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Synthesis, solubility and antitumor activity of maslinic acid derivatives

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ABSTRACT

Maslinic acid (MA), a pentacyclic triterpenoid obtained from olives that is characterized by its antiproliferative activity in tumor cells, has become a promising molecule that could be modified to improve cancer treatment. In this work we have synthesized in good yields several new MA conjugates, including glycerin, *oligo*(ethylene glycol), and amino acid derivatives (compounds **3a-f**). The synthesis offers the possibility of recovering unreacted MA, and thus the scaling up of the process. For the tyramine-MA conjugate, compound **3f** or TMA, the preparation has been optimized to a one-pot reaction. Solubility of conjugates in polar solvents has been measured, showing a marked increase of solubility with respect to MA. Moreover, we selected the tyramidyl maslinic acid conjugate (**3f** or TMA) to determine antitumor capacity over a wide range of cancer cell lines, including glioblastoma, melanoma, breast, lung, colorectal and pancreatic cancer. Our results clearly demonstrated that TMA induced higher cytotoxicity in all cancer cell types compared to MA. TMA was more effective than MA, especially in breast cancer cells (MCF-7) and melanoma cells (B16–F10) where IC_{50} reductions of 4.12 and 4.72, respectively, was detected. Interestingly, TMA showed a remarkable antitumor ability against the resistant HCT-15 colon cancer cell line. Furthermore, we demonstrated for the first time a relevant effect of a MA derivative against glioblastoma cells (A172 and SF-268). These results suggest that TMA is able to improve the antitumor characteristics of MA in a wide range of cancers and that it may be a promising compound for various tumor types, including resistant cancer.

1. Introduction

Pentacyclic triterpenes (PT) are naturally occurring secondary metabolites from plants, fungi, marine invertebrates, and many other organisms [1]. Recent studies show that the connection of these pentacyclic compounds to an appropriate modifying molecule, results in extremely useful semisynthetic products with a high potential to treat for example cancer or viral infections, or results in designed compounds that are useful for the study of its mechanism of action at the molecular level [1]. Within the group of pentacyclic triterpenoids with an oleanane skeleton, maslinic acid (MA, **1**) appears abundantly in industrial olive oil waste [2] attracting attention as a pharmacologically active product due to its varied biological properties, especially as an antitumor [3,4], antioxidant [5], antiviral, against HIV-1 protease [6] and anti-inflammatory agent [7,8]. However, MA presents very low solubility in water solvents.

In recent years, the synthesis of conjugates of PT with other molecules improved their biological properties [1]. It is very common the use of a linker to connect PT to different molecules, for example, betulinic acid has been modified with azidothymidine under Click reaction conditions [9], with amino acids and polyphenols [1], or even with Darunavir in a direct coupling with the drug [10]. However, few cases are reported for the preparation of MA derivatives, *i*) MA has been directly connected to coumarin and used to study the conformational changes of human serum albumin since the conjugate forms an excellent fluorescence resonance energy transfer pair with the tryptophan residue of the protein [11], and *ii*), recently, to the coumarin skeleton using an alkene as linker [12]. In

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case on cancer, some of MA conjugates have been tested as potential antitumor agents, and some exhibit cytotoxicity with submicromolar values and reach a total apoptosis rate greater than 95% [6,13,14].

In recent decades, cancer has acquired increasing importance as the leading cause of premature death worldwide, leading in 57 countries ahead of cardiovascular diseases [15]. Concretely, lung cancer being the leading cause of death from this disease (18%) followed by colorectal cancer [16]. Pancreatic cancer (4.7% of cancer deaths) has the worst survival data of all solid tumors [17]. Finally, melanoma is responsible for most skin cancer-related deaths and glioblastoma is the most frequent and lethal brain tumor [18].

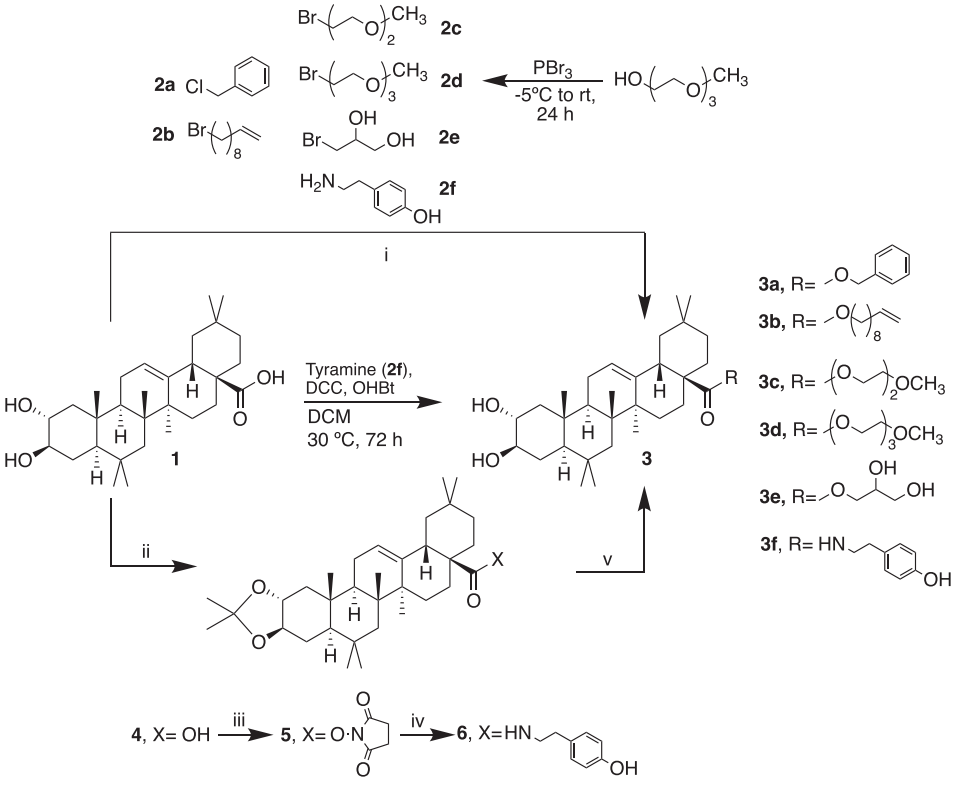
As mentioned, MA has already demonstrated a significant antitumor effect in very diverse types of cancer such as colorectal, breast, lung and melanoma, among others. In fact, MA inhibited cell proliferation and induced apoptosis via the mitochondrial pathway in the human adenocarcinoma cell line HT29 [19]. Furthermore, the antitumor effect of this compound was related to the adenosine monophosphate (AMP)-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR) pathway in in vitro and in vivo models of colorectal cancer [20]. Besides, MA can induce apoptosis in human lung cancer cell line by regulating the cleavage of caspases as well as Smac, c-IAP1, c-IAP2, XIAP and Survivin proteins [21]. On the other hand, Tian et al. showed that pancreatic cancer cells activated autophagy through down-regulation of HSPA8 protein when treated with MA [22]. Successful results have also been obtained in breast cancer, in which MA induced caspase-independent apoptosis in estrogen-positive and triple-negative breast cancer cell lines. Therefore, the ERK signaling pathway was involved in this process [23]. Likewise, Wang et al. showed that the triple-negative breast carcinoma cell line MDA-MB-231 was more sensitive to Docetaxel when combined treatment with MA was performed due to the regulation of the MELK-FoxM1-ABCB1 signaling cascade [24]. MA derivatives have also been synthesized showing an 80% of pro-apoptotic effect in B16–F10 murine melanoma cells [25]. In this context, the synthesis of MA derivatives emerges as a promising and unexplored area that could afford a series of molecules with improved cancer activities.

