α,3β-dihydroxyolean-12-en-28-oic acid, MA) (Fig. 1) are naturally occurring pentacyclic triterpenes present in high proportions in olive-oil wastes. These compounds can be isolated by different extraction methods, using solvents of different polarity, such as methanol, ethyl acetate and/or n-hexane [34,35].



I: R_1 = H, R_2 = OH (oleanolic acid, **OA**)
II: R_1 = R_2 = OH, (maslinic acid, **MA**)

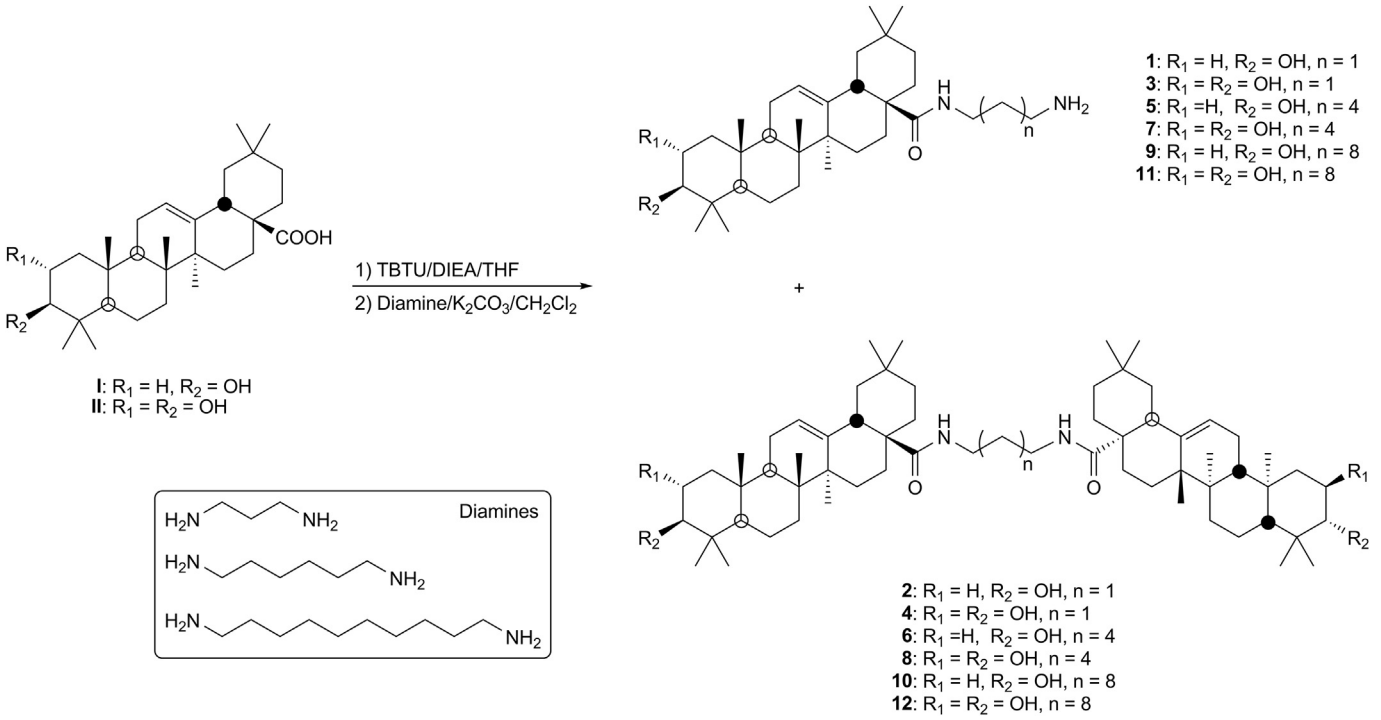
Fig. 1. Structures of oleanolic and maslinic acid.

These triterpene acids (OA or MA) have been used as substrates for the amidation and PEGylation reactions described below.

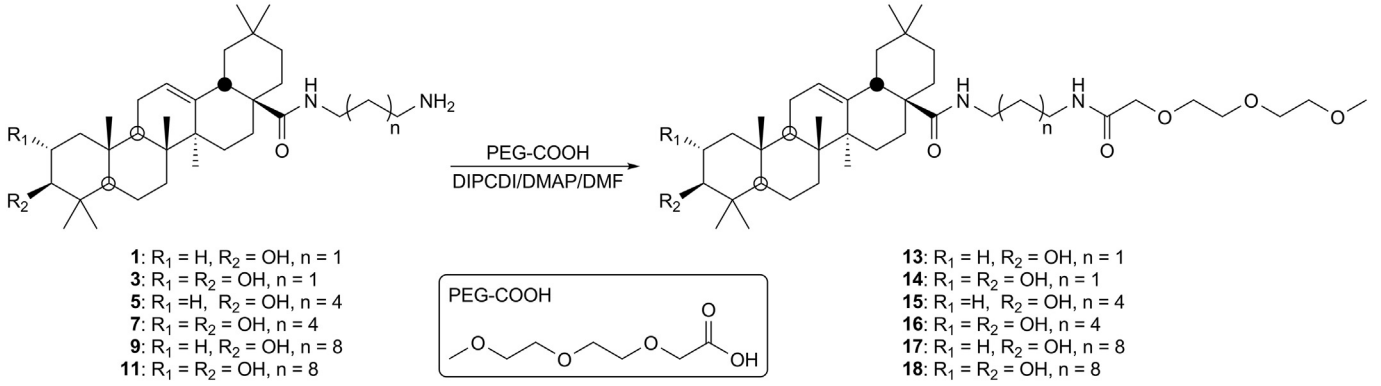
Several C-28 amide derivatives of oleanolic or maslinic acid were semi-synthesised to evaluate their biological properties. These OA- or MA-diamine conjugates (**1**–**12**) were prepared attaching propane-1,3-diamine, hexane-1,6-diamine, or decane-1,10-diamine to one or two units of oleanolic or maslinic acid, through the carboxyl group of C-28 of these natural triterpenes (Scheme 1). The diamine reagents were selected with different chain lengths to evaluate their influence on the biological activities of the triterpenic derivatives formed (**1**–**12**). As a means of improving the effectiveness of this amidation reaction between the different diamine reagents and the C-28 carboxyl group of the triterpenic compounds (OA or MA), this carboxyl group was activated with *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU). The reaction to produce the TBTU derivatives of OA or MA was performed by adding TBTU to each of the solutions of these compounds in dry THF, in the presence of DIEA, at rt [24]. Then, each TBTU derivative (OA or MA) was dissolved in CH_2Cl_2 and each solution was split into three parts. The corresponding diamine reagent (propane-1,3-diamine, hexane-1,6-diamine, or decane-1,10-diamine) was then added in the presence of K_2CO_3 to each of the six new solutions, yielding the derivatives **1**–**12** (Scheme 1). Each reaction yielded two compounds: **1** (65%) and **2** (28%), **3** (63%) and **4** (27%), **5** (62%) and **6** (29%), **7** (64%) and **8** (28%), **9** (63%) and **10** (29%), and **11** (62%) and **12** (30%).

The difference in the atomic mass between an amide derivative of OA and its dimeric analogue (**1** and **2**, **5** and **6**, or **9** and **10**) was consistently 438 Da, whereas this difference between the pairs of amide derivatives of MA (**3** and **4**, **7** and **8**, or **11** and **12**) was 454 Da. These atomic-mass differences confirmed that compounds **2**, **4**, **6**, **8**, **10**, and **12** were the corresponding dimeric analogues of compounds **1**, **3**, **5**, **7**, **19**, and **11**, in which a second molecule of the corresponding triterpene had coupled to the free amino group (Scheme 1). The ^{1}H NMR spectra of these diamine conjugates (**1**–**12**) were similar to that of their corresponding precursor (OA or MA). The main differences observed regarding the ^{1}H NMR spectra of derivatives **1**, **3**, **5**, **7**, **9**, and **11** (one unit of a triterpene compound) were two multiplet signals of the protons of the carbon atom (C-1′) attached to the amide group, at δ_H 3.50–3.30 and at δ_H 3.15–2.95, and another triplet signal at approximately δ_H 2.75, corresponding to the protons of the last carbon atom (C-3′, C-6′, or C-10′) attached to the free amino group. However, for the dimeric analogues (**2**, **4**, **6**, **8**, **10**, and **12**), the main differences observed were only two multiplet signals of the protons of the carbon atoms attached to the amide groups, at δ_H 3.40–3.25 and at δ_H 3.15–2.95, since these compounds are symmetrical. In the ^{13}C NMR spectra of these diamine conjugates, the main differences observed were also in the chemical shifts of the terminal carbon atoms of the diamine chain. Thus, in the ^{13}C NMR spectra of derivatives **1**, **3**, **5**, **7**, **9**, and **11** (one unit of a triterpene compound), the signal of the last carbon atom of the diamine chain (C-3′, C-6′, or C-10′) was situated at δ_C 40–42, while the signal of the carbon atom (C-1′) attached to the amide group always appeared between 38 and 40 ppm. However, for the dimeric analogues (**2**, **4**, **6**, **8**, **10**, and **12**), the signals of both terminal carbons were situated at δ_C 36 for the propane-1,3-diamine conjugates (**2** and **4**), and at δ_C 39 for the hexane-1,6-diamine and the decane-1,10-diamine conjugates (**6**, **8**, **10**, and **12**).

Several PEGylated derivatives (**13**–**18**) were synthesised from the OA- or MA-diamine conjugates with a free amino group (**1**, **3**, **5**, **7**, **9**, and **11**) (Scheme 2). Thus, a new amide bond was formed by coupling each of these diamine conjugates with the commercial PEG-acid reagent (3,6,9-trioxadecanoic acid, PEG-COOH), yielding the corresponding PEGylated-diamine conjugates of OA or MA: **13** (91%), **14** (88%), **15** (89%), **16** (91%), **17** (94%), and **18** (89%).



Scheme 1. Synthesis of the diamine conjugates of OA or MA and their corresponding dimeric analogues.



Scheme 2. Synthesis of the PEGylated-diamine conjugates of OA or MA.

The main differences observed between the ^{1}H NMR spectra of these PEGylated derivatives (**13**–**18**) and those of their corresponding precursors (**1**, **3**, **5**, **7**, **9**, and **11**) were the signal of a new amide NH proton, between 7.35 and 7.00 ppm, the signals of five methylene protons of the PEG group, between 4.00 and 3.50 ppm, and that of the methyl protons of the same PEG group at δ_H 3.35. In the ^{13}C NMR spectra, the same types of differences were observed between these PEGylated derivatives (**13**–**18**) and their corresponding precursors (**1**, **3**, **5**, **7**, **9**, and **11**). Thus, the signals of a new carboxamide carbon atom (δ_C 171.0–170.0), five methylene carbon atoms (δ_C 72.0–70.0), and a methyl carbon atom (δ 59) of the PEG group, were present in the ^{13}C NMR spectra of these PEGylated derivatives (**13**–**18**).

2.2. Effects of diamine and PEGylated-diamine conjugates on cancer-cell proliferation

The effects of 12 OA- or MA-diamine conjugates (**1**–**12**) and 6 PEGylated-diamine conjugates (**13**–**18**), compared with those of their precursors (OA or MA), on the proliferation of three cancer-cell lines (B16-F10, mouse murine melanoma cells, HT29, human Caucasian colon adenocarcinoma cells, and Hep G2, human Caucasian hepatocyte carcinoma cells) were investigated by an MTT assay. Cell viability was determined by uptake of formazan dye and expressed as a percentage of untreated control cells. In these three cell lines, the concentration of those compounds at which the response (or binding) was reduced by half (IC_{50}) was determined (Table 1).

