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Novel merosesquiterpene exerts a potent antitumor activity against breast cancer cells *in vitro* and *in vivo*

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ABSTRACT

This article describes the antitumor properties of a new family of merosesquiterpenes, which were synthesized by Diels–Alder cycloaddition of the labdane diene *trans*-communic acid, highly abundant in *Cupressus sempervirens*, or its methyl ester, with the appropriate dienophile. These compounds demonstrated potent cytotoxic activity *in vitro* against human breast, colon, and lung tumor cells. We highlight the elevated activity (IC_{50}: 0.35 ± 0.10 μM) and specificity (TI: 9) of compound ***13*** against the MCF-7 line, which corresponds to the most prevalent breast cancer cell subtype, luminal A. It was found that compound ***13*** exerts its anti-tumor action by inducing oxidative stress, arresting the cell cycle in stages G_0–G_1, and activating apoptosis, which are all associated with low cyclin D1 regulation, pRb hypophosphorylation, increased expression of p27 and p53, and poly (ADP-ribose) polymerase (PARP) fractioning. Epithelial–mesenchymal transition, a phenomenon associated with metastasis promotion and a worsened prognosis also appeared to be inhibited by compound ***13***. In addition, it markedly reduced tumor development in immunocompetent C57BL/6 mice with allografts of E0771 mouse breast tumor cells (luminal A subtype). According to these findings, this new family of compounds, especially compound ***13***, may be highly useful in the treatment of human breast cancer.

1. Introduction

The term cancer defines a group of diseases characterized by the uncontrolled growth of cells and their dissemination to other body tissues through blood and lymphatic vascular systems. Cancer is one of the main causes of death worldwide, with 12.7 million new cases and 7.6 million deaths in 2008 according to the World Health Organization [1,2]. Generally, cancers of the breast, lung, colorectal, and prostate cancer have been the most frequent types in developed countries and cancers of the stomach, liver, oral cavity, and cervix cancer the most frequent in developing countries, although these patterns are changing, especially due to population aging and life style changes [3].

Breast cancer is the most frequent cancer among women worldwide, with 1.38 million new cases being diagnosed in 2008. Although the mortality rate is much lower than the incidence (around 6–19 per 100,000), it remains the first cause of death by cancer in females [1]. Breast cancer is currently treated with surgical resection, chemotherapy, radiotherapy, and/or hormone therapy, but there is an elevated rate of recurrence and development of metastatic disease, largely attributable to the onset of resistance to some chemotherapies or other treatments. There is a need to develop more effective and selective treatments that produce lesser systemic toxicity and do not generate resistance [4,5].

Medicinal plants have been used as a source of therapeutic agents against all types of disease for thousands of years. For instance, natural compounds and their derivatives have been used as anticancer agents, including many widely administered “standard” chemotherapeutics such as paclitaxel, vinblastine, or vincristine, among others [6]. Attention first centered on terrestrial organisms, given their accessibility, and then turned to marine

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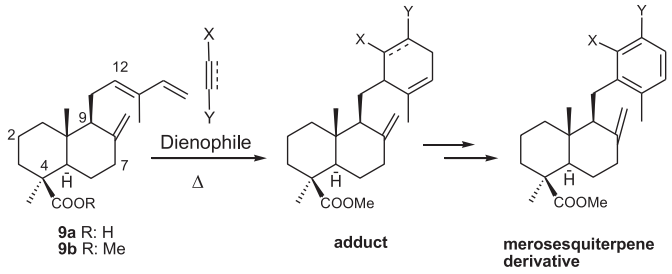
organisms and microorganisms, aided by advances in analysis techniques [7].

Natural products of mixed biosynthetic origin (polyketide–terpenoid) containing a sesquiterpene unit joined to a phenolic or quinone moiety are generally named "merosesquiterpenes" [8]. The most important metabolites of this family are the compounds bearing a bicyclic terpene (drimane) moiety, mainly due to their potent biological activities. Drimenyl phenols include the cholesteryl ester transfer protein (CETP) inhibitors wiedendiol A (***1***) and wiedendiol B (***2***), isolated from the marine sponge *Xestospongia wiedenmayeri* [9]. Drimenyl quinones include: the antitumor tauranin (***3***), isolated from *Phyllosticta spinarum*, a fungal strain endophytic in *Platycladus orientalis* [10], which also inhibits cholesterol biosynthesis; the anti-HIV (+)-hyatellaquinone (***4***), found in the sponge *Hyatella intestinalis* [11]; and the recently reported discomycete metabolite (−)-F-12509 A (**5**), which exhibits sphingosine kinase inhibitory activity [12] (Fig. 1).