In the present study, conjugates of MA have been prepared through the C-28 carboxyl group of this pentacyclic triterpene without using linker at that position. Coupling has been carried out with several compounds, from benzyl chloride and the amino acid tyramine to *oligo*(-ethylene glycol) derivatives. We chose tyramine as model amino acid since *i*) the conjugate will present an improved solubility in aqueous solvents [12], *ii*) among others active compounds tested, tyramine derivatives showed the highest activity against cholangiocarcinoma cell lines, which was nearly equal to that of the 5-fluorouracil standard [26], and *iii*) amino acid derivatives of PT have been reported as prodrugs that improve intestinal permeability and pharmacokinetics of the drug [27]. In order to determine the enhancement of the antitumor effect of these maslinic acid derivatives, a cytotoxic assay has been carried out for the tyramine conjugate (**3f** or TMA) on some cancer cell lines including breast, lung, colorectal and pancreatic cancer and glioblastoma and melanoma cells.

2. Result and discussion

2.1. Synthesis

The reaction of MA (**1**) with benzyl chloride (**2a**) has been chose to stablish the optimal conditions for the coupling. Dimethylformamide as solvent, K_2CO_3 as a base and heating at 65 °C during 12 h have been found to produce the higher yields. Under these conditions, MA (**1**) has been coupled a series of halogen derivatives (**2a-e**) to obtain the corresponding **1**-carboxyl substituted derivatives (**3a-e**) in good yields (Scheme 1). Bromine-alkene derivative **2b** add to the conjugate an alkene group and allow the photo-activated grafting of the **1**-alkene compound



i) **2a-e**, K_2CO_3, DMF; ii) Dimethoxypropane, DCM, rt, 48 h, 91 %; iii) NHS, DCC, EtOAc, rt, 24 h, 99 %
iv) Tyramine (**2f**), DMAP, DCM, rt, 72 h, 23 %; v) PTSA, MeOH, rt, 1 h, 78 %

Scheme 1. i) **2a-e**, K_2CO_3, DMF; ii) Dimethoxypropane, DCM, 25 °C, 48 h, 91%; iii) NHS, DCC, EtOAc, rt, 24 h, 99%; iv) Tyramine (**2f**), DMAP, DCM, rt, 72 h, 23%; v) PTSA, MeOH, rt, 1 h, 78%.

onto hydrogen-terminated silicon surfaces [28]. Reagents **2c,d**, consisting in two and three ethylene glycol units, respectively, and reagent **2e**, a glycerin derivative, have been used with the purpose of improving solubility of the MA conjugates. In case of reagent **2d**, this compound was obtained in good yield by solvent-free reaction of tri(ethylene glycol) monomethyl ether with PBr_3 (Fig. 1).

The introduction of a nitrogen atom in the carbonyl group of **1**, to obtain compound **3f**, was carried out initially in three steps, firstly, protection of vicinal hydroxyl groups by treatment of **1** with 2,2-dimethoxypropane to give **4**, secondly, activation of carboxyl group with *N*-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) and treatment with tyramine (**2f**) to give **6**, and finally, deprotection of **6** with PTSA to obtain **3f** (TMA). This sequence afforded compound **3f** with a global yield of 24%.

For the improved efficiency of the amidation reaction between the primary amine reagent group of **2f** and the C-28 carboxylic acid group of **1**, this functionality was activated previously with DCC and OHBt [29]. The intermediate MA−Bt derivative was formed by adding OHBt to a solution of this triterpene in dry dichloromethane (DCM), in the presence of DCC, at 30 °C for 72 h. Then, the mixture was diluted with brine and decanted, yielding compound **3f** (TMA) in 78% yield (Fig. 1).

2.2. Solubility

Solubility is a major issue in drug design [30]. Solubility in water and hydrophilic solvents is necessary for biological applications of active compounds. This physicochemical property is a major issue with MA and its conjugates, since the triterpene skeleton in MA has a markedly reduced solubility. In order to analyze this point, we measured the solubility of the conjugates **3a-f** in polar solvents as water, methanol, di(ethylene glycol), propylene glycol and glycerin (Table 1). The solubility of **1** in hydrophilic solvents is very poor, except in propylene glycol (3.1 mg/mL, Table 1, entry 1). As expected, compounds **3a,b**, having a benzyl group and alkenyl chain, respectively, in the structure, do not improve the solubility (Table 1, entries 2 and 3). However, conjugates **3c,d**, presenting di(ethylene glycol) and tri(ethylene glycol) moieties in the structure, respectively, show solubilities in water, di(ethylene glycol) and propylene glycol remarkably better than **1**, reaching values around 10 mg/mL in methanol (Table 1, entries 4 and 5). In this sense, it is important to remark that the presence of the *oligo*(ethylene glycol) chain in compounds **3c,d** increase the water solubility. Surprisingly, glycerin derivative **3e**, having a total of four hydroxyl groups, do not improve the mentioned solubility values (Table 1, entry 6). Finally, tyramine derivative 3f shows good solubility in the tested polar solvents (Table 1, entry 7). It is worth to mention that compounds **3a-f** are very soluble in organic

Table 1
Solubility of **1** and its conjugates **3a-f** in mg/mL of solvent.