All compounds tested induced a dose-dependent decrease in the viability of cells after 72 h of treatment, ranging from 0 to 200 μg/mL. According to the analysis of the different types of compounds tested, the best results were achieved by the OA- or MA-diamine conjugates (**1**, **3**, **5**, **7**, **9**, and **11**), with IC_{50} concentrations lower than 10 μM in the three cancer-cell lines. The PEGylated-diamine conjugates of OA or MA (**13**–**18**) also showed low cytotoxicity data with IC_{50} concentrations, which in most cases

Table 1
Growth-inhibitory effects of the diamine conjugates and their dimer analogues of OA or MA (**1**–**12**), and the PEGylated-diamine conjugates of OA or MA (**13**–**18**), on B16-F10, HT29, and Hep G2 cells.

Type of compound	Compd#	B16-F10	[a]IC_{50} of precursor / IC_{50} of compd#	HT29	[a]IC_{50} of precursor / IC_{50} of compd#	Hep G2	[a]IC_{50} of precursor / IC_{50} of compd#
Precursor	OA (**I**)	106.4 ± 3.7	1.0	429.9 ± 0.7	1.0	211.8 ± 0.5	1.0
Diamine conjugate	**1**	0.76 ± 0.03	140.0	3.97 ± 0.19	108.3	4.75 ± 0.01	44.6
	5	2.15 ± 0.15	49.5	4.66 ± 0.01	92.3	4.56 ± 0.01	46.5
	9	1.75 ± 0.08	60.8	4.72 ± 0.36	91.1	5.85 ± 0.10	36.2
Diamine conjugate dimer	**2**	66.22 ± 0.97	1.6	72.95 ± 2.06	5.9	53.13 ± 3.53	4.0
	6	80.73 ± 0.09	1.3	73.74 ± 1.91	5.8	105.34 ± 0.28	2.0
	10	104.78 ± 0.73	1.0	109.24 ± 1.25	3.9	128.59 ± 2.86	1.6
PEGylated diamine conjugate	**13**	9.14 ± 1.38	11.6	125.15 ± 1.21	3.4	120.09 ± 2.87	1.8
	15	11.34 ± 0.66	9.4	11.74 ± 0.33	36.6	19.32 ± 0.12	11.0
	17	9.41 ± 0.04	11.3	11.90 ± 0.27	36.1	12.99 ± 0.01	16.3
Precursor	MA (**II**)	36.2 ± 2.5	1.0	32.2 ± 3.8	1.0	99.2 ± 15.5	1.0
Diamine conjugate	**3**	1.56 ± 0.04	23.2	5.80 ± 0.03	5.6	4.85 ± 0.17	20.5
	7	6.25 ± 0.21	5.8	7.84 ± 0.27	4.1	8.64 ± 0.03	11.5
	11	1.76 ± 0.02	20.6	4.48 ± 0.03	7.2	4.13 ± 0.01	24.0
Diamine conjugate dimer	**4**	43.48 ± 5.34	0.8	77.23 ± 1.84	0.4	73.48 ± 1.16	1.4
	8	72.73 ± 1.53	0.5	53.82 ± 0.81	0.6	105.90 ± 2.72	0.9
	12	92.19 ± 1.43	0.4	70.88 ± 2.19	0.5	96.37 ± 6.54	1.0
PEGylated diamine conjugate	**14**	17.65 ± 0.27	2.1	37.31 ± 0.65	0.9	31.57 ± 0.41	3.1
	16	20.27 ± 0.01	1.8	20.34 ± 0.27	1.6	19.04 ± 0.04	5.2
	18	12.26 ± 0.39	3.0	13.25 ± 0.13	2.4	13.68 ± 0.17	7.3

The IC_{50} values (μM) were calculated considering control untreated cells as 100% of viability. Cell-growth inhibition was analysed by the MTT assay, as described in Experimental section.
[a] Relationship between IC_{50} of OA or MA and the IC_{50} of each compound. All assays were made two times using three replicates. Values, means ± S.E.M.

did not exceed 20 μM. Since the OA- or MA-diamine conjugate dimers (**2**, **4**, **6**, **8**, **10**, and **12**) showed IC_{50} values higher than those of the other types of derivatives, these compounds were discarded for the following cytometric studies. In general, the best cytotoxic results were produced by the OA- or MA-diamine conjugates (**1**, **3**, **9**, and **11**), with the shortest and the longest diamine chain (3 or 10 carbon atoms), on the B16-F10 cell line (0.76 μM, 1.56 μM, 1.75 μM, and 1.76 μM, respectively), being between 140- and 20-fold more effective than their corresponding precursors (OA or MA). The IC_{50} values of these derivatives (**1**, **3**, **9**, and **11**) are slightly higher with the other two cancer-cell lines (HT29 and Hep G2), around 5 μM, being between 108- and 6-fold more effective than OA or MA (Table 1, Fig. 2).

Only the diamine conjugates (**1**, **3**, **5**, **7**, **9**, and **11**) and the PEGylated-diamine conjugates (**13**–**18**) of OA or MA were selected to compare the growth-inhibitory effects of these compounds on murine melanoma cells (B16-F10) with those of a non-tumour cell line from a similar tissue, such as human epithelial pulmonary fibroblasts (HPF). In the same way, we compare the growth-inhibitory effects of these compounds on colon-cancer cells (HT29) and hepatoma cells (Hep G2) with those of non-tumour cell lines of the same tissue, such as normal rat ileum cells (IEC-18) and human embryonic hepatic cells (WRL68), respectively.

For the comparison of the cytotoxic effects that these compounds exert on the non-tumour cells (HPF, IEC-18, and WRL68) vs. the cancer cells (B16-F10, HT29, and Hep G2), the percentage of viability of the non-tumour cells was calculated using the corresponding IC_{50} concentrations of the triterpenic derivatives for the cancer cells, i.e. the percentage of non-tumour cells living at the IC_{50} values of the triterpene compounds in the corresponding cancer cells (Table 2). Almost all OA- or MA-diamine conjugates (**1**, **3**, **7**, **9**, and **11**), except **5**, and the PEGylated diamine conjugate of MA (**18**), showed cell-viability values of between 81% and 94% for the non-tumour HPF cell line, at the corresponding IC_{50} concentrations of the B16-F10 cancer-cell line. Only the MA-diamine conjugate (**7**) and the PEGylated diamine conjugate of OA (**17**) exceeded 80% of cell viability for the non-tumour IEC-18 cell line, at the corresponding IC_{50} concentrations of the HT29 cancer-cell line. Finally, only the OA-diamine conjugate (**9**) and the PEGylated diamine conjugates of MA (**14** and **16**) have a percentage of cell

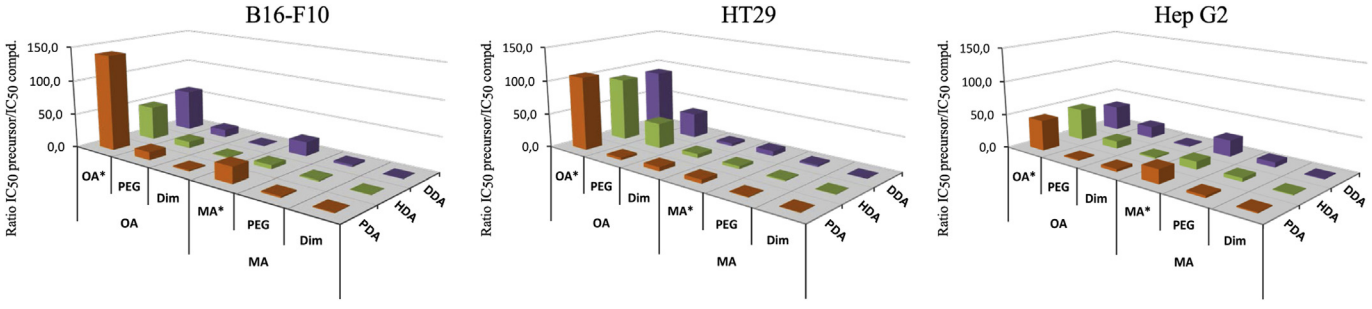


Fig. 2. Relationship between IC_{50} of OA or MA and the IC_{50} of each triterpenic derivative. OA* or MA* = Oleanolic or Maslinic acid derivatives, PEG = PEGylated derivatives (**13**–**18**), Dim = dimer derivatives (**2**, **4**, **6**, **8**, **10**, and **12**), PDA = propane-1,3-diamine, HAD = hexane-1,3-diamine, DDA = decane-1,10-diamine.

Table 2
The percentage of viability of the non-tumour cells at the corresponding IC_{50} concentrations for the cancer cells of the diamine conjugates (**1**, **3**, **5**, **7**, **9**, and **11**) and the PEGylated-diamine conjugates (**13**–**18**) of OA or MA.

Type of compound	Compd#	% viability of HPF cells	% viability of IEC-18 cells	% viability of WRL68 cells
Diamine conjugate (OA)	**1**	91.87 ± 0.84	<50	54.25 ± 1.64
	5	<50	<50	<50
	9	93.89 ± 0.76	60.56 ± 1.38	88.52 ± 0.62
PEGylated diamine conjugate (OA)	**13**	65.95 ± 1.12	<50	<50
	15	50.28 ± 1.44	78.14 ± 0.73	<50
	17	70.66 ± 0.64	81.46 ± 1.36	51.44 ± 0.13
Diamine conjugate (MA)	**3**	83.29 ± 1.09	72.97 ± 1.20	55.13 ± 1.17
	7	92.94 ± 1.31	87.63 ± 0.67	64.03 ± 2.40
	11	81.26 ± 0.76	51.51 ± 1.16	<50
PEGylated diamine conjugate (MA)	**14**	74.00 ± 1.56	76.38 ± 0.98	92.74 ± 0.77
	16	<50	60.18 ± 0.91	85.77 ± 3.84
	18	82.12 ± 0.94	72.54 ± 1.18	75.77 ± 1.36

viability equal or greater than 80% for the non-tumour WRL68 cell line, at the corresponding IC_{50} concentrations of the Hep G2 cancer-cell line (Table 2).

2.3. Characterization of apoptotic effects by flow cytometry

Taking into account the cytotoxic results of these triterpenic derivatives with cancer cells as well as non-tumour cells, 4 diamine conjugates (**1**, **3**, **9**, and **11**) and 4 PEGylated-diamine conjugates (**13**, **14**, **17**, and **18**) of OA or MA were selected for the following cytometric studies on the B16-F10 cell line. Thus, the apoptotic determination assays were conducted through double staining with Annexin V (An-V), conjugated fluorescein isothiocyanate (FITC), and propidium iodide (PI). These assays on the B16-F10 cell line were measured at 24, 48, and 72 h after treatment with the selected 8 triterpene derivatives at their corresponding IC_{50} concentration. The percentages of apoptosis were determined with Annexin V–FICT/PI by flow-activated cell sorter (FACS) cytometry analysis (Fig. 3).

The four OA- or MA-diamine conjugates (**1**, **3**, **9**, and **11**) showed apoptotic effects on treated cells, with total apoptosis rates at 72 h, ranging from 73% to 90% relative to control. These percentages of total apoptosis were lower for the four PEGylated-diamine conjugates of OA or MA (**13**, **14**, **17**, and **18**), under the same conditions, ranging from 32% to 80% relative to control. In addition, the analysis of these apoptosis results at the different times tested (24, 48, and 72 h), revealed that at 24 h the percentages of total apoptosis were very low for both types of derivatives (between 3% and 21%), although slightly higher for the PEGylated-diamine conjugates (Fig. 3). These percentages of total apoptosis underwent a major change at 48 h of the assay. While the diamine conjugates (**1**, **3**, **9**, and **11**) had percentages of between 60% and 80%, the PEGylated-diamine conjugates (**13**, **14**, **17**, and **18**) did not exceed 30%. All compounds reached the highest percentage of apoptosis at 72 h. Compound **1**, the propane-1,3-diamine conjugate of OA, registered the best total apoptosis value (>90% over control), although compounds **9** and **11**, the decane-1,10-diamine conjugates of OA or MA, and compound **18**, the PEGylated decane-1,10-diamine conjugate of MA, had total apoptosis values greater than 80% (Fig. 3). The length of the diamine chain of these derivatives did not have a clear influence on the apoptosis results. Obtaining new cytotoxic compounds to restore the ability of cancer cells to undergo apoptosis may be a key strategy in new cancer therapies.