More recently, merosesquiterpenoids with different functionalities on the C-4 of the drimane moiety were reported. These include: the 15-human lipoxygenase (15-HLO) inhibitor jaspic acid (***6***), found in the Papua New Guinea marine sponge *Jaspis* cf. *johnstoni* [13]; the antileishmanial disulfated meroterpenoid ilhabrene (***7***), isolated from the Brazilian marine sponge *Callyspongia* sp. [14]; and the fungitoxic terpenoid-quinone pycnanthuquinone C (***8***), isolated from the stem bark of *Pycnanthus angolensis* (Welw.) [15].

The two-synthon strategy is the most frequently applied method for synthesizing this type of compound and usually involves the reaction of a drimane electrophile with a nucleophilic phenol derivative [16]. However, this strategy has some drawbacks: a nucleophilic aromatic synthon must be utilized, preventing the straightforward generation of compounds bearing electrophilic groups, such as COOR, COR, and CN in the phenolic moiety, i.e., the immediate precursors of metabolites ***3*** and ***6***. In addition, the two-synthon strategy involves the use of a drimane electrophile, which is usually prepared in a multistep synthetic sequence from a labdane diterpene.



Scheme 1. Reaction protocol for synthesis of the new merosesquiterpenes.

Recently, Alvarez-Manzaneda et al. reported a new procedure for producing this type of compound. The merosesquiterpene skeleton of these compounds is constructed by Diels–Alder cycloaddition of the labdane diene *trans*-communic acid (***9a***), highly abundant in *Cupressus sempervirens*, or its methyl ester (***9b***), with the appropriate dienophile. The Diels–Alder adduct was converted into the corresponding merosesquiterpene derivative after aromatization (Scheme 1) [17].

Fig. 2 shows some Diels–Alder adducts, aromatization products (merosesquiterpene derivatives), and other compounds obtained after functionalization of the aromatic compounds.

When the methyl *trans*-communate (***9b***) was treated with 2-chloroprop-2-enenitrile in refluxing toluene for 48 h, the α-chloronitrile ***10*** was obtained (88% yield). The treatment of ester ***9b*** with methyl prop-2-ynoate in xylene under reflux for 12 h and the subsequent treatment of the resulting crude product with DDQ (1.1 eq) in refluxing dioxane rendered a 1:1 mixture of regioisomers ***11a*** and ***11b*** (94% yield). Compound ***11a*** was isolated unreacted after treating the mixture with MeMgBr. When ester ***9b*** was heated with dimethyl but-2-ynedioate in xylene under reflux for 6 h and then aromatized by treating with DDQ (1.1 eq) in refluxing dioxane, the result was diester ***12*** (95% yield). The heating of ester ***9b*** with cyclohexa-2,5-diene-1,4-dione or 2-methoxycyclohexa-2,5-diene-

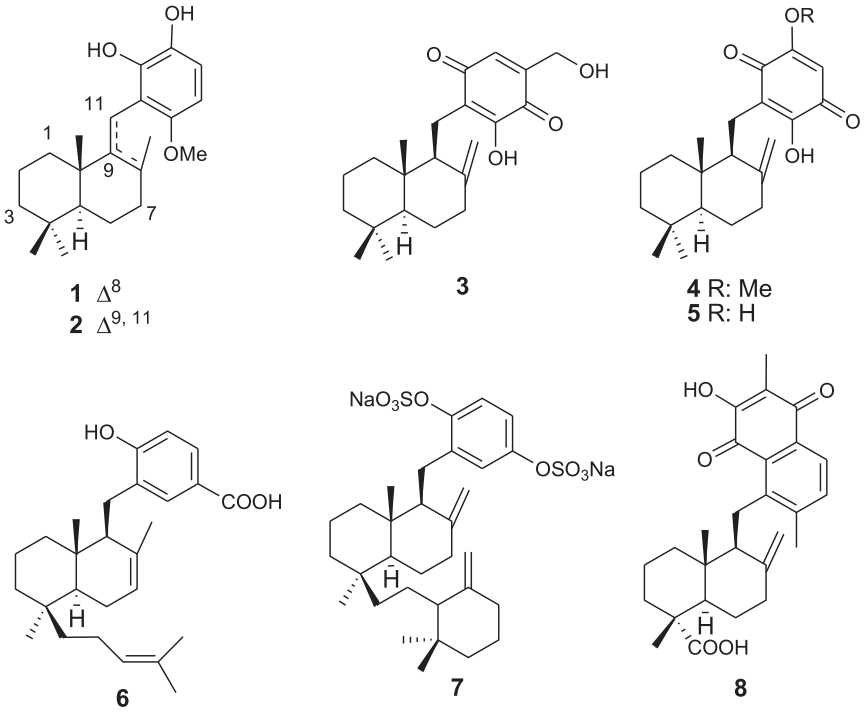


Fig. 1. Chemical structures of compounds **1**–**8**.