N°	Product	Methanol	Agua	Ethyleneglycol	Gly	Propyleneglycol
1	3a	8	0	7	0	8
2	3b	0	0	0	0	0
3	3c	15	0	3	0	4
4	3d	0	0	1	0	1
5	3e	8	0.3	4	4	5
6	3f	9	0.2	7	2	9

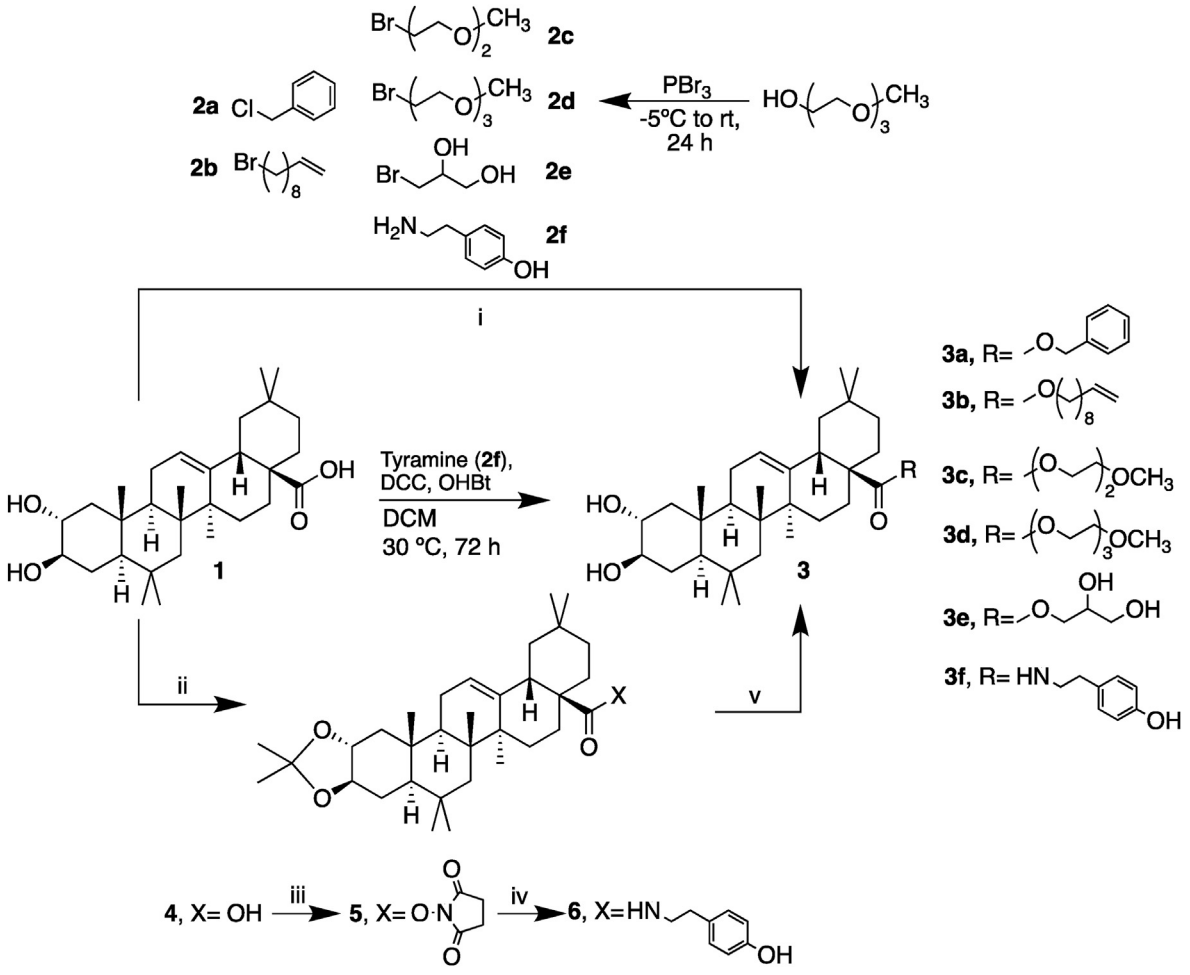


Fig. 1. Preparation of maslinic acid derivatives.

solvent as CH_2Cl_2 and $CHCl_3$. The good solubility in water and polar solvents of conjugates, remarkably improving that of MA, makes them potential useful semisynthetic drugs.

Solubilization essays were carried out of by adding compound **3a-f** to the solvent (1 mL) at rt. Samples were sonicated during 10 min to obtain a clear solution. A value of 0 means that after 10 min sonicating there is still a suspension.

2.3. In vitro antiproliferative assays

The IC_{50} of MA and the selected MA derivative tyramidyl maslinic acid (TMA) were determined in all cell lines. As shown in Table 2, significant differences were found between both MA and TMA in all of cancer cells ($p < 0.05$). TMA exhibited higher cytotoxic effect than MA in all cell lines tested. In fact, TMA was especially effective in MCF-7 human breast cancer (Fig. 2), SF-268 human glioblastoma and B16–F10 melanoma cell lines (Fig. 3) with an IC_{50} decrease of 4.12, 3 and 4.72, respectively ($p < 0.001$). In the rest of the cell lines tested, TMA showed a nearly 2-fold reduction of the IC_{50} in relation to MA. Only in the H520 human lung cancer cell line, an IC_{50} decrease of 1.78-fold was detected after TMA exposure in comparison to MA ($p < 0.001$). Furthermore, cells were treated with equivalent doses of DMSO to determine the solvent toxicity. No cytotoxicity was observed with most of the DMSO concentrations in all cell lines tested. Only with very high doses of DMSO was determined some toxicity in the human glioblastoma cell line SF-268, the human breast cancer cell line MCF-7 and the murine melanoma cell line B16–F10.

Our results suggest that MA have highly cytotoxic activity in all the cancer cell lines tested including colon, lung, breast and pancreatic cancer, melanoma and glioblastoma. Previous studies showed the MA activity against melanoma [31,32], pancreatic [22,33], breast [23,34], lung [21] and colon cancers [3,35]. However, our results showed a significant difference in antitumor activity when IC_{50}s were compared. In fact, MA showed the best results in colon cancer cells, while in breast cancer cells it had less antitumor activity. Furthermore, to our knowledge, this is the first time that MA activity has been demonstrated in glioblastoma cell lines A172 and SF-268. This differentiated antitumor activity may be related to the cancer cell type sensitivity to MA action mechanisms. In fact, it has been reported that MA plays different roles in inducing cell death. Specifically, MA modulates the caspases −3, −8 and −9 that induce cellular apoptosis and acts against the inhibitor of apoptosis family proteins (IAP) that inhibits caspase activity [21,23]. Furthermore, MA can produce an arrest in different phases of the cycle depending of the cell line such as MDA-MB-231, MDA-MB-468 and Caco-2 MA cells (arrest in G0/G1) or MCF-7 cells (arrest in the S and G2/M phases) [23,35].