2.4. Cell-cycle arrest and distribution

Since inhibition of cell proliferation can be related to cytotoxic and cytostatic effects in response to treatment with these triterpenic derivatives, we analysed cell-cycle distribution and cell-cycle arrest. Flow cytometry by stained propidium iodide (PI) was used to measure DNA ploidy as well as alteration in cell-cycle profiles. The percentages of cells in the different phases of the cell cycle were analysed at 48 h. These tests were not performed at 72 h because under these conditions there were not enough live cells for the results to be reliable. The cancer-cell line B16-F10 was treated with the selected diamine (**1**, **3**, **9**, and **11**) and PEGylated-diamine conjugates (**13**, **14**, **17**, and **18**) of OA or MA at their respective IC_{50} concentrations.

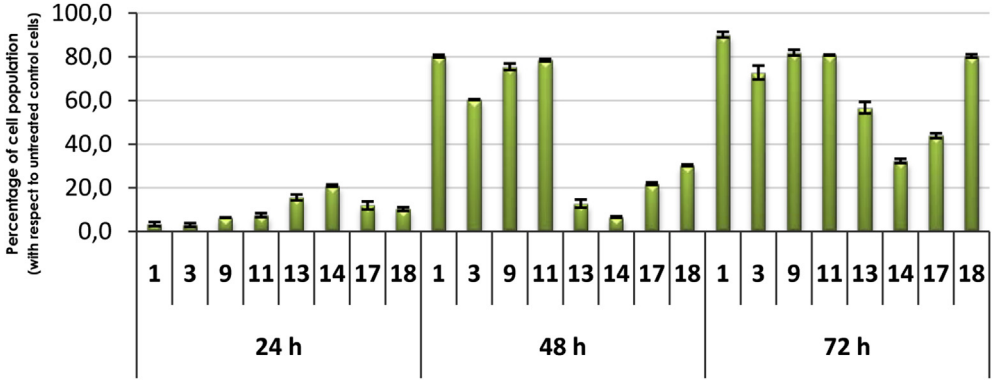


Fig. 3. Flow-cytometry analysis of Annexin V-FITC staining and PI accumulation (total apoptosis) after exposure of B16-F10 to OA or MA derivatives for 24, 48, and 72 h. Cell lines were treated at concentrations equal to their corresponding IC_{50} values. Values are expressed as means ± S.E.M. of at least two experiments in duplicate.

The DNA-histogram analyses revealed that all compounds tested produced cell-cycle arrest in B16-F10 cells, increasing the number of these cells in the S phase. These increases were accompanied by a decrease in the percentage of proliferating cells in the G0/G1 phase, changes in the G2/M phase being less significant (Fig. 4). The S phase is the stage when DNA replication occurs.

Fig. 5 shows the cell-cycle histograms of B16-F10 cancer cells, after 48 h of treatment with compounds **1**, **3**, **9**, and **11**, at their corresponding IC_{50} concentration. These diamine conjugates of OA or MA were the most apoptotic compounds at 48 h. Cell-cycle arrest in this S phase had absolute populations values of 52.84%, 45.43%, 54.63%, and 52.17%, respectively, for compounds **1**, **3**, **9**, and **11**, while the control showed a 19.70% of population in this S phase (Fig. 5).

2.5. Effects on changes in mitochondrial-membrane potential

Apoptosis can occur through two essential pathways: one causes mitochondrial disruption (the intrinsic pathway) and leads to loss of mitochondrial-membrane potential (MMP), and the other (the extrinsic pathway) induces apoptosis without MMP changes. We analysed the MMP to elucidate the possible mechanism involved in the apoptotic responses of the selected diamine (**1**, **3**, **9**, and **11**) and PEGylated-diamine conjugates (**13**, **14**, **17**, and **18**) of OA or MA in the B16-F10 cell line, at their respective IC_{50} concentration, for 48 h. Changes in MMP were analysed by monitoring the cell fluorescence after a double staining with rhodamine 123 (Rh123) and propidium iodide (PI). Only compound **1** clearly showed a negative Rh123 staining, causing the disruption of the mitochondrial membrane with the loss of MMP. This result suggests the activation of an intrinsic apoptotic pathway triggered by this propane-1,3-diamine conjugate of OA (**1**). However, the other derivatives tested (**1**, **3**, **9**, **11**, **13**, **14**, **17**, and **18**) showed positive Rh123 staining, and the mitochondrial membrane was not affected, suggesting that the apoptosis caused by these OA or MA derivatives could occur through the activation of an extrinsic pathway (Fig. 6).

3. Conclusions

A total of 18 diamine or PEGylated-diamine conjugates of OA or MA were tested for cytotoxicity in three cancer-cell lines (B16-F10, HT29, and Hep G2), and except for the dimer compounds they were more cytotoxic than natural triterpenes. Percentages of live HPF non-tumour cells at the IC_{50} values of the B16-F10 cancer cells ranged from 81% to 94% for almost all diamine conjugates tested. The best cytotoxic results were achieved using the diamine conjugates of OA or MA with the shortest and the longest diamine chain (IC_{50} values from 0.76 μM to 1.76 μM), on the B16-F10 cell line, being between 140- and 20-fold more effective than their corresponding precursors (OA or MA).

The four OA- or MA-diamine conjugates (**1**, **3**, **9**, and **11**) showed the best apoptotic effects on treated cells at 72 h on the B16-F10 cell line, with total apoptosis rates between 73% and 90%, being between lower (between 32% and 80%) for the four PEGylated-diamine conjugates (**13**, **14**, **17**, and **18**). These percentages of total apoptosis were very low at 24 h for both types of derivatives (between 3% and 21%), increasing at 48 h between 60% and 80% for the diamine conjugates, and not exceeding 30% for the PEGylated-diamine conjugates. Compound **1** showed the best total apoptosis value (>90% relative to the control), followed by compounds **9**, **11**, and **18** with values greater than 80%. The length of the diamine chain of these derivatives did not seem to have a clear influence on these apoptosis results.

In the B16-F10 cell line, all the compounds tested caused cell-cycle arrest in the S phase. The MMP results suggested the activation of the extrinsic pathway during the apoptosis of B16-F10 cancer cells, for almost all the compounds tested, except for **1**, the most cytotoxic and apoptotic compound on this cancer-cell line, suggesting that the intrinsic pathway was activated by this compound (**1**).

The induction of apoptosis at very low concentrations exhibited by some of these diamine conjugates of OA or MA in the melanoma cell line, and the high percentage of viability of the corresponding non-tumour cells at these concentrations, suggest that these compounds could be used in the future as safe and effective anticancer agents that could reduce the side effects associated with current anticancer treatments in the clinic. Additional molecular studies as well as in vivo studies will be necessary.

4. Experimental

4.1. General experimental chemical procedures

Measurements of NMR spectra were made in VARIAN direct drive (400 and 500 MHz ^{1}H NMR) spectrometers. The ^{13}C chemical shifts were assigned with the aid of distortion less enhancement by polarization transfer (DEPT) using a flip angle of 135°. IR spectra were recorded on a MATTSON SATELLITE FTIR spectrometer. Optical

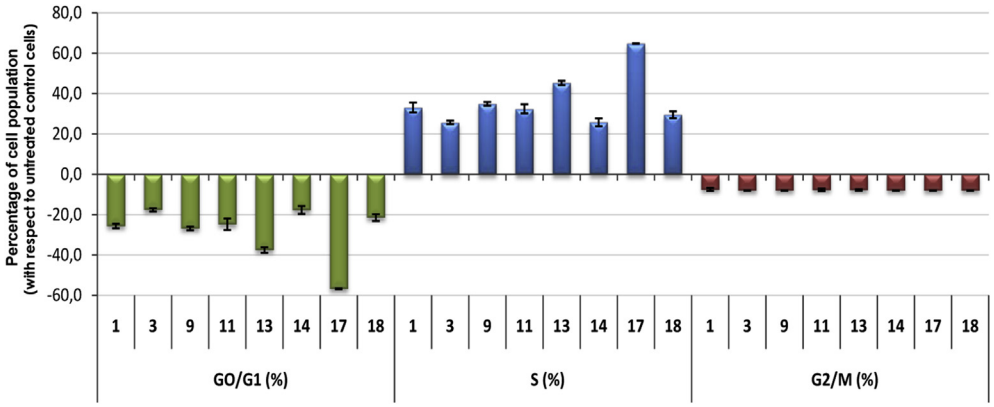


Fig. 4. Changes in the percentages of cells, in each of the cell-cycle phase, were performed with respect to untreated control cells. B16-F10 was treated with OA or MA derivatives, at their corresponding IC_{50} concentrations. Cell-cycle analysis was conducted after propidium iodide staining, G0/G1 phase (green bars), S phase (blue bars), and G2/M phase (red bars). Values represent means ± S.E.M. of at least two independent experiments performed in triplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

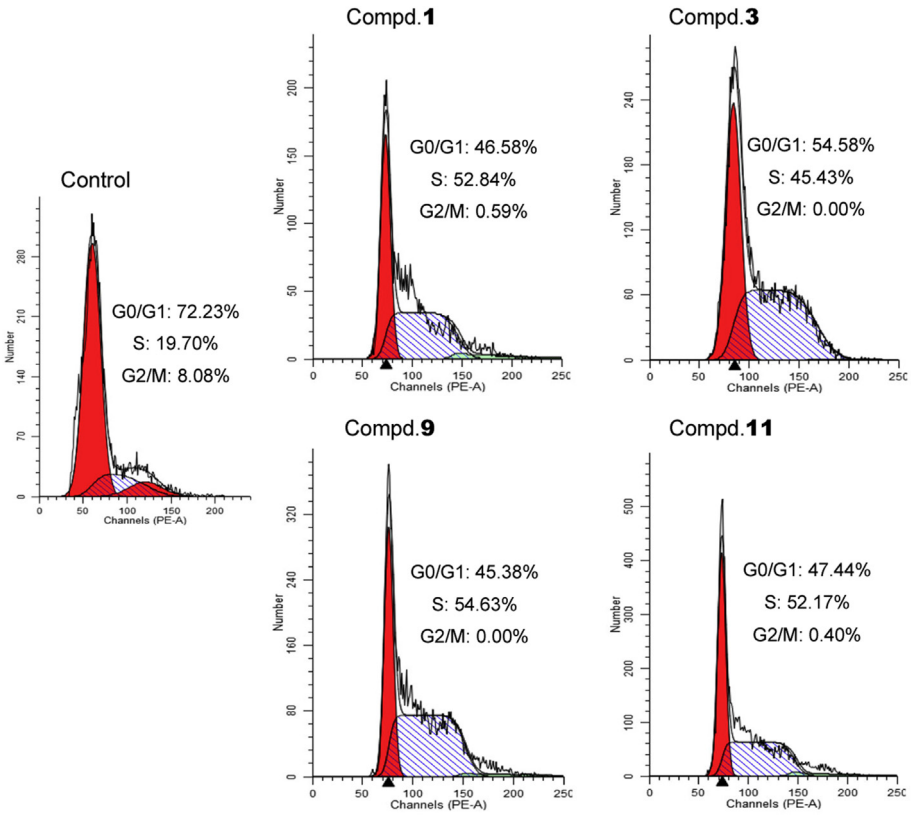


Fig. 5. Cell-cycle histograms of B16-F10 cancer cells, after 48 h of treatment with compounds **1**, **3**, **9**, and **11**, at their corresponding IC_{50} concentration.