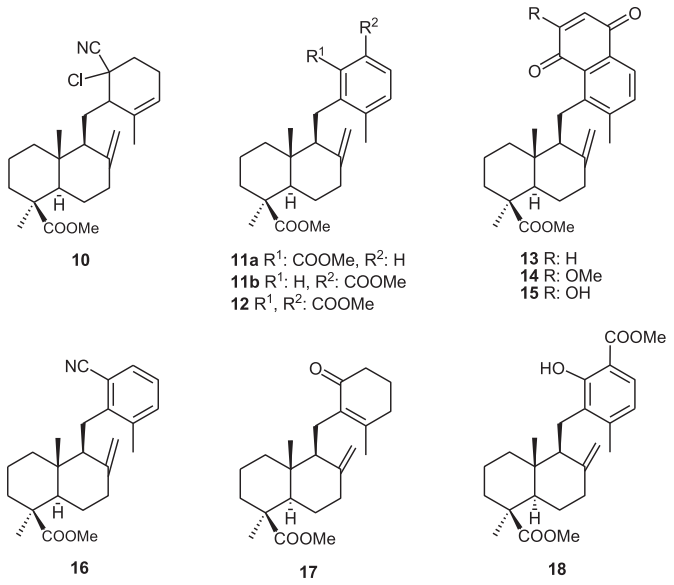


Fig. 2. Chemical structure of novel synthetic merosesquiterpenes.

1,4-dione in toluene, under appropriate reaction conditions, and further treatment with DDQ (2.2 eq) in dioxane under reflux gave a good yield of naphthoquinones ***13*** and ***14***, respectively. Methoxyquinone ***14*** was efficiently converted into hydroxyquinone ***15*** after treatment with KOH in MeOH at room temperature.

Cycloadduct ***10*** was transformed into aromatic compounds ***16*** and ***17***. The α-chloronitrile ***10*** was readily transformed into the aromatic nitrile ***16*** after dehydrohalogenation, by treating with DBU in refluxing benzene and subsequent aromatization with DDQ in dioxane under reflux. Alternatively, chloronitrile ***10*** was converted into the corresponding α,β-unsaturated ketone ***17*** by refluxing with KOH in t-BuOH. This ketone was transformed into salicylate ***18*** after methoxycarbonylation and further aromatization.

This study investigated the antitumor properties of this new family of synthetic merosesquiterpenes, whose synthesis and antitumor effects are protected under international patents WO/2009/112622 [18] and WO/2010/076358 [19], respectively. After an initial screening in breast, lung, and colon cancer cells, the compound showing the highest activity and therapeutic index, compound ***13***, was selected for study of its mechanism of action against breast tumor cells, which were found to be the most susceptible cells to this compound. We studied its effects on oxidative stress, the cell cycle, and apoptosis, given their high involvement in the action of chemotherapeutics. Finally, we examined the effect of this compound on the development of *in vivo* tumors in a murine model of breast cancer allograft.

2. Results and discussion

2.1. In vitro cytotoxicity assay

In a first phase, we assessed the cytotoxic action of the compounds listed in Fig. 2 against human breast (MCF-7), lung (A-549), and colon (T-84) tumor lines. In general, activity was observed by all of these molecules, with inhibitory concentration 50 (IC_{50}) values < 50 μM in all cases (Table 1). The optimal results were observed for compounds ***13*** and ***14***. The highest activity was evidenced by ***13***, with an $IC_{50} < 1$ μM in both MCF-7 and T-84 cells. Although the structure–activity relationship for this type of compound is poorly understood, the active compound usually possesses a quinone moiety. It has been postulated that the activity of this group may be attributable to the Michael acceptor (electrophile) character of the unsaturated carbonyl system present in this type of compound; this proposal was supported by our observation of the activity of quinones ***13***, ***14***, and ***15***. Thus, compounds ***14*** and ***15***, which have methoxy and hydroxyl groups, respectively, in the double bond, thereby diminishing the electrophilic character of the quinone system, exerted a markedly lower activity in comparison to compound ***13*** (IC_{50} = of 38.37 μM for ***15*** *versus* 0.35 μM for ***13*** in MCF-7 cells).

Table 1
Antiproliferative activity of merosesquiterpenes against MCF-7, A-549, and T-84 cells.