Table 2
IC_{50} values of MA and TMA in cancer cell lines.

Cell line	Tumor	MA IC_{50} (μg/mL)	TMA IC_{50} (μg/mL)	Times that TMA reduces IC_{50} (μg/mL)
A172	Human glioblastoma	30.36 ± 8.69	16.35 ± 2.73	1.86
SF-268	Human glioblastoma	57.34 ± 7.71	19.11 ± 1.77	3.00
PANC-1	Human pancreatic cancer	47.19 ± 2.06	18.56 ± 0.72	2.54
MIA PaCa-2	Human pancreatic cancer	47.12 ± 3.48	16.73 ± 2.89	2.82
MCF-7	Human breast cancer	64.80 ± 4.14	15.71 ± 1.86	4.12
MDA-MB-231	Human breast cancer	32.65 ± 3.86	17.98 ± 1.00	1.82
A549	Human lung cancer	24.01 ± 3.78	12.05 ± 2.95	1.99
H520	Human lung cancer	29.43 ± 9.87	16.56 ± 1.56	1.78
T84	Human colon cancer	28.53 ± 7.46	12.02 ± 1.24	2.37
HCT15	Human colon cancer	33.97 ± 2.37	16.43 ± 1.29	2.07
MC38	Murine colon cancer	22.55 ± 1.51	10.97 ± 0.75	2.06
B16–F10	Murine melanoma	38.07 ± 2.16	8.06 ± 0.10	4.72

IC_{50} was calculated relativizing respect to toxic values of DMSO. Data are showed as mean ± SD of triplicate cultures.

Although a great effort has been made in the synthesis of new antitumor agents, cancer mortality has increased by 40% in the last 40 decades [36]. Therefore, the development of new anticancer compounds is necessary in order to achieve new effective and safe treatments. In this context, MA has been found to be a promising anti-tumor agent. Interestingly, its chemical modifications have shown cytotoxic activity against cancer cells even better than native drug [37,38]. In our study, synthesized TMA showed a significantly higher cytotoxicity in all cell lines tested compared to MA. In fact, the greater IC_{50} decreases were observed in breast cancer (MCF-7 cell line) (4.12 times) and melanoma (B16–F10 cell line) (4.72 times). In the latter type of tumor, melanoma, some MA derivatives also induced decrease in cell proliferation. In fact, Medina-O'Donnell et al. and Chouaïb et al. developed MA modifications based on diamine and PEGylated diamine with strong antitumor activity against B16 –F10 melanoma cells (IC_{50} reductions between 0.4 and 23.2 times) [6,36]. In addition, Pavel et al. a benzylamide modification of MA was cytotoxic in B164A5 melanoma cells at 50 and 100 μM and in A375 melanoma cells at 100 μM [39]. Besides, our TMA derivative also showed high antitumor activity against pancreatic cancer cells (PANC-1 MIA PaCa-2 with an IC_{50} reduction of 2.54 and 2.82 times, respectively) and colon cancer cells (T84 and HCT15, with an IC_{50} reduction of 2.37 and 2.07 times, respectively). The remarkable results in the HCT15 colon cancer cells are very relevant since this cell line is characterized by the overexpression of P-glycoprotein (P-gp) [40], a resistance mechanism that represents one of the main causes of chemotherapy failure in colon cancer [41]. However, future studies will be necessary to demonstrate the connection with MA derivatives. Finally, our derivative MA was active against human glioblastoma cells, a fact that has been described for the first time, which represents a possible therapeutic alternative for a tumor for which there are very few therapeutic tools.

3. Conclusion

In summary, by following the optimized protocol, several new MA conjugates have been synthesized in good yields (**3a-f**). The protocol offers the possibility of recovery unreacted MA and thus the scale up of the process. Tyramine-MA conjugate, compound TMA/**3f**, has been prepared in a one-pot reaction. Solubility of conjugates in polar solvents has been measured, showing a marked increase of solubility with respect to MA. In addition, the selected tyramidyl maslinic acid conjugate (TMA/**3f**) showed great cytotoxicity in various types of cancer cell lines, including glioblastoma, melanoma, breast, lung, colorectal and pancreatic cancer. The antitumor activity was especially relevant against breast cells, melanoma and resistant colon. Interestingly, we demonstrated for the first time a remarkable effect against glioblastoma cells. Therefore, TMA could improve cancer therapy of different types of tumors, although future studies including in vivo assays will be necessary to determine its effectiveness.

4. Experimental

4.1. General remarks

Diethyl ether, dimethoxyethane (DME) and tetrahydrofuran (THF) were distilled from sodium/benzophenone under argon atmosphere, while dichloromethane (DCM) was distilled over CaH_2. All other reagents and solvents were purchased from commercial sources and used without