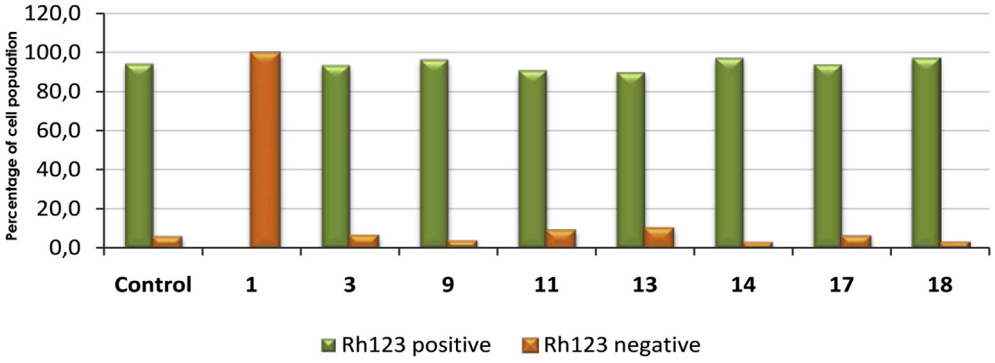


Fig. 6. Flow-cytometry analysis of rhodamine 123 and PI staining after exposure of B16-F10 cells to the selected diamine or PEGylated-diamine conjugates of OA or MA, for 48 h with respect to the untreated control cells. Cell lines were treated at concentrations equal to their corresponding IC_{50} values. Rh123 positive cells (green bars) were rhodamine 123^+ with PI^+ or PI^-. Rh123 negative cells (orange bars) were rhodamine 123^- with PI^- or PI^+. Values are expressed as means ± S.E.M. of at least two experiments in duplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

rotations were measured with a Perkin-Elmer 241 polarimeter at 25 °C. The purity of new compounds was determined by a WATERS ACQUITY UPLC system (ultra-performance liquid chromatography), coupled with a WATERS SYNAPT G2 HRMS spectrometer (high-resolution mass spectra), with ESI (electrospray ionization). The purities of all compounds were confirmed to be ≥ 95%. Melting points (mp) were determined using a Kofler (Reichter) apparatus and are uncorrected. All reaction solvents and chromatography solvents were distilled prior to use. Commercially available reagents were used without further purification. Merck silica-gel 60 aluminium sheets (ref. 1.16835) were used for TLC, and spots were rendered visible by spraying with H_2SO_4–AcOH, followed by heating to 120 °C, and also visualized under UV at 254 nm. Merck silica-gel 60 (0.040–0.063 mm, ref. 1.09385) was used for flash chromatography. CH_2Cl_2 (Fisher, ref. D/1852/17), $CHCl_3$ (Fisher, ref. C/4960/17), or n-hexane (Merck, ref. 1.04374), with increasing amounts of Me_2CO (Fisher, ref. A/0600/17), MeOH (Fisher, ref. M/4000/17), or EtOAc (Fisher, ref. E/0900/17), were used as eluents (all the solvents had an analytical reagent-grade purity). The PEG reagent 3,6,9-trioxadecanoic acid (PEG-COOH, CAS Number 16024-58-1) and TBTU (CAS Number 125700-67-6) were purchased from Sigma-Aldrich.

4.2. Isolation of OA (*I*) and MA (*II*)

OA (**I**) and MA (**II**) were isolated from solid olive-oil-production

wastes, which were extracted successively in a Soxhlet with hexane and EtOAc. Hexane extracts were a mixture of OA and MA (80:20), whereas this relationship was (20:80) for the EtOAc extracts. Both products were purified from these mixtures by column chromatography over silica gel, eluting with a $CHCl_3$-MeOH or CH_2Cl_2-Me_2CO mixtures of increasing polarity [36]. These natural compounds can also be extracted more efficiently using an alternative method such as microwave-assisted extraction [35].

4.3. Amidation reactions on the C-28 carboxylic group of OA (*I*) or MA (*II*)

DIEA (0.3 mmol each) and TBTU (0.66 mmol each) were added to a solution of OA (**I**) and to a solution of MA (**II**) (0.44 mmol of each) in THF (20 mL each). The reaction mixtures were maintained at rt for 12 h, and then diluted with water and extracted three times with CH_2Cl_2. The organic layers were dried with dry Na_2SO_4 and the solvent was removed under reduced pressure. Finally, each residue was purified by column chromatography using hexane/EtOAc as eluents, yielding the corresponding TBTU derivatives [24]. These two TBTU derivatives (0.35 mmol of each) were dissolved, respectively, in CH_2Cl_2 (15 mL each), and each of these two solutions were split into three new solutions, whereupon K_2CO_3 (1 mmol each) and the corresponding diamine reagent (3 mmol of propane-1,3-diamine or hexane-1,6-diamine or decane-1,10-diamine each) were added. These reaction mixtures were maintained at rt for 5 h. Thereafter, CH_2Cl_2 was added to each reaction mixture, and then were washed several times with water. Each organic layer was treated with dry Na_2SO_4 and the solvent was removed under reduced pressure. Finally, each residue was purified in a chromatography column using CH_2Cl_2/acetone as eluent, yielding the following pairs of derivatives: **1** (65%) and **2** (28%), **3** (63%) and **4** (27%), **5** (62%) and **6** (29%), **7** (64%) and **8** (28%), **9** (63%) and **10** (29%), and **11** (62%) and **12** (30%).

4.3.1. Compound *1*

White solid, mp 97–99 °C; $[\alpha]^{25}_D$ + 55 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3356, 2922, 1633, 1524, 1463, 1386, 1030 and 997; δ_H ($CDCl_3$, 400 MHz): 6.41 (dd, 1H, $J_1 = J_2 = 5.0$ Hz, NHCO group), 5.35 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 3.49–3.44 (m, 1H, H-1′), 3.21 (dd, 1H, $J_1 = 4.6$, $J_2 = 8.8$ Hz, H-3), 3.13–3.10 (m, 1H, H-1′), 2.77 (t, 2H, $J = 6.0$ Hz, H-3′), 2.52 (dd, 1H, $J_1 = 3.4$, $J_2 = 13.0$ Hz, H-18), 1.25, 1.15, 0.98, 0.90, 0.90, 0.78, 0.76 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.7 (C-28), 145.0 (C-13), 122.9 (C-12), 79.1 (C-3), 55.3 (C-5), 47.7 (C-9), 47.0 (C-19), 46.4 (C-17), 42.3 (C-18), 42.2 (C-14), 40.0 (C-3′), 39.5 (C-8), 38.9 (C-4), 38.7 (C-1), 37.6 (C-1′), 37.1 (C-10), 34.3 (C-21), 33.2 (Me), 32.9 (C-7), 32.6 (C-22), 32.3 (C-2′), 30.9 (C-20), 28.3 (Me), 27.5 (C-2), 27.3 (C-15), 26.0 (Me), 23.9 (C-16), 23.8 (Me), 23.7 (C-11), 18.5 (C-6), 17.1 (Me), 15.7 (Me), 15.5 (Me); ESI-HRMS *m*/*z* calcd for $C_{33}H_{57}N_2O_2$ $[M+1]^+$ 513.4420, found 513.4426.

4.3.2. Compound *2*

White solid, mp 118–120 °C; $[\alpha]^{25}_D$ + 49 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3354, 2942, 1634, 1520, 1455, 1386, 1030 and 997; δ_H ($CDCl_3$, 400 MHz): 6.41 (dd, 1H, $J_1 = J_2 = 6.0$ Hz, NHCO group), 5.40 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 3.28–3.24 (m, 1H, H-1′), 3.21 (dd, 1H, $J_1 = 4.8$, $J_2 = 11.21$ Hz, H-3), 3.14–3.10 (m, 1H, H-1′), 2.64 (dd, 1H, $J_1 = 3.2$, $J_2 = 14.4$ Hz, H-18), 1.25, 1.15, 0.98, 0.91, 0.90, 0.77, 0.74 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.5 (C-28), 144.7 (C-13), 122.9 (C-12), 79.1 (C-3), 55.3 (C-5), 47.7 (C-9), 46.9 (C-19), 46.4 (C-17), 42.1 (C-14), 42.0 (C-18), 39.5 (C-8), 38.9 (C-4), 38.6 (C-1), 37.1 (C-10), 36.2 (C-1′ and C-3′), 34.4 (C-21), 33.2 (Me), 33.1 (C-7), 32.6 (C-22), 30.9 (C-20), 29.8 (C-2′), 28.2 (Me), 27.5 (C-2), 27.3 (C-15), 25.9 (Me), 23.9 (C-16), 23.8 (Me), 23.7 (C-11), 18.5 (C-6), 17.1 (Me), 15.7 (Me), 15.5 (Me); ESI-HRMS *m*/*z* calcd for $C_{63}H_{101}N_2O_4$ $[M-1]^-$ 949.7761, found 949.7772.

4.3.3. Compound *3*

White solid, mp 110–112 °C; $[\alpha]^{25}_D$ + 41 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3354, 2942, 1634, 1525, 1462, 1365, 1049 and 749; δ_H ($CDCl_3$, 400 MHz): 6.43 (dd, 1H, $J_1 = J_2 = 5.4$ Hz, NHCO group), 5.35 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 3.70 (ddd, 1H, $J_1 = 4.8$, $J_2 = 9.6$, $J_3 = 14.0$ Hz, H-2), 3.50–3.41 (m, 1H, H-1′), 3.13–3.10 (m, 1H, H-1′), 2.98 (d, 1H, $J = 9.6$ Hz, H-3), 2.75 (t, 1H, $J = 6.4$ Hz, H-3′), 2.52 (dd, 1H, $J_1 = 3.6$, $J_2 = 12.8$ Hz, H-18), 1.15, 1.02, 0.97, 0.90, 0.90, 0.81, 0.75 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.5 (C-28), 145.0 (C-13), 122.7 (C-12), 84.0 (C-3), 68.9 (C-2), 55.4 (C-5), 47.7 (C-9), 46.9 (C-19), 46.5 (C-1), 46.4 (C-17), 42.2 (C-14), 42.3 (C-18), 40.2 (C-3′), 39.6 (C-8), 39.3 (C-10), 38.3 (C-4), 37.8 (C-1′), 34.3 (C-21), 33.2 (Me), 32.9 (C-7), 32.6 (C-22), 32.5 (C-2′), 30.9 (C-20), 28.8 (Me), 27.5 (C-15), 26.0 (Me), 23.8 (C-16), 23.9 (Me), 23.8 (C-11), 18.5 (C-6), 17.2 (Me), 17.0 (Me), 16.8 (Me); ESI-HRMS *m*/*z* calcd for $C_{33}H_{57}N_2O_3$ $[M+1]^+$ 529.4369, found 529.4336.

4.3.4. Compound *4*

White solid, mp 78–79 °C; $[\alpha]^{25}_D$ + 45 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3356, 1634, 1525, 1462, 1365, 1050 and 751; δ_H ($CDCl_3$, 400 MHz): 6.43 (dd, 1H, $J_1 = J_2 = 6.0$ Hz, NHCO group), 5.35 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 3.70 (ddd, 1H, $J_1 = 4.0$, $J_2 = 9.2$, $J_3 = 14.0$ Hz, H-2), 3.28–3.23 (m, 1H, H-1′), 3.15–3.10 (m, 1H, H-1′), 3.01 (d, 1H, $J = 9.2$ Hz, H-3), 2.65 (dd, 1H, $J_1 = 3.6$, $J_2 = 13.2$ Hz, H-18), 1.16, 1.03, 0.98, 0.92, 0.91, 0.83, 0.74 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.6 (C-28), 144.8 (C-13), 122.7 (C-12), 84.1 (C-3), 69.0 (C-2), 55.3 (C-5), 47.7 (C-9), 46.8 (C-19), 46.5 (C-1), 46.4 (C-17), 42.2 (C-14), 42.0 (C-18), 39.6 (C-8), 39.3 (C-10), 38.4 (C-4), 36.1 (C-1′ and C-3′), 34.4 (C-21), 33.2 (Me), 33.1 (C-7), 32.5 (C-22), 30.9 (C-20), 29.9 (C-2′), 28.7 (Me), 27.5 (C-15), 26.0 (Me), 23.7 (C-16), 23.8 (Me), 23.8 (C-11), 18.5 (C-6), 17.1 (Me), 16.9 (Me), 16.8 (Me); ESI-HRMS *m*/*z* calcd for $C_{63}H_{103}N_2O_6$ $[M+1]^+$ 983.7816, found 983.7781.