Compound	IC_{50} (μM)[a]		
	MCF-7	A-549	T-84
11a	40.41 ± 9.75	28.14 ± 0.45	29.02 ± 0.48
12	33.01 ± 2.79	19.89 ± 0.22	25.04 ± 0.24
13	0.35 ± 0.10	1.38 ± 0.83	0.56 ± 0.13
14	16.02 ± 0.40	17.51 ± 0.75	16.74 ± 0.62
15	38.37 ± 5.30	41.17 ± 2.70	30.01 ± 3.92
16	39.63 ± 6.00	20.17 ± 0.21	23.66 ± 4.40
18	45.06 ± 6.5	26.96 ± 0.14	40.02 ± 0.23

[a] Data are means ± SD of four determinations.

Lipophilicity (log *P*) is a key parameter of the biological effect developed by these compounds, because it can determine their capacity to penetrate biological membranes and therefore influence intracellular bioavailability [20]. The log *P* of the studied molecules was determined by using Marvin 6.0.5 software (ChemAxon Ltd., 2013). Table 2 shows the resulting theoretical values. A very strong and inversely proportional relationship was found between the IC_{50} in the three tumor lines treated with compounds ***13***, ***14***, and ***15*** and the log *P* values of the lines (Spearman's correlation coefficient -1, $p = 0$), although no major differences in these values were observed. This increase in compound activity with higher log *P* was not observed in the remaining compounds.

Based on the initial screening results, we elected to study the effect of ***13*** on other breast and colon cell lines, comparing the results with those obtained under the same experimental conditions with 5-fluorouracil (5-Fu), a commercial pharmaceutical widely administered to treat colon and breast cancer, among other solid tumors [21,22]. The IC_{50} values of these two compounds were determined in human breast tumor lines MCF-7, MDA-MB-231, and T-47D, mouse breast tumor line E0771, and human colon tumor lines T-84, HT-29, RKO, and SW-480. Their IC_{50} was also obtained in normal human breast (MCF-10A) and colon (CCD18Co) cell lines (Table 3) in order to calculate their *in vitro* therapeutic index (TI).

The IC_{50} of compound ***13*** was <1 μM in all lines with the exception of HT-29 (1.97 μM). In general, the activity of ***13*** was highly superior to that of 5-Fu. Thus, the mean IC_{50} was 0.48 μM for ***13*** *versus* 10.51 μM for 5-Fu in human breast tumor lines and 1.04 μM for ***13*** *versus* 3.44 μM for 5-Fu in human colon tumor lines. Exceptionally, the activity of 5-Fu was almost 30% higher than that of ***13*** in the mouse E0771 line, which has an elevated proliferative rate, with a three-fold shorter duplication time in comparison to MCF-7 cells. Hence, E0771 cells would require high rates of

Table 2
Lipophilicity values of synthetic merosesquiterpenes.

Compound	***11a***	***12***	***13***	***14***	***15***	***16***	***18***
Log *P*	6.21	6.22	5.73	5.32	5.21	6.07	6.56

Table 3
IC_{50} and TI of ***13*** and 5-fluorouracil against breast and colon cancer cells.

	Cell line	***13***		5-Fu	
		IC_{50} (μM)[a]	TI	IC_{50} (μM)[a]	TI
Breast	MCF-7	0.35 ± 0.10	9.00	2.86 ± 0.21	2.82
	MDA-MB-231	0.46 ± 0.18	6.85	18.12 ± 1.41	0.44
	T-47D	0.63 ± 0.08	5.00	10.56 ± 0.92	0.76
	E0771	0.39 ± 0.04	8.08	0.28 ± 0.01	28.79
	MCF-10A	3.15 ± 0.08	1.00	8.06 ± 0.18	1.00
Colon	T-84	0.56 ± 0.13	0.59	3.35 ± 0.58	2.76
	HT-29	1.97 ± 0.22	0.17	2.58 ± 0.68	3.60
	RKO	0.59 ± 0.24	0.56	4.39 ± 0.22	2.11
	SW-480	0.92 ± 0.03	0.36	3.69 ± 0.49	2.51
	CCD18Co	0.33 ± 0.24	1.00	9.26 ± 1.28	1.00

[a] Data are means ± SD of four determinations.

nucleoside and nucleotide synthesis to include them in their nucleic acids and metabolic mediators. 5-Fu inhibits thymidilate synthase enzyme, responsible for the *de novo* synthesis of thymidylate, a nucleotide required for DNA synthesis and repair. 5-Fu can also introduce errors during DNA and RNA synthesis through its inclusion in the chain instead of thymidine or uracil [22,23]. These effects of the drug on nucleic acid metabolism may explain why its effect on the highly proliferative E0771 cells was greater than that of ***13***.