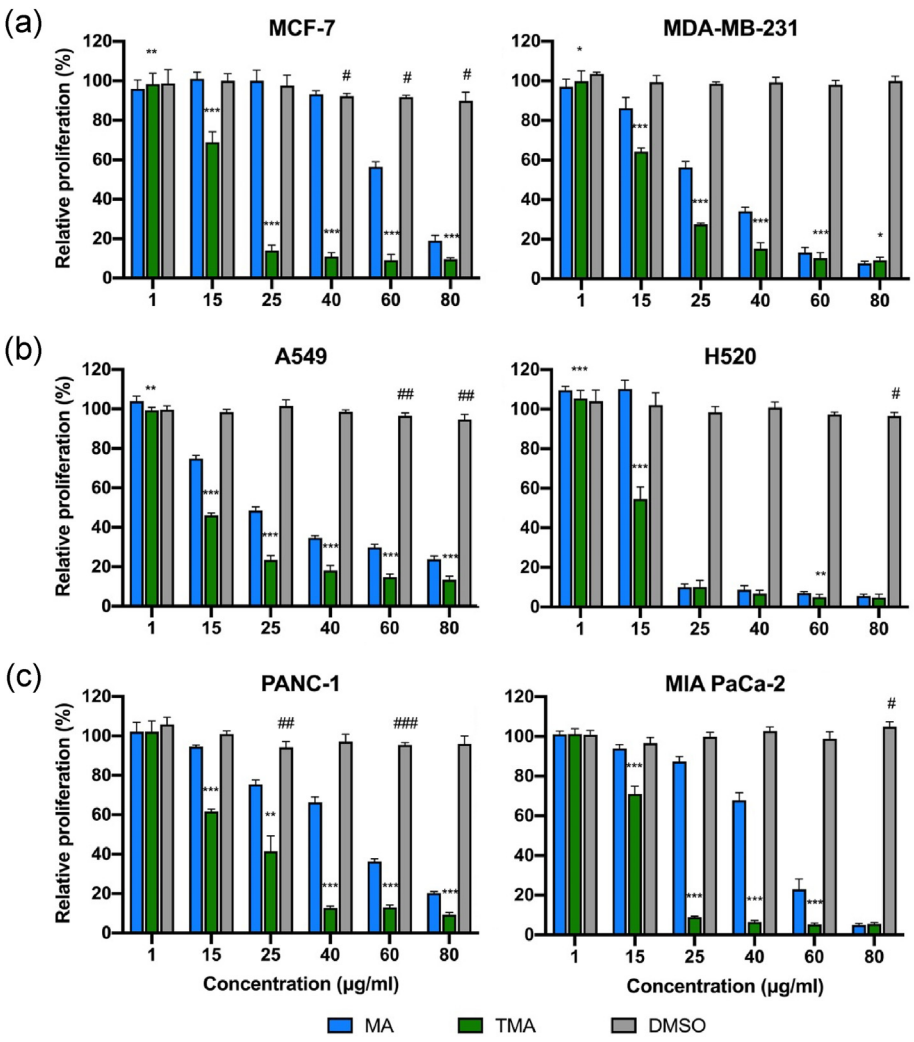


Fig. 2. *In vitro* antiproliferative assays of MA and TMA in breast, lung and pancreatic cancer cells. Antitumor effect and toxicity of equivalent concentrations of DMSO after 72 h of exposure in a) MCF-7 and MDA-MB-231 human breast cancer cell lines; b) A549 and H520 human lung cancer cell lines; and c) PANC-1 and MIA PaCa-2 human pancreatic cancer cell lines. Data represent the mean values ± SD (n = 3). (*) $p < 0.05$; (**) $p < 0.01$, and (***) $p < 0.001$ compared with MA. (#) $p < 0.05$; (##) $p < 0.01$, and (###) $p < 0.001$ compared with untreated control where DMSO showed some cytotoxicity.

further purification. All solvents were evaporated at reduced pressure by using rotary evaporator. Millipore water was used in all experiments. Melting points (mp) were determined with a Gallenkamp instrument. Optical rotations were measured on a Jasco P2000 polarimeter at 20 °C. Ultraviolet (UV) spectra were recorded on a Biochrom Libra S22 spectrophotometer. Infrared (IR) spectra were recorded on a Jasco FTIR spectrophotometer. Mass spectrometry (MS) measurements were carried out in a PerkinElmer Clarus SQ8C spectrometer coupled to a PerkinElmer Gas Chromatograph Clarus 690, and using 70 eV electron impact ionization (EI). High-resolution mass spectrometry (HRMS) was performed in a VG Autospec spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with a 400 MHz ARX 400 Bruker spectrometer by using the residual solvent peak in $CDCl_3$ (δ 7.24 ppm, 400 MHz, for ^{1}H and $\delta = 77.0$ ppm, 100 MHz, for ^{13}C). The multiplicity of the signals is indicated as: s (singlet), d (doublet), t (triplet), m (multiplet) and for any of them, br (broad). Coupling constants *J* are given in Hz. TLC analyses were performed on Merck silica gel 60 F 254 plates and column chromatography was performed on silica gel 60 (0.040–0.063 mm).

4.2. Synthesis

4.2.1. Synthesis of bromo tri(ethylene glycol) monomethyl ether (2d)

Under an Ar atmosphere, a round bottom flask containing tri(-ethylene glycol) monomethyl ether (10 g, 60.9 mmol) was cooled at −5 °C. Over this product, PBr_3 (1.9 mL, 20.3 mmol) was slowly added. The reaction mixture was stirred at −5 °C for 2 h. After this period, the mixture was left to reach rt, and stirred for 24 h more at 80 °C. The residue was vacuum distilled to obtain **2d**, which was used without more purification [42].

4.2.2. Synthesis of MA conjugates 3a-d

In a round bottom flask (50 mL), under an Ar atmosphere, **1** (4.08 g, 8.85 mmol) and K_2CO_3 (0.65 g, 4.69 mmol) in dimethylformamide (40 mL) were heated at 65 °C. Then, benzyl chloride (**2a**, 0.13 mL, 1.05 mmol), 10-bromo-1-decene (**2b**, 0.21 mL, 1.05 mmol), **2c** (0.28 mL, 2.10 mmol), **2d** (0.11 mL, 1.05 mmol) or 1-bromoglycerin (**2e**, 3-bromo-1,2-propanodiol, 0.85 mL, d = 1.771 g/mL, 9.74 mmol) was added. The reaction mixture was stirred at 65 °C for 12 h (72 h for **2e**). After this period, the mixture was concentrated to dryness under vacuum and the residue was treated with *t*-buthylmethyl ether (250 mL). Remaining solid (unreacted **1**, around 1 g) was removed by filtration. Then, the filtrates were concentrated to dryness and the residue was treated with a mixture of diethyl ether (300 mL) and methanol (0.5 mL). Remaining solid (**1**, around 0.5 g) was removed by filtration. The residue was crystallized from diethyl ether to give a solid (**3a-e**).

3a. 95.1%; white solid; mp 161–2 °C; $[\alpha]_D$ +21 (c 0.5, MeOH); IR (KBr) ν (cm^{-1}) 3320, 1730, 1487, 1052; MS *m/z* (%): 562 (M^+, 25), 544 (100), 455 (75); ^{1}H NMR ($CDCl_3$) δ (ppm): 7.28 (m, 5H, Ar–H), 5.23 (brt, 1H, H-12), 5.01 (dd, $J = 20.0, 12.8$, 2H, benzylic CH_2), 3.62 (m, 1H, H-2), 2.84 (d, $J = 9.6$, 1H, H-3), 2.88 (dd, $J = 14.4, 4.0$, 1H, H-18), 2.01–0.40 (m, 20H, aliphatic H), 1.06, 0.96, 0.88, 0.84, 0.82, 0.75, 0.62 (s, 21H, 7 x Me); ^{13}C NMR ($CDCl_3$) δ (ppm): 177.4, 143.7, 136.4, 128.4, 127.93,