4.3.5. Compound *5*

White solid, mp 80–82 °C; $[\alpha]^{25}_D$ + 35 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3343, 2939, 1634, 1526, 1462, 1386, 1030 and 751; δ_H ($CDCl_3$, 400 MHz): 5.96 (dd, 1H, $J_1 = J_2 = 6.0$ Hz, NHCO group), 5.37 (dd, 1H, $J_1 = J_2 = 2.8$ Hz, H-12), 3.37–3.32 (m, 1H, H-1′), 3.21 (dd, 1H, $J_1 = 4.4$, $J_2 = 10.8$ Hz, H-3), 2.98–2.94 (m, 1H, H-1′), 2.78 (t, 2H, $J = 6.0$ Hz, H-6′), 2.50 (dd, 1H, $J_1 = 2.8$, $J_2 = 13.2$ Hz, H-18), 1.15, 0.98, 0.91, 0.90, 0.90, 0.78, 0.75 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.4 (C-28), 145.2 (C-13), 122.9 (C-12), 79.1 (C-3), 55.3 (C-5), 47.7 (C-9), 47.0 (C-19), 46.4 (C-17), 42.2 (C-14), 42.5 (C-18), 40.3 (C-6′), 39.5 (C-8), 39.4 (C-1′), 38.9 (C-4), 38.6 (C-1), 37.1 (C-10), 34.3 (C-21), 33.2 (Me), 32.7 (C-7), 32.6 (C-22), 30.9 (C-20), 30.0 (C-5′), 29.5 (C-2′), 28.3 (Me), 27.5 (C-2), 27.3 (C-15), 26.4 (C-3′), 25.9 (Me), 25.9 (C-4′) 23.8 (C-16), 23.8 (Me), 23.7 (C-11), 18.5 (C-6), 17.1 (Me), 15.8 (Me), 15.6 (Me); ESI-HRMS *m*/*z* calcd for $C_{36}H_{63}N_2O_2$ $[M+1]^+$ 555.4890, found 555.4874.

4.3.6. Compound *6*

White solid, mp 84–86 °C; $[\alpha]^{25}_D$ + 41 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/m^{-1} 2941, 1738, 1637, 1440, 1366, 1216 and 527; δ_H ($CDCl_3$, 400 MHz): 5.95 (dd, 1H, $J_1 = J_2 = 5.2$ Hz, NHCO group), 5.36 (dd, 1H, $J_1 = J_2 = 3.0$ Hz, H-12), 3.35–3.30 (m, 1H, H-1′), 3.20 (dd, 1H, $J_1 = 4.2$, $J_2 = 11.0$ Hz, H-3), 3.00–2.93 (m, 1H, H-1′), 2.48 (dd, 1H, $J_1 = 3.2$, $J_2 = 13.2$ Hz, H-18), 1.14, 0.97, 0.89, 0.89, 0.88, 0.77, 0.74 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.3 (C-28), 145.2 (C-13), 122.8 (C-12), 79.0 (C-3), 55.2 (C-5), 47.7 (C-9), 46.9 (C-19), 46.4 (C-17), 42.2 (C-14), 42.5 (C-18), 39.5 (C-8), 39.4 (C-1′ and C-6′), 38.6 (C-4), 38.6 (C-1), 37.1 (C-10), 34.3 (C-21), 33.1 (Me), 32.6 (C-7), 32.5 (C-

22), 30.8 (C-20), 29.5 (C-2′ and C-5′), 28.2 (Me), 27.4 (C-2), 27.4 (C-15), 26.8 (C-3′ and C-4′), 25.9 (Me), 23.9 (C-16), 23.7 (Me), 23.7 (C-11), 18.4 (C-6), 17.1 (Me), 15.7 (Me), 15.5 (Me); ESI-HRMS *m*/*z* calcd for $C_{66}H_{107}N_2O_4$ [M-1]$^{\rightarrow}$ 991.8231, found 991.8235.

4.3.7. Compound **7**

White solid, mp 100–102 °C; $[\alpha]^{25}_D$ + 38 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 2927, 1738, 1526, 1634, 1455, 1365, 1217, 1050 and 733; δ_H ($CDCl_3$, 400 MHz): 5.90 (dd, 1H, $J_1 = J_2 = 5.4$ Hz, NHCO group), 5.36 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 3.68 (ddd, 1H, $J_1 = 4.8$, $J_2 = 9.2$, $J_3 = 14.0$ Hz, H-2), 3.35–3.32 (m, 1H, H-1′), 3.19–3.15 (m, 1H, H-1′), 2.98 (d, 1H, $J = 9.2$ Hz, H-3), 2.69 (t, 2H, $J = 6.4$ Hz, H-6′), 2.49 (dd, 1H, $J_1 = 3.6$, $J_2 = 12.8$ Hz, H-18), 1.15, 1.02, 0.98, 0.90, 0.90, 0.82, 0.75 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.2 (C-28), 145.3 (C-13), 122.6 (C-12), 83.9 (C-3), 68.9 (C-2), 55.3 (C-5), 47.7 (C-9), 46.9 (C-19), 46.5 (C-1), 46.4 (C-17), 42.5 (C-18), 42.3 (C-14), 39.6 (C-8), 39.5 (C-1′), 39.5 (C-6′), 39.3 (C-10), 38.3 (C-4), 34.3 (C-21), 33.1 (Me), 32.7 (C-7), 32.4 (C-22), 30.9 (C-20), 29.5 (C-2′), 29.5 (C-5′), 28.8 (Me), 27.4 (C-15), 26.8 (C-3′), 26.8 (C-4′), 25.9 (Me), 23.9 (C-16), 23.9 (Me), 23.9 (C-11), 18.5 (C-6), 17.1 (Me), 16.9 (Me), 16.9 (Me); ESI-HRMS *m*/*z* calcd for $C_{36}H_{63}N_2O_3$ [M+1]$^+$ 571.4839, found 571.4842.

4.3.8. Compound **8**

White solid, mp 81–83 °C; $[\alpha]^{25}_D$ + 41 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 2970, 1738, 1639, 1365, 1216, 1049 and 528; δ_H ($CDCl_3$, 400 MHz): 5.94 (dd, 1H, $J_1 = J_2 = 5.4$ Hz, NHCO group), 5.38 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 3.70 (ddd, 1H, $J_1 = 4.4$, $J_2 = 9.2$, $J_3 = 13.6$ Hz, H-2), 3.38–3.30 (m, 1H, H-1′), 3.01–2.94 (m, 1H, H-1′), 3.00 (d, 1H, $J = 9.2$ Hz, H-3), 2.50 (dd, 1H, $J_1 = 3.2$, $J_2 = 12.8$ Hz, H-18), 1.25, 1.16, 1.03, 0.99, 0.90, 0.83, 0.76 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.3 (C-28), 145.4 (C-13), 122.6 (C-12), 84.0 (C-3), 69.0 (C-2), 55.3 (C-5), 47.7 (C-9), 46.9 (C-19), 46.5 (C-1), 46.4 (C-17), 42.3 (C-14), 42.5 (C-18), 39.5 (C-1′ and C-6′), 39.4 (C-8), 39.3 (C-10), 38.3 (C-4), 34.3 (C-21), 33.1 (Me), 32.7 (C-7), 32.4 (C-22), 30.9 (C-20), 29.5 (C-2′ and C-5′), 28.8 (Me), 27.4 (C-15), 26.8 (C-3′ and C-4′), 25.9 (Me), 23.9 (C-16), 23.9 (Me), 23.9 (C-11), 18.5 (C-6), 17.1 (Me), 16.9 (Me), 16.9 (Me); ESI-HRMS *m*/*z* calcd for $C_{66}H_{109}N_2O_6$ [M+1]$^+$ 1025.8286, found, 1025.8268.

4.3.9. Compound **9**

White solid, mp 62–64 °C; $[\alpha]^{25}_D$ + 42 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3351, 2924, 1738, 1641, 1530, 1455, 1365, 1216 and 528; δ_H ($CDCl_3$, 400 MHz): 6.41 (dd, 1H, $J_1 = J_2 = 5.2$ Hz, NHCO group), 5.35 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 3.49–3.39 (m, 1H, H-1′), 3.21 (dd, 1H, $J_1 = 4.6$, $J_2 = 11.1$ Hz, H-3), 3.14–3.07 (m, 1H, H-1′), 2.77 (t, 2H, $J = 6.0$ Hz, H-10′), 2.52 (dd, 1H, $J_1 = 3.6$, $J_2 = 13.2$ Hz, H-18), 1.25, 1.15, 0.98, 0.91, 0.90, 0.78, 0.76 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.2 (C-28), 145.3 (C-13), 122.8 (C-12), 79.1 (C-3), 55.3 (C-5), 47.7 (C-9), 47.0 (C-19), 46.4 (C-17), 42.4 (C-10′), 42.3 (C-14), 42.6 (C-18), 39.6 (C-1′), 39.5 (C-8), 38.9 (C-4), 38.7 (C-1), 37.1 (C-10), 34.3 (C-21), 33.9 (C-9′), 33.2 (Me), 32.7 (C-7), 32.6 (C-22), 31.1 (C-2′), 30.9 (C-20), 29.8 (C-4′), 29.7 (C-5′), 29.6 (C-6′), 29.4 (C-7′), 28.3 (Me), 27.5 (C-2), 27.5 (C-15), 27.3 (C-3′), 27.1 (C-8′), 25.9 (Me), 24.0 (C-16), 23.8 (Me), 23.7 (C-11), 18.5 (C-6), 17.1 (Me), 15.7 (Me), 15.5 (Me); ESI-HRMS *m*/*z* calcd for $C_{40}H_{71}N_2O_2$ [M+1]$^+$ 611.5516, found 611.5534.

4.3.10. Compound **10**

White solid, mp 60–62 °C; $[\alpha]^{25}_D$ + 51 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3415, 2920, 1737, 1636, 1528, 1450, 1365, 1216 and 519; δ_H ($CDCl_3$, 400 MHz): 5.90 (dd, 1H, $J_1 = J_2 = 4.8$ Hz, NHCO group), 5.36 (dd, 1H, $J_1 = J_2 = 2.8$ Hz, H-12), 3.37–3.32 (m, 1H, H-1′), 3.21 (dd, 1H, $J_1 = 4.4$, $J_2 = 10.8$ Hz, H-3), 3.00–2.96 (m, 1H, H-1′), 2.49 (dd, 1H, $J_1 = 2.6$, $J_2 = 12.6$ Hz, H-18), 1.25, 1.15, 0.98, 0.91, 0.90, 0.78, 0.76 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.2 (C-28), 145.3 (C-13), 122.8 (C-12), 79.1 (C-3), 55.3 (C-5), 47.7 (C-9), 47.0 (C-19), 46.4 (C-17), 42.6 (C-18), 42.3 (C-14), 39.6 (C-1′ and C-10′), 39.5 (C-8), 38.7 (C-1), 38.6 (C-4), 37.1 (C-10), 34.3 (C-21), 33.1 (Me), 32.7 (C-7), 32.5 (C-22), 30.9 (C-20), 29.7 (C-2′ and C-9′), 29.5 (C-3′ and C-8′), 29.4 (C-4′ and C-7′), 28.3 (Me), 27.5 (C-2), 27.5 (C-15), 27.3 (C-5′ and C-6′), 25.9 (Me), 24.0 (C-16), 23.8 (Me), 23.7 (C-11), 18.5 (C-6), 17.1 (Me), 15.7 (Me), 15.5 (Me); ESI-HRMS *m*/*z* calcd for $C_{70}H_{117}N_2O_4$ [M+1]$^+$ 1049.9013, found 1049.8990.

4.3.11. Compound **11**

White solid, mp 75–77 °C; $[\alpha]^{25}_D$ + 36 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 2925, 1737, 1635, 1527, 1455, 1365, 1216 and 528; δ_H ($CDCl_3$, 400 MHz): 5.91 (dd, 1H, $J_1 = J_2 = 4.8$ Hz, NHCO group), 5.36 (dd, 1H, $J_1 = J_2 = 2.8$ Hz, H-12), 3.68 (ddd, 1H, $J_1 = 4.0$, $J_2 = 9.6$, $J_3 = 14.0$ Hz, H-2), 3.36–3.28 (m, 1H, H-1′), 3.06–2.99 (m, 1H, H-1′), 3.00 (d, 1H, $J = 9.6$ Hz, H-3), 2.68 (t, 2H, $J = 6.4$ Hz, H-10′), 2.50 (dd, 1H, $J_1 = 2.4$, $J_2 = 12.4$ Hz, H-18), 1.28, 1.16, 1.03, 0.99, 0.90, 0.82, 0.76 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.2 (C-28), 145.5 (C-13), 122.6 (C-12), 83.8 (C-3), 68.7 (C-2), 55.3 (C-5), 47.6 (C-9), 46.9 (C-19), 46.7 (C-1), 46.4 (C-17), 42.7 (C-18), 42.3 (C-14), 42.3 (C-10′), 39.6 (C-8), 39.6 (C-1′), 39.3 (C-10), 38.4 (C-4), 34.3 (C-21), 33.7 (C-9′), 33.2 (Me), 32.7 (C-7), 32.4 (C-22), 30.9 (C-20), 29.9 (C-2′), 29.8 (C-5′), 29.8 (C-4′), 29.5 (C-6′), 29.3 (C-7′), 28.8 (Me), 27.5 (C-15), 27.5 (C-3′), 27.2 (C-8′), 25.9 (Me), 23.9 (C-11), 23.8 (C-16), 23.8 (Me), 18.5 (C-6), 17.2 (Me), 17.0 (Me), 16.9 (Me); ESI-HRMS *m*/*z* calcd for $C_{70}H_{71}N_2O_3$ [M+1]$^+$ 627.5465, found 627.5478.

4.3.12. Compound **12**

White solid, mp 69–71 °C; $[\alpha]^{25}_D$ + 27 (*c* 1 in $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 2928, 1738, 1634, 1535, 1449, 1366, 1217 and 528; δ_H ($CDCl_3$, 400 MHz): 5.94 (dd, 1H, $J_1 = J_2 = 5.2$ Hz, NHCO group), 5.37 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 3.69 (ddd, 1H, $J_1 = 4.4$, $J_2 = 9.6$, $J_3 = 14.0$ Hz, H-2), 3.35–3.29 (m, 1H, H-1′), 3.02–2.96 (m, 1H, H-1′), 3.51 (d, 1H, $J = 9.6$ Hz, H-3), 2.47 (dd, 1H, $J_1 = 3.2$, $J_2 = 12.4$ Hz, H-18), 1.25, 1.16, 1.03, 0.98, 0.90, 0.83, 0.76 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.4 (C-28), 145.4 (C-13), 122.6 (C-12), 83.7 (C-3), 68.8 (C-2), 55.2 (C-5), 47.6 (C-9), 46.8 (C-19), 46.6 (C-1), 46.4 (C-17), 42.6 (C-18), 42.3 (C-14), 39.7 (C-8), 39.6 (C-1′ and C-10′), 39.3 (C-10), 38.4 (C-4), 34.2 (C-21), 33.1 (Me), 32.6 (C-7), 32.4 (C-22), 30.9 (C-20), 29.8 (C-2′ and C-9′), 29.6 (C-3′ and C-8′), 29.3 (C-4′ and C-7′), 28.7 (Me), 27.4 (C-15), 27.4 (C-5′ and C-6′), 25.8 (Me), 23.8 (C-11), 23.7 (C-16), 23.7 (Me), 18.4 (C-6), 17.1 (Me), 16.9 (Me), 16.8 (Me); ESI-HRMS *m*/*z* calcd for $C_{70}H_{71}N_2O_3$ [M+1]$^+$ 1081.8912, found 1081.8900.

4.4. PEGylation reaction on the free amino group of the OA- or MA-diamine conjugates

DIPCDI (4 mmol each) and 3,6,9-trioxadecanoic acid (PEG-COOH, 4 mmol each) were added to six solutions of compounds **1**, **3**, **5**, **7**, **9**, or **11** (1 mmol of each) in THF (20 mL each). The reaction mixtures were stirred at rt for 4 h, and then filtered and diluted with CH_2Cl_2. These organic layers were separately washed several times with water, treated with dry Na_2SO_4, and the solvent removed under reduced pressure. Finally, each residue was purified on a chromatography column using CH_2Cl_2/acetone as eluent, isolating the following PEGylated-diamine conjugates of OA or MA: **13** (91%), **14** (88%), **15** (89%), **16** (91%), **17** (94%), and **18** (89%).

4.4.1. Compound **13**

Transparent syrup; $[\alpha]^{25}_D$ + 48 (*c* 1 in $CHCl_3$); IR ν_{max}(film)/cm^{-1} 2926, 1738, 1639, 1530, 1365, 1216, 1106 and 528; δ_H ($CDCl_3$, 500 MHz): 7.32 (dd, 1H, $J_1 = J_2 = 6.5$ Hz, NHCO group), 6.54 (dd, 1H, $J_1 = J_2 = 6.0$ Hz, NHCO group), 5.38 (dd, 1H, $J_1 = J_2 = 3.5$ Hz, H-12),

4.00 (s, 2H, CH_2 PEG group), 3.74–3.53 (m, 8H, CH_2 PEG group), 3.35 (s, 3H, CH_3 PEG group), 3.34–3.17 (m, 4H, H-3, H-1′, and H-3′), 3.08–3.03 (m, 1H, H-1′), 2.64 (dd, 1H, $J_1 = 3.2$, $J_2 = 12.8$ Hz, H-18), 1.13, 0.96, 0.90, 0.88, 0.87, 0.76, 0.72 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 125 MHz): 178.3 (C-28), 170.8 (C-1″), 144.6 (C-13), 122.8 (C-12), 79.0 (C-3), 71.9 (C-2″), 71.2 (C-3″), 70.5 (C-4″), 70.4 (C-5″), 70.2 (C-6″), 59.0 (C-7″), 55.2 (C-5), 47.7 (C-9), 46.7 (C-19), 46.4 (C-17), 42.0 (C-14), 41.9 (C-18), 39.5 (C-8), 38.9 (C-4), 38.5 (C-1), 37.1 (C-10), 35.9 (C-1′), 35.8 (C-3′), 34.3 (C-21), 33.1 (C-7), 32.6 (C-22), 32.6 (Me), 30.8 (C-20), 29.7 (C-2′), 28.2 (Me), 27.5 (C-2), 27.2 (C-15), 25.9 (Me), 23.6 (C-16), 23.7 (Me), 23.6 (C-11), 18.4 (C-6), 17.1 (Me), 15.7 (Me), 15.4 (Me); ESI-HRMS m/z calcd for $C_{40}H_{69}N_2O_6$ $[M+1]^+$ 673.5156, found 673.5144.

4.4.2. Compound **14**

Transparent syrup; $[\alpha]^{25}_D + 23$ (c 1 in $CHCl_3$); IR ν_{max}(film)/cm^{-1} 3343, 2923, 1737, 1645, 1532, 1365, 1104 and 558; δ_H ($CDCl_3$, 500 MHz): 7.34 (dd, 1H, $J_1 = J_2 = 6.5$ Hz, NHCO group), 6.58 (dd, 1H, $J_1 = J_2 = 5.5$ Hz, NHCO group), 5.40 (dd, 1H, $J_1 = J_2 = 3.5$ Hz, H-12), 4.00 (s, 2H, CH_2 PEG group), 3.77–3.54 (m, 9H, H-2 and 4 CH_2 PEG group), 3.37 (s, 3H, CH_3 PEG group), 3.35–3.24 (m, 3H, H-3′ and H-1′), 3.08–3.04 (m, 1H, H-1′), 3.0 (d, 1H, $J_1 = 9.5$ Hz, H-3), 2.67 (dd, 1H, $J_1 = 3.5$, $J_2 = 13.0$ Hz, H-18), 1.14, 1.02, 0.97, 0.92, 0.89, 0.81, 0.73 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 125 MHz): 178.3 (C-28), 170.9 (C-1″), 144.6 (C-13), 122.7 (C-12), 84.0 (C-3), 72.0 (C-2″), 71.3 (C-3″), 70.6 (C-4″), 70.5 (C-5″), 70.3 (C-6″), 69.1 (C-2), 59.1 (C-7″), 55.4 (C-5), 47.7 (C-9), 46.7 (C-1), 46.5 (C-19), 46.4 (C-17), 42.1 (C-14), 42.0 (C-18), 39.5 (C-8), 39.3 (C-10), 38.3 (C-4), 35.9 (C-1′), 35.9 (C-3′), 34.4 (C-21), 33.2 (C-7), 32.6 (C-22), 33.2 (Me), 30.8 (C-20), 29.8 (C-2′), 28.8 (Me), 27.5 (C-15), 26.0 (Me), 23.8 (Me), 23.7 (C-16), 23.7 (C-11), 18.5 (C-6), 17.2 (Me), 16.9 (Me), 16.8 (Me); ESI-HRMS m/z calcd for $C_{40}H_{69}N_2O_7$ $[M+1]^+$ 689.5105, found 689.5112.

4.4.3. Compound **15**

Transparent syrup; $[\alpha]^{25}_D + 35$ (c 1 in $CHCl_3$); IR ν_{max}(film)/cm^{-1} 3349, 2926, 1655, 1531, 1454, 1364, 1104 and 558; δ_H ($CDCl_3$, 500 MHz): 7.02 (dd, 1H, $J_1 = J_2 = 5.5$ Hz, NHCO group), 5.93 (dd, 1H, $J_1 = J_2 = 6.0$ Hz, NHCO group), 5.35 (dd, 1H, $J_1 = J_2 = 3.5$ Hz, H-12), 3.96 (s, 2H, CH_2 PEG group), 3.74–3.52 (m, 8H, CH_2 PEG group), 3.35 (s, 3H, CH_3 PEG group), 3.34–3.17 (m, 4H, H-3, H-6′, and H-1′), 2.98–2.93 (m, 2H, H-1′), 2.50 (dd, 1H, $J_1 = 3.5$, $J_2 = 13.0$ Hz, H-18), 1.13, 0.96, 0.90, 0.88, 0.88, 0.76, 0.73 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 125 MHz): 178.3 (C-28), 170.0 (C-1″), 145.2 (C-13), 122.8 (C-12), 79.0 (C-3), 72.0 (C-2″), 71.1 (C-3″), 70.6 (C-4″), 70.5 (C-5″), 70.3 (C-6″), 59.1 (C-7″), 55.2 (C-5), 47.6 (C-9), 46.9 (C-19), 46.3 (C-17), 42.2 (C-14), 42.4 (C-18), 39.5 (C-1′), 39.4 (C-8), 38.8 (C-4), 38.6 (C-1), 38.9 (C-6′), 37.1 (C-10), 34.3 (C-21), 32.6 (C-7), 32.5 (C-22), 33.1 (Me), 30.8 (C-20), 29.7 (C-2′), 29.4 (C-5′), 28.2 (Me), 27.4 (C-2), 27.2 (C-15), 26.9 (C-3′), 26.7 (C-4′), 25.8 (Me), 23.9 (C-11), 23.7 (C-16), 23.7 (Me), 18.4 (C-6), 17.0 (Me), 15.7 (Me), 15.5 (Me); ESI-HRMS m/z calcd for $C_{43}H_{75}N_2O_6$ $[M+1]^+$ 715.5625, found 715.5634.