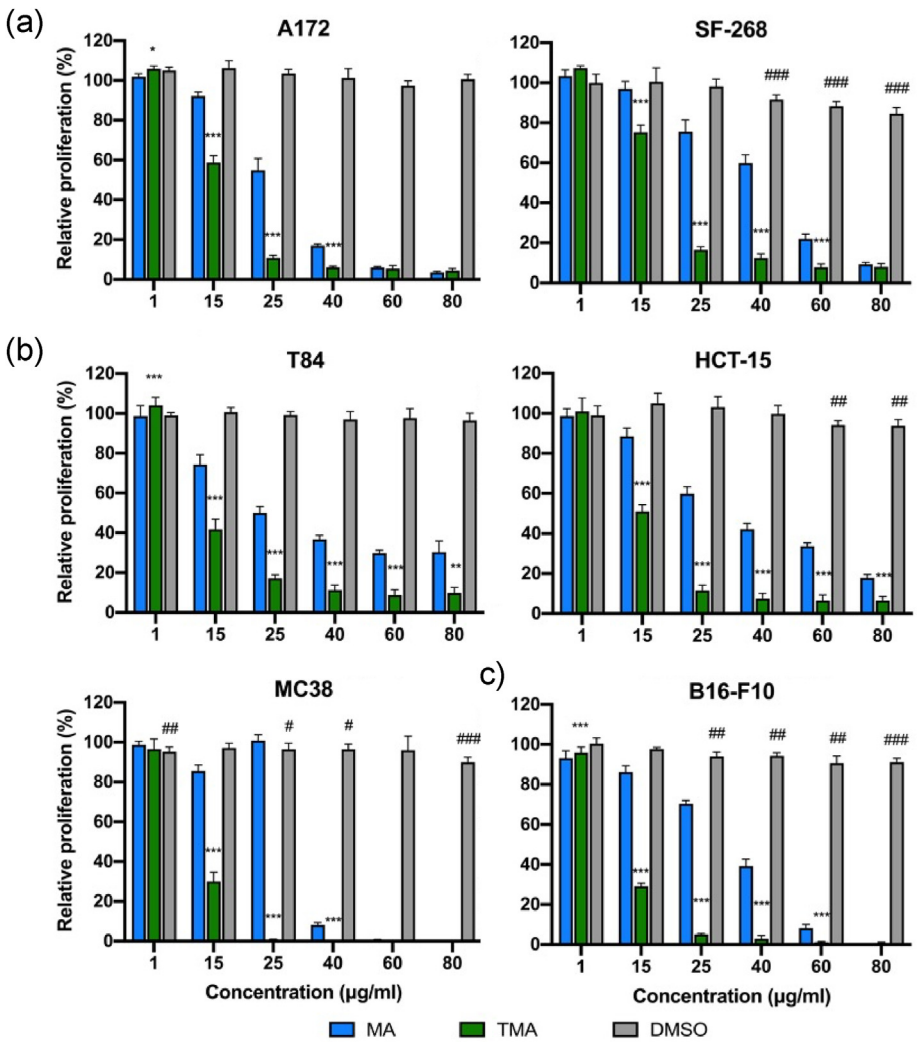


Fig. 3. *In vitro* antiproliferative assays of MA and TMA in glioblastoma colon and melanoma cancer cells. Antitumor effect and toxicity of DMSO after 72 h of exposure in a) A172 and SF-268 glioblastoma cell lines; b) T84, HCT-15 and MC38 colorectal cancer cell lines; and c) B16–F10 murine melanoma cell line. Data represent the mean values ± SD (n = 3). (*) $p < 0.05$; (**) $p < 0.01$, and (***) $p < 0.001$ compared with MA. (#) $p < 0.05$; (##) $p < 0.01$, and (###) $p < 0.001$ compared with untreated control where DMSO showed some cytotoxicity.

127.88, 122.3, 83.9, 68.9, 65.9, 55.21, 55.16, 47.5, 46.7, 46.3, 45.8, 41.7, 41.3, 39.3, 39.1, 38.2, 33.8, 33.1, 32.5, 32.3, 30.7, 28.6, 27.5, 25.9, 23.6, 23.5, 23.3, 22.9, 18.2, 16.8, 16.7, 16.5. HRMS *m/z:* $[M + H]^+$ calcd. for $C_{37}H_{54}O_4$ 563.4022, found 563.4030.

3b. 84.7%; white solid; mp 140–2 °C; $[\alpha]_D$ +37 (c 1, $CHCl_3$); IR (KBr) ν (cm^{-1}) 3100, 1717, 1610, 1510, 1033; MS *m/z* (%): 610 (M^+, 15), 592 (100), 455 (95); ^1H NMR ($CDCl_3$) δ (ppm): 5.75 (m, 1H, vinyl H), 5.23 (br t, 1H, H-12), 4.90 (m, 2H, vinyl H), 3.94 (m, 2H, $COOCH_2$), 3.62 (m, 1H, H-1), 2.94 (d, $J = 9.6$, 1H, H-3), 2.81 (dd, $J = 14.4$, 4.0, 1H, H-18), 2.0–0.8 (m, 34H, aliphatic H), 1.07, 0.97, 0.92, 0.86, 0.84, 0.76, 0.62 (s, 21H, 7 x Me); ^{13}C NMR ($CDCl_3$) δ (ppm): 177.7, 143.8, 139.0, 122.0, 114.1, 83.8, 68.8, 64.2, 55.1, 47.5, 46.5, 41.6, 41.2, 41.1, 39.3, 39.0, 38.2, 33.8, 33.7, 33.0, 32.5, 32.4, 30.6, 29.3, 29.1, 29.0, 28.8, 28.5, 28.0, 27.5, 26.0, 25.8, 23.5, 23.5, 22.0, 18.0, 17.0, 16.5, 16.4. HRMS *m/z:* $[M + H]^+$ calcd. for $C_{40}H_{67}O_4$ 611.5039, found 611.5041.

3c. 85.2%; white solid; mp 136–8 °C; $[\alpha]_D$ +47 (c 1, $CHCl_3$); IR (KBr) ν (cm^{-1}) 3400–3000, 1715, 1510, 1030; MS *m/z* (%): 574 (M^+, 5), 543 (70), 456 (100); ^1H NMR ($CDCl_3$) δ (ppm): 5.23 (br t, 1H, H-12), 4.10 (m, 2H, CH_2O), 3.7–3.4 (m, 7H, H-2, 3 x CH_2), 3.30 (s, 3H, OMe), 2.88 (d, $J =$ 9.6, 1H, H-3), 2.80 (dd, J = 14.4, 4.0, 1H, H-18), 1.98–0.80 (m, 20H, aliphatic H), 1.07, 0.97, 0.92, 0.86, 0.84, 0.76, 0.62 (s, 21H, 7 x Me); ^{13}C NMR ($CDCl_3$) δ (ppm): 177.5, 143.6, 122.1, 83.8, 71.8, 70.4, 69.1, 68.8, 63.3, 59.0, 55.2, 47.5, 46.6, 46.2, 45.7, 41.6, 41.1, 39.3, 39.1, 38.2, 33.8, 33.0, 32.5, 32.2, 30.6, 28.5, 27.5, 25.8, 23.5, 23.4, 23.0, 22.5, 16.8, 16.7, 16.5. HRMS *m/z:* $[M + H]^+$ calcd. for $C_{35}H_{59}O_6$ 575.4312, found 575.4309.

3d. 88.7%; white solid; mp 132–5 °C; $[\alpha]_D$ +40 (c 1, $CHCl_3$); IR (KBr) ν (cm^{-1}) 3400–3000, 1712, 1510, 1035; MS *m/z* (%): 618 (M^+, 15), 587 (65), 456 (100); ^1H NMR ($CDCl_3$) δ (ppm): 5.23 (br t, 1H, H-12), 4.13 (m, 2H, CH_2O), 3.63 (m, 11H, H-2, 5 x CH_2), 3.33 (s, 3H, OMe), 2.82 (d, $J =$ 9.6, 1H, H-3), 2.79 (dd, J = 14.4, 4.0, 1H, H-18), 1.98–0.80 (m, 20H, aliphatic H), 1.07, 0.97, 0.92, 0.86, 0.84, 0.76, 0.62 (s, 21H, 7 x Me); ^{13}C NMR ($CDCl_3$) δ (ppm): 177.5, 143.6, 122.1, 83.7, 71.8, 70.5, 69.10, 69.09, 63.3, 59.0, 55.1, 47.5, 47.4, 46.6, 46.5, 46.2, 45.7, 41.6, 41.1, 39.0, 38.6, 38.1, 33.7, 33.0, 32.4, 32.2, 30.6, 28.5, 27.5, 25.8, 23.5, 23.3, 22.8, 18.2, 16.8, 16.7, 16.5. HRMS *m/z:* $[M + H]^+$ calcd. for $C_{37}H_{63}O_7$ 619.4574, found 619.4578.

3e. 93.0%; White solid: mp 180–2 °C; $[\alpha]_D$ +28 (c 0.5, $CHCl_3$); IR (KBr) ν (cm^{-1}) 3400–3000, 1709, 1521, 1030; MS *m/z* (%): 545 (M^+, 37), 546 (25), 456 (100); ^1H NMR ($CDCl_3$) δ (ppm): 5.28 (br t, 1H, H-12), 4.11 (m, 2H, $COOCH_2$), 3.80 (m, 1H, CHOH), 3.65 (m, 3H, H-1 y CH_2OH), 2.96 (d, $J = 9.6$, 1H, H-3), 2.83 (dd, $J = 14.4$, 4.0, 1H, H-18), 2.0–0.7 (m, 20H, aliphatic H), 1.07, 0.97, 0.92, 0.86, 0.84, 0.76, 0.62 (s, 21H, 7 x Me); ^{13}C NMR ($CDCl_3$) δ (ppm): 178.1, 143.7, 122.1, 83.2, 69.8, 68.3, 64.8, 63.3, 55.0, 47.4, 46.7, 46.1, 45.7, 45.5, 41.5, 41.1, 41.0, 39.12, 39.07, 37.9, 33.6, 32.8, 32.5, 30.4, 28.3, 27.4, 25.6, 23.2, 22.7, 18.1, 16.7, 16.5, 16.2. HRMS *m/z:* $[M + H]^+$ calcd. for $C_{33}H_{55}O_6$ 547.3999, found 547.3993.

*4.2.3. Synthesis of conjugate **3f**/TMA through diol protection*

Preparation of 4. Under an Ar atmosphere, in a round bottom flask containing MA (1.5 g, 3.1 mmol) a 1:1 (v/v) mixture of 2,2-

dimethoxypropane and DCM (30 mL) was added. The mixture was strongly stirred to reach a white suspension. Then PTSA (0.015 mg, 0.08 mmol) was added. The reaction mixture was stirred at 25 °C during 48 h. After this period, brine (15 mL), $NaHCO_3$ (10%, 15 mL) and DCM (20 mL) were added. Organic phase was decanted, dried over $MgSO_4$ and concentrated under vacuum to dryness to give **4** (1.45 g, 91%) which was used without further purification. ^{1}H NMR ($CDCl_3$) δ (ppm): 5.20 (br t, 1H, H-12), 3.41 (m, 2H, H-2,3), 2.35–0.40 (m, 22H, aliphatic H), 1.23, 1.19, 1.08, 0.99, 0.97, 0.87, 0.85, 0.80, 0.69 (s, 27H, 9 x Me).

Preparation of 6. Under an Ar atmosphere, in a round bottom flask was dissolved **4** (1.45 g, 2.83 mmol) and NHS (1.14 g, 9.91 mmol) in EtOAc (40 mL). Then, a solution of DCC (2.39 g, 11.60 mmol) in EtOAc (12 mL) was added. The reaction mixture was stirred at 25 °C during 24 h (tlc). After this period, a white solid was filtered, and filtrates were concentrated to dryness to give **5** (3.87 g, 6.3 mmol), which was used in the next step without purification. Over this solid in a round bottom flask and under an Ar atmosphere, tyramine (**2f**, 1.73 g, 12.6 mmol) and DCM (35 mL) were added, and the mixture was strongly stirred to reach a white suspension. Then DMAP (catalytic) was added. The reaction mixture was stirred at 25 °C during 72 h (tlc). After this period, brine (15 mL) and DCM (20 mL) were added. Organic phase was decanted, washed with $NaHCO_3$ solution (10%, 15 mL) dried over $MgSO_4$ and concentrated under vacuum to dryness to give **6** (0.50 g, 24%) as yellowish solid, mp 159–61 °C. UV (MeOH): 270, 248 nm;^{1}H NMR ($CDCl_3$) δ (ppm): 6.91 (dd, $J = 11.1$, 4.5, 2H, ArH), 6.78 (dd, $J = 11.1$, 4.5, 2H, ArH), 5.25 (br t, 1H, H-12), 3.47 (m, 2H, H-2,3), 3.30 (br t, 2H, CH_2–N), 2.90 (br t, 2H, CH_2-Ar), 2.35–0.40 (m, 22H, aliphatic H), 1.25, 1.20, 1.10, 0.89, 0.88, 0.85, 0.83, 0.76, 0.71 (s, 27H, 9 x Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.9, 155.5, 143.8, 129.6, 123.1, 120.0, 115.6, 83.3, 68.7, 60.4, 55.1, 47.4, 46.7, 46.2, 46.1, 41.9, 41.7, 40.4, 39.2, 39.1, 37.9, 34.2, 33.8, 33.6, 32.7, 32.1, 32.0, 30.5, 28.6, 26.8, 26.3, 26.0, 25.2, 24.3, 23.6, 23.2, 20.8, 18.2, 16.7, 16.3, 13.8.