4.4.4. Compound **16**

Transparent syrup; $[\alpha]^{25}_D + 29$ (c 1 in $CHCl_3$); IR ν_{max}(film)/cm^{-1} 3352, 2926, 1736, 1635, 1530, 1365, 1106 and 528; δ_H ($CDCl_3$, 400 MHz): 7.00 (dd, 1H, $J_1 = J_2 = 6.2$ Hz, NHCO group), 5.91 (dd, 1H, $J_1 = J_2 = 5.2$ Hz, NHCO group), 5.37 (dd, 1H, $J_1 = J_2 = 3.2$ Hz, H-12), 4.00 (s, 2H, CH_2 PEG group), 3.71–3.54 (m, 8H, CH_2 PEG group), 3.71–3.54 (m, 1H, H-2), 3.37 (s, 3H, CH_3 PEG group), 3.34–3.24 (m, 3H, H-1′ and H-6′), 3.00 (d, 1H, $J_1 = 9.6$ Hz, H-3), 2.96–2.93 (m, 1H, H-1′), 2.50 (dd, 1H, $J_1 = 3.4$, $J_2 = 13.4$ Hz, H-18), 1.15, 1.03, 0.98, 0.90, 0.90, 0.82, 0.75 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 100 MHz): 178.2 (C-28), 170.0 (C-1″), 145.3 (C-13), 122.6 (C-12), 84.0 (C-3), 72.0 (C-2″), 71.1 (C-3″), 70.7 (C-4″), 70.6 (C-5″), 70.3 (C-6″), 69.0 (C-2), 59.1 (C-7″), 55.3 (C-5), 47.7 (C-9), 46.9 (C-1), 46.5 (C-19), 46.4 (C-17), 42.4 (C-18), 42.3 (C-14), 39.6 (C-8), 39.5 (C-1′), 39.3 (C-10), 38.9 (C-6′), 38.3 (C-4), 34.3 (C-21), 32.7 (C-7), 32.4 (C-22), 33.1 (Me), 30.9 (C-20), 29.8 (C-2′), 28.7 (C-5′), 28.7 (Me), 27.4 (C-15), 25.9 (C-3′), 25.9 (C-4′), 25.9 (Me), 23.9 (C-11), 23.7 (C-16), 23.7 (Me), 18.4 (C-6), 17.1 (Me), 16.9 (Me), 16.8 (Me); ESI-HRMS m/z calcd for $C_{43}H_{75}N_2O_7$ $[M+1]^+$ 731.5574, found 731.5560.

4.4.5. Compound **17**

Transparent syrup; $[\alpha]^{25}_D + 38$ (c 1 in $CHCl_3$); IR ν_{max}(film)/cm^{-1} 3354, 2924, 1659, 1531, 1463, 1385, 1105 and 571; δ_H ($CDCl_3$, 500 MHz): 7.00 (dd, 1H, $J_1 = J_2 = 5.0$ Hz, NHCO group), 6.00 (dd, 1H, $J_1 = J_2 = 5.5$ Hz, NHCO group), 5.36 (dd, 1H, $J_1 = J_2 = 3.3$ Hz, H-12), 4.00 (s, 2H, CH_2 PEG group), 3.75–3.54 (m, 8H, CH_2 PEG group), 3.37 (s, 3H, CH_3 PEG group), 3.35–3.18 (m, 4H, H-3, H-1′, and H-10′), 3.00–2.96 (m, 2H, H-1′), 2.50 (dd, 1H, $J_1 = 3.5$, $J_2 = 13.0$ Hz, H-18), 1.15, 0.97, 0.90, 0.90, 0.90, 0.77, 0.75 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 125 MHz): 178.2 (C-28), 169.9 (C-1″), 145.3 (C-13), 122.8 (C-12), 79.0 (C-3), 72.0 (C-2″), 71.1 (C-3″), 70.7 (C-4″), 70.6 (C-5″), 70.3 (C-6″), 59.2 (C-7″), 55.3 (C-5), 47.7 (C-9), 47.0 (C-19), 46.4 (C-17), 42.2 (C-14), 42.5 (C-18), 39.6 (C-1′), 39.5 (C-8), 39.1 (C-10′), 39.0 (C-4), 38.6 (C-1), 37.1 (C-10), 34.3 (C-21), 32.7 (C-7), 32.5 (C-22), 33.1 (Me), 30.9 (C-20), 29.8 (C-2′), 29.8 (C-9′), 29.7 (C-3′), 29.6 (C-8′), 29.5 (C-4′), 29.5 (C-7′), 28.3 (Me), 27.5 (C-2), 27.3 (C-15), 27.1 (C-5′), 27.1 (C-6′), 25.9 (Me), 23.7 (C-16), 23.7 (Me), 23.9 (C-11), 18.4 (C-6), 17.1 (Me), 15.7 (Me), 15.5 (Me); ESI-HRMS m/z calcd for $C_{47}H_{83}N_2O_6$ $[M+1]^+$ 771.6251, found 711.6266.

4.4.6. Compound **18**

Transparent syrup; $[\alpha]^{25}_D + 21$ (c 1 in $CHCl_3$); IR ν_{max}(film)/cm^{-1} 3349, 2924, 1658, 1532, 1455, 1365, 1104 and 560; δ_H ($CDCl_3$, 500 MHz): 7.03 (dd, 1H, $J_1 = J_2 = 5.5$ Hz, NHCO group), 5.90 (dd, 1H, $J_1 = J_2 = 5.5$ Hz, NHCO group), 5.33 (dd, 1H, $J_1 = J_2 = 3.5$ Hz, H-12), 3.93 (s, 2H, CH_2 PEG group), 3.66–3.51 (m, 9H, H-2 and 4 CH_2 PEG group), 3.34 (s, 3H, CH_3 PEG group), 3.31–3.20 (m, 3H, H-1′ and H-10′), 2.95 (d, 1H, $J_1 = 9.5$ Hz, H-3), 2.98–2.94 (m, 1H, H-1′), 2.45 (dd, 1H, $J_1 = 3.5$, $J_2 = 12.5$ Hz, H-18), 1.24, 1.12, 1.00, 0.94, 0.86, 0.78, 0.72 (s, 3H each, methyl groups); δ_C ($CDCl_3$, 125 MHz): 178.1 (C-28), 170.0 (C-1″), 145.2 (C-13), 122.6 (C-12), 83.6 (C-3), 71.9 (C-2″), 71.0 (C-3″), 70.6 (C-4″), 70.5 (C-5″), 70.3 (C-6″), 68.7 (C-2), 59.1 (C-7″), 55.3 (C-5), 47.6 (C-9), 46.8 (C-1), 46.5 (C-19), 46.2 (C-17), 42.5 (C-18), 42.1 (C-14), 39.5 (C-1′), 39.4 (C-8), 39.1 (C-10), 39.1 (C-10′), 38.9 (C-4), 34.2 (C-21), 32.6 (C-7), 32.4 (C-22), 33.1 (Me), 30.7 (C-20), 29.8 (C-2′), 29.7 (C-9′), 29.6 (C-3′),29.6 (C-8′), 29.5 (C-4′), 29.4 (C-7′), 28.8 (Me), 27.3 (C-15), 27.1 (C-5′), 27.0 (C-6′), 25.8 (Me), 23.8 (C-11), 23.7 (C-16), 23.7 (Me), 18.4 (C-6), 17.1 (Me), 16.9 (Me), 16.8 (Me); ESI-HRMS m/z calcd for $C_{47}H_{83}N_2O_7$ $[M+1]^+$ 787.6200, found 787.6211.

4.5. Biological experimental procedures

4.5.1. Drugs

The OA or MA derivatives employed in cell treatment were dissolved before use at a concentration of 5 mg/mL in DMSO. They were stored at −20 °C, constituting the stock solution from which solutions were made for the different tests. Prior to the experiments, this solution was diluted in cell-culture medium.

4.5.2. Cell cultures

Mouse melanoma cells B16-F10 (ATTC no. CRL-6475), human colorectal adenocarcinoma cells HT29 (ECACC no. 9172201; ATTC no. HTB-38), human hepatocarcinoma cells Hep G2 (ECACC no. 85011430), non-tumour human lung fibroblast cells HPF (ScienCell cno. 3300), non-tumour rat epithelium cells IEC-18 (ECACC no. 88011801), and non-tumour human embryo liver cells WRL68 (ECACC no. 89121403), were cultured in DMEM medium supplemented with 2 mM glutamine, 10% heat-inactivated foetal bovine

serum (FCS), 10,000 units/mL of penicillin and 10 mg/mL of streptomycin, being incubated at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. The culture media were changed every 48 h and the confluent cultures were separated with a trypsin solution (0.25%-EDTA). Subconfluent monolayer cells were used in all experiments. All cell lines used were provided by the cell bank of the University of Granada (Spain), except for the HPF cell line that was purchased from ScienCell Research Laboratories.

4.5.3. Cell-proliferation activity assay

The effect of the synthesised compounds on the viability of tumour and non-tumour cells was performed using the MTT assay, based on the ability of living cells to cleave the tetrazolium ring, thus producing formazan, which absorbs at 570 nm. All these compounds were assayed against the selected cancer-cell lines, and only the diamine and PEGylated-diamine conjugates were also tested against the selected non-tumour cell lines. To examine the cytotoxic effects of these compounds, the cells were seeded in 96-well plates at an initial cell density of $5 \cdot 10^3$ B16-F10 cells, $6 \cdot 10^3$ HT29 cells, $15 \cdot 10^3$ Hep G2 cells, $8 \cdot 10^3$ HPF, $15 \cdot 10^3$ IEC-18 cells, and $11 \cdot 10^3$ WRL68 cells, per well. For their growth, they were incubated for 24 h and subsequently treated with different compounds in triplicate, at different concentrations (0–200 μg/mL), and incubated for 72 h. Thereafter, 100 μL of MTT solution (0.5 mg/mL) were added to each well. After 2 h of incubation, the cells were washed twice with phosphate buffered saline (PBS), and the formazan was resuspended in 100 μL of DMSO. Relative cell viability, with respect to untreated control cells, was measured by absorbance at 550 nm on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN, Austria). Compounds with low IC_{50} values (**1**, **3**, **9**, **11**, **13**, **14**, **17**, and **18**) were selected for several cytometry assays, such as apoptosis, cell cycle, and mitochondrial-membrane potential. These experiments were measured and compared to the control after 24, 48, or 72 h of treatment.

4.5.4. Annexin V-FICT/propidium iodide flow-cytometry analysis

Apoptosis was assessed by flow cytometry using a FACScan (fluorescence-activated cell sorter) flow cytometer (Coulter Corporation, Hialeah, FL, USA). For this assay, $5 \cdot 10^4$ B16-F10 cells were plated in 24-well plates with 1.5 mL of medium and incubated for 24 h. Subsequently, the cells were treated with the selected compounds in triplicate for 24, 48, and 72 h at their corresponding IC_{50} concentration. The cells were collected and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC conjugate (1 μg/mL) was then added and incubated for 15 min at rt in darkness. Just before the analysis by flow cytometry, cells were stained with 5 μL of 1 mg/mL PI solution. In each experiment, approximately $10 \cdot 10^3$ cells were analysed and the experiment was duplicated twice.