Deprotection of 6 to give 3f. Under an Ar atmosphere, in a round bottom flask, **7** (0.26 g, 0.41 mmol) was dissolved in MeOH (5 mL). Over this solution, PTSA (0.086, 0.45 mmol) was added. The reaction mixture was stirred at 25 °C during 1 h (tlc). After this period, brine (5 mL) and EtOAc (10 mL) were added. Organic phase was decanted, dried over $MgSO_4$ and concentrated under vacuum to dryness. The residue was separated by flash column chromatography (cyclohexane:EtOAc, 3:7 to 2:8) to give **3f** (0.24 g, 78%) as a white solid, mp 175–7 °C. $[\alpha]_D$ +37 (c 1, $CHCl_3$); UV (MeOH): 262, 238 nm, basic media: 288, 248 nm; IR (KBr) ν (cm^{-1}) 2990, 1608, 1600, 1051, 1027; MS *m/z* (%): 591 (100, M^+), 573 (49), 455 (61); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.90 (dd, $J = 11.1$, 4.5, 2H), 6.68 (dd, $J = 11.1$, 4.6, 2H), 4.86 (s, 1H), 3.71–3.62 (m, 1H), 3.25 (dt, $J = 3.3$, 1.6, 1H), 2.96–2.85 (m, 1H), 2.81 (d, $J = 9.4$, 1H), 2.72–2.63 (m, 1H), 2.53–2.42 (m, 1H), 2.17 (d, $J = 12.7$, 1H), 1.93 (s, 1H), 1.79 (d, $J = 13.0$, 2H), 1.56 (d, $J = 13.5$, 3H), 1.41 (dd, $J = 11.2$, 6.7, 3H), 1.30 (s, 1H), 1.29 (s, 1H), 1.26 (s, 2H), 1.21 (s, 1H), 1.14 (s, 2H), 1.10 (s, 2H), 0.97 (s, 3H), 0.88 (s, 3H), 0.81 (s, 3H), 0.77 (s, 6H), 0.68 (s, 3H), 0.40 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.86, 155.61, 143.91, 129.64, 123.08, 115.48, 83.26, 68.37, 60.54, 55.00, 47.40, 46.58, 46.21, 46.05, 41.89, 41.73, 40.37, 39.21, 39.12, 37.86, 34.11, 33.96, 33.58, 32.69, 32.14, 31.97, 30.49, 28.37, 26.98, 25.53, 24.78, 23.55, 23.20, 20.76, 18.17, 16.53, 16.30, 13.88. HRMS *m/z:* $[M + H]^+$ calcd. for $C_{38}H_{58}NO_4$ 592.4366, found 592.4360.

4.2.4. One-step synthesis of tyramidyl maslinic acid: conjugate *3f/TMA*

In a round bottom flask (500 mL), under an Ar atmosphere, **1** (3.50 g, 7.4 mmol), tyramine (1.22 g, 8.89 mmol), 1-hydroxy-benzotriazole (HOBt, 0.99 g, 7.4 mmol) and dicyclohexylcarbodiimide (DCC, 1.53 g, 7.4 mmol) where dissolved in anhydrous DCM (300 mL). Then, TEA (1.63 mL) was added. The reaction mixture was stirred at 30 °C for 72 h (tlc). After this period, brine was added. The mixture was decanted, and organic phase was washed with HCl (5%), dried over $MgSO_4$ and concentrated under vacuum. The residue was separated by flash column chromatography (cyclohexane:EtOAc, 3:7 to 2:8) to give **3f** (78%).

4.3. Cell culture

The T84 and HCT-15 human colon adenocarcinoma, A549 human lung cancer and B16–F10 murine melanoma cell lines were purchased from the American Type Culture Collection (ATCC, USA). The MC38 murine colon adenocarcinoma cells were kindly provided by Dr. J. Scholl (Public Health Service, National Institutes of Health, Bethesda, MD, USA). The A172 and SF-268 human glioblastoma, PANC-1 and MIA PaCa-2 human pancreatic adenocarcinoma, H520 human lung cancer and MCF-7 and MDA-MB-231 human breast cancer cell lines were provided by the Centre for Scientific Instrumentation of the University of Granada (CIC-UGR, Spain). All the cell lines, except MDA-MB-231 human breast cancer cells, were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin-streptomycin (Sigma–Aldrich, Madrid, Spain). MDA-MB-231 cell line was grown in Dulbecco′s Modified Eagle′s Medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich, Madrid, Spain) also supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% of penicillin-streptomycin. All of them were maintained at 37 °C in an atmosphere containing 5% CO_2.

4.4. Cell viability assay

Cells were seeded in 48-well plates at densities of 5×10^3 cells/well for T84, HCT-15, A172, A549 and H520; 3×10^3 cells/well in MC-38 and MIA PaCa-2; $1{,}5 \times 10^3$ cells/well in B16–F10, MCF-7 and SF-268; and 7×10^3 cells/well in MDA-MB-231 and PANC-1 with 300 μL of supplemented culture medium. After 24 h, cell cultures were exposed to increasing concentrations of MA and TMA, previously resuspended in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Madrid, Spain). DMSO was also tested in cell cultures at equivalent concentrations. After 72 h, cells were fixed with trichloroacetic acid (TCA) at 10% for 20 min at 4 °C. Once dried, the plates were stained with 0.4% sulforhodamine B (SRB) in 1% acetic acid during 20 min in agitation. After washing with 1% acetic acid, SRB was solubilized with Trizma® (10 mM, pH 10.5). Finally, the optical density (OD) at 492 nm was measured in a spectrophotometer EX-Thermo Multiskan and the percentages of proliferation were calculating with the following equation: Proliferation (%) = (Treated cells OD-Blank)/(Untreated cells OD-Blank) × 100. In DMSO assays, values < 95% of relative proliferation were considered toxic.

4.5. Statistical analysis

All the results were presented as mean ± standard deviation (SD) of triplicate cultures. Statistical analysis was performed using Student's t-tests with the Statistical Package for the Social Sciences (SPSS) v.26 software. Data with $p < 0.05$ were considered as statistically significant.

Author contributions

Conceptualization: J.M.L-R., J.P. and C.M.; investigation: D.F-R. and A.C., M.G-C. and R.C–C.; data analysis: R.L. and L.C.; software and figures: L.C.; supervision: M.G-C. and R.C–C.; writing—original draft, J.M.L-R., J.P.; writing— review and editing, J.M.L-R., J.P. and C.M.; funding acquisition, J.M.L-R. and J.P. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmcr.2022.100032.

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