4.5.5. Cell cycle

The method used to quantify the amount of DNA in the different phases of the cell cycle (G0/G1, S, and G2/M) was performed by flow cytometry, using a fluorescence-activated cell sorter (FACS) at 488 nm in an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL, USA). For this assay, $5 \cdot 10^4$ B16-F10 cells were plated in 24-well plates with 1.5 mL of medium and incubated for 24 h. After this time, the cells were treated with the different compounds selected for 48 h at their corresponding IC_{50} concentration. After treatment, the cells were washed twice with PBS, trypsinized and resuspended in 1 × TBS (10 mM Tris and 150 mM NaCl), and thereafter Vindelov buffer (100 mM Tris, 100 mM NaCl, 10 mg/mL Rnase, and 1 mg/mL PI, at pH 8) was added. Cells were stored on ice, and just before measurement, were stained with 10 mL of 1 mg/mL PI solution. Approximately, $10 \cdot 10^3$ cells were analysed in each experiment. The experiments were performed three times with two replicates per assay.

4.5.6. Flow-cytometry analysis of the mitochondrial-membrane potential

The electrochemical gradient across the mitochondrial membrane was studied by analytical flow cytometry, using dihydrorhodamine (DHR). DHR is oxidized in contact with living cells, forming a highly fluorescent product called rhodamine (Rh 123). The fluorescence emitted can be monitored by fluorescence spectroscopy using excitation and emission wavelengths of 500 and 536 nm, respectively. B16-F10 cells ($5 \cdot 10^4$) were plated in 24-well plates, incubated for 24 h and treated with the compounds selected for 48 h at their corresponding IC_{50} concentration. After treatment, the culture medium was renewed with fresh medium by adding 0.5 mL of DHR, for a final concentration of 5 mg/mL. Cells were incubated for 1 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidity, and subsequently washed and resuspended in PBS with 5 mg/mL of PI. The fluorescence intensity was measured using a FACScan flow cytometer (fluorescence-activated cell sorter). The experiments were performed three times with two replicates per assay.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2018.02.044.

References

[1] J.J. Monsuez, J.C. Charniot, N. Vignat, J.Y. Artigou, Cardiac side-effects of cancer chemotherapy, Int. J. Cardiol. 144 (2010) 3–15.
[2] B. Guillot, D. Bessis, O. Dereure, Mucocutaneous side effects of antineoplastic chemotherapy, Expet Opin. Drug Saf. 3 (2004) 579–587.
[3] A.D. Kinghorn, Anticancer Agents from Natural Products, second ed., CRC Press/Taylor & Francis Group, Boca Raton, FL, 2012.
[4] G.M. Cragg, P.G. Grothaus, D.J. Newman, Impact of natural products on developing new anti-cancer agents, Chem. Rev. 109 (2009) 3012–3043.
[5] M. Trendowski, Recent advances in the development of antineoplastic agents derived from natural products, Drugs 75 (2015) 1993–2016.
[6] K.H. Lee, Discovery and development of natural product-derived chemotherapeutic agents based on a medicinal chemistry approach, J. Nat. Prod. 73 (2010) 500–516.
[7] E.E. Rufino-Palomares, A. Pérez-Jiménez, F.J. Reyes-Zurita, L. García-Salguero, K. Mokhtari, A. Herrera-Merchán, P.P. Medina, J. Peragón, J.A. Lupiáñez, Anticancer and anti-angiogenic properties of various natural pentacyclic triterpenoids and some of their chemical derivatives, Curr. Org. Chem. 19 (2015) 919–947.
[8] M. Chudzik, I. Korzonek-Szlacheta, W. Król, Triterpenes as potentially cytotoxic compounds, Molecules 20 (2015) 1610–1625.
[9] J.A.R. Salvador, A.S. Leal, D.P.S. Alho, B.M.F. Goncalves, A.S. Valdeira, V.I.S. Mendes, Y. Jing, Highlights of pentacyclic triterpenoids in the cancer settings, Stud. Nat. Prod. Chem. 41 (2014) 33–73.
[10] S.M. Kamble, S.N. Goyal, C.R. Patil, Multifunctional pentacyclic triterpenoids as adjuvants in cancer chemotherapy: a review, RSC Adv. 4 (2014) 33370–33382.
[11] M.K. Shanmugam, A.H. Nguyen, A.P. Kumar, B.K.H. Tan, G. Sethi, Targeted inhibition of tumor proliferation, survival, and metastasis by pentacyclic triterpenoids: potential role in prevention and therapy of cancer, Canc. Lett. 320 (2012) 158–170.
[12] J.M.R. Patlolla, C.V. Rao, Triterpenoids for cancer prevention and treatment: current status and future prospects, Curr. Pharmaceut. Biotechnol. 13 (2012) 147–155.
[13] B. Eignerova, M. Tichy, J. Krasulova, M. Kvasnica, L. Rarova, R. Christova, M. Urban, B. Bednarczyk-Cwynar, M. Hajduch, J. Sarek, Synthesis and antiproliferative properties of new hydrophilic esters of triterpenic acids, Eur. J.

Med. Chem. 140 (2017) 403–420.
[14] A. Garcia-Granados, A. Martinez, J.N. Moliz, A. Parra, F. Rivas, 3β-hydroxyolean-12-en-28-oic acid (oleanolic acid), Molecules 3 (1998) M87.
[15] A. Garcia-Granados, A. Martinez, J.N. Moliz, A. Parra, F. Rivas, 2α,3β-dihydroxyolean-12-en-28-oic acid (maslinic acid), Molecules 3 (1998) M88.
[16] A. Fernandez-Hernandez, A. Martinez, F. Rivas, J.A. Garcia-Mesa, A. Parra, Effect of the solvent and the sample preparation on the determination of triterpene compounds in two-phase olive-mill-waste samples, J. Agric. Food Chem. 63 (2015) 4269–4275.
[17] N.R. Parikh, A. Mandal, D. Bhatia, K.S. Siveen, G. Sethi, A. Bishayee, Oleanane triterpenoids in the prevention and therapy of breast cancer: current evidence and future perspectives, Phytochemistry Rev. 13 (2014) 793–810.
[18] G. Lozano-Mena, M. Sánchez-González, M.E. Juan, J.M. Planas, Maslinic acid, a natural phytoalexin-type triterpene from olives — a promising nutraceutical? Molecules 19 (2014) 11538–11559.
[19] J.A.R. Salvador, A.S. Leal, A.S. Valdeira, B.M.F. Goncalves, D.P.S. Alho, S.A.C. Figueiredo, S.M. Silvestre, V.I.S. Mendes, Oleanane-, ursane-, and quinone methide friedelane-type triterpenoid derivatives: recent advances in cancer treatment, Eur. J. Med. Chem. 142 (2017) 95–130.
[20] M. Masullo, C. Pizza, S. Piacente, Oleanane derivatives for pharmaceutical use: a patent review (2000-2016), Expert Opin. Ther. Pat. 27 (2017) 237–255.
[21] K.T. Liby, M.B. Sporn, Synthetic oleanane triterpenoids: multifunctional drugs with a broad range of applications for prevention and treatment of chronic disease, Pharmacol. Rev. 64 (2012) 972–1003.
[22] A. Parra, S. Martin-Fonseca, F. Rivas, F.J. Reyes-Zurita, M. Medina-O'Donnell, A. Martinez, A. Garcia-Granados, J.A. Lupiañez, F. Albericio, Semi-synthesis of acylated triterpenes from olive-oil industry wastes for the development of anticancer and anti-HIV agents, Eur. J. Med. Chem. 74 (2014) 278–301.
[23] A. Parra, S. Martin-Fonseca, F. Rivas, F.J. Reyes-Zurita, M. Medina-O'Donnell, E.E. Rufino-Palomares, A. Martinez, A. Garcia-Granados, J.A. Lupiañez, F. Albericio, Solid-phase library synthesis of bi-functional derivatives of oleanolic and maslinic acids and their cytotoxicity on three cancer cell lines, ACS Comb. Sci. 16 (2014) 428–447.
[24] M. Medina-O'Donnell, F. Rivas, F.J. Reyes-Zurita, A. Martinez, S. Martin-Fonseca, A. Garcia-Granados, R.M. Ferrer-Martin, J.A. Lupianez, A. Parra, Semisynthesis and antiproliferative evaluation of PEGylated pentacyclic triterpenos, Eur. J. Med. Chem. 118 (2016) 64–78.
[25] F.J. Reyes-Zurita, M. Medina-O'Donnell, R.M. Ferrer-Martin, E.E. Rufino-Palomares, S. Martin-Fonseca, F. Rivas, A. Martinez, A. Garcia-Granados, A. Perez-Jimenez, L. Garcia-Salguero, J. Peragon, K. Mokhtari, P.P. Medina, A. Parra, J.A. Lupiañez, The oleanolic acid derivative, 3-O-succinyl-28-O-benzyl oleanolate, induces apoptosis in B16-F10 melanoma cells via the mitochondrial apoptotic pathway, RSC Adv. 6 (2016) 93590–93601.
[26] M. Medina-O'Donnell, F. Rivas, F.J. Reyes-Zurita, A. Martinez, F. Galisteo-González, J.A. Lupiañez, A. Parra, Synthesis and in vitro antiproliferative evaluation of PEGylated triterpene acids, Fitoterapia 120 (2017) 25–40.
[27] T. Tian, X. Liu, E.S. Lee, J. Sun, Z. Feng, L. Zhao, C. Zhao, Synthesis of novel oleanolic acid and ursolic acid in C-28 position derivatives as potential anticancer agents, Arch. Pharm. Res. 40 (2017) 458–468.
[28] S. Sommerwerk, L. Heller, C. Kerzig, A.E. Kramell, R. Csuk, Rhodamine B conjugates of triterpenoic acids are cytotoxic mitocans even at nanomolar concentrations, Eur. J. Med. Chem. 127 (2017) 1–9.
[29] L. Heller, A. Knorrscheidt, F. Flemming, J. Wiemann, S. Sommerwerk, I.Z. Pavel, A. Al-Harrasi, R. Csuk, Synthesis and proapoptotic activity of oleanolic acid derived amides, Bioorg. Chem. 68 (2016) 137–151.
[30] J.J. Swidorski, Z. Liu, S.Y. Sit, J. Chen, Y. Chen, N. Sin, B.L. Venables, D.D. Parker, B. Nowicka-Sans, B.J. Terry, T. Protack, S. Rahematpura, U. Hanumegowda, S. Jenkins, M. Krystal, I.B. Dicker, N.A. Meanwell, A. Regueiro-Ren, Inhibitors of HIV-1 maturation: development of structure-activity relationship for C-28 amides based on C-3 benzoic acid-modified triterpenoids, Bioorg. Med. Chem. Lett 26 (2016) 1925–1930.
[31] B. Siewert, E. Pianowski, A. Obernauer, R. Csuk, Towards cytotoxic and selective derivatives of maslinic acid, Bioorg. Med. Chem. 22 (2014) 594–615.
[32] B. Siewert, E. Pianowski, R. Csuk, Esters and amides of maslinic acid trigger apoptosis in human tumor cells and alter their mode of action with respect to the substitution pattern at C-28, Eur. J. Med. Chem. 70 (2013) 259–272.
[33] K.G. Cheng, C.H. Su, L.D. Yang, J. Liu, Z.F. Chen, Synthesis of oleanolic acid dimers linked at C-28 and evaluation of anti-tumor activity, Eur. J. Med. Chem. 89 (2015) 480–489.
[34] K. Cheng, J. Liu, H. Sun, J. Xie, Synthesis of oleanolic acid dimers as inhibitors of glycogen phosphorylase, Chem. Biodivers. 7 (2010) 690–697.
[35] I. Fernandez-Pastor, A. Fernandez-Hernandez, S. Perez-Criado, F. Rivas, A. Martinez, A. Garcia-Granados, A. Parra, Microwave-assisted extraction versus Soxhlet extraction to determine triterpene acids in olive skins, J. Sep. Sci. 40 (2017) 1209–1217.
[36] A. Martinez, F. Rivas, A. Perojil, A. Parra, A. Garcia-Granados, A. Fernandez-Vivas, Biotransformation of oleanolic and maslinic acids by Rhizomucor miehei, Phytochemistry 94 (2013) 229–237.