The TI values of ***13*** were ≥5 in the breast cell lines and were markedly higher than those of 5-Fu with the exception again of the E0771 line, in which the value was 28.79. In the colon cell lines, however, the TI was <0.6 for ***13*** *versus* >***2*** for 5-Fu. According to these findings, ***13*** is more selective against human breast tumor lines and 5-Fu is more selective against colon tumor lines. Taking the IC_{50} and TI values together, the best results were obtained using ***13*** against the MCF-7 line, with values of 0.35 μM and 9.00, respectively. We therefore selected this line for in-depth study of the action mechanism of compound ***13***.

The MCF-7 cell line was the first human breast cancer line to be isolated and permanently maintained in culture (in 1970), and it has been one of the most widely used for the study of breast cancer biology and treatment. Its phenotypic and genotypic properties and characteristics are well documented. It is known to be wild-type for p53 and to express estrogen and progesterone receptors, making it a good model for studying the most prevalent breast cancer subtype, luminal A. These features made the MCF-7 line ideal for our study [24,25].

The assays described below were conducted under conditions of high cell density, 5×10^4 cells/cm^2, to obtain sufficient biomass. Hence, for the induction of the cultures, the IC_{50} calculated under these conditions was taken as the reference value, i.e., 2.09 ± 0.25 μM for compound ***13*** and 18.21 ± 1.66 μM for 5-Fu.

2.2. Study of oxidative stress induction

Reactive oxygen species (ROS) intervene in signal transduction and gene transcription, activating different pathways involved in biological processes, including cell growth, proliferation, differentiation, and apoptosis. They appear as metabolic by-products of oxygen and present unpaired electrons; therefore, ROS can accept electrons from other biological molecules, such as lipids, proteins, and nucleic acids, altering their function. Antioxidants act to avoid this, being responsible for neutralizing ROS by electron donation and establishing a dynamic balance (oxidative balance). Oxidative stress develops when this balance is altered in favor of ROS [26].

These free radicals have a dual role in cancer and can either favor or inhibit its development. Because of their cell toxicity, their accumulation can damage the DNA, producing the instability and the mutation of genes (e.g., tumor suppressor gene p53) associated with cancer development, and it can also alter proteins involved in signaling pathways related to cell proliferation. Conversely, when ROS levels are regulated and oxidative balance is maintained, they can increase p53 expression and even induce apoptosis, inhibiting the tumor process [27]. ROS levels are higher in cancer cells than in normal cells due to the elevated metabolic activity of the former, which leads to a chronic state of oxidative stress. It has been suggested that the action mechanism of numerous anticancer drugs is based on the promotion of ROS production, contributing to oxidative imbalance and the death of tumor cells. The first known anticancer drug to act by ROS induction and oxidative stress was elesclomol, whose antitumor activity is strongly reduced when administered with the antioxidant N-acetylcysteine (NAC), as in the case of paclitaxel [27,28]. Moreover, some drugs, e.g., endostatin, only act when used with other conventional chemotherapeutic drugs capable of generating ROS [29].

In order to determine whether the mechanism of action of ***13*** on line MCF-7 is mediated by oxidative stress induction, MCF-7 cells were induced with this compound in the presence or absence of NAC at 20 mM. After 3 days, the cultures were processed for calorimetric quantification with sulforhodamine B, following the procedure reported in Material and Methods. A dose-dependent decrease in cell viability was observed in the cultures induced at concentrations of 1, 2, and 3 μM, with reductions of 90.21%, 46.09%, and 23.26%, respectively, *versus* controls (Fig. 3). In the presence of NAC, however, the compound showed no cytotoxicity, and no significant differences were found with controls, indicating that the action of ***13*** is based on oxidative stress induction.

These findings were confirmed by measuring the intracellular ROS production in cultures induced with 2 μM ***13*** for 12 h in the presence or absence of NAC. Fig. 4 shows that the intracellular ROS concentration was increased by ***13*** in the absence but not in the presence of NAC, when the fluorescence intensity was very similar to that of the control group.

These results allow us to become involved in the continuing debate on the benefits of antioxidants for cancer patients. Evidence has been published since the 1990s on the chemopreventive effect of antioxidants consumed as food supplements or as part of the diet, and many of them have been attributed with action against some types of cancer, e.g., resveratrol, folic acid, vitamins A and E, among many others. However, there has been an increase in studies reporting a negative effect of antioxidants on cancer therapy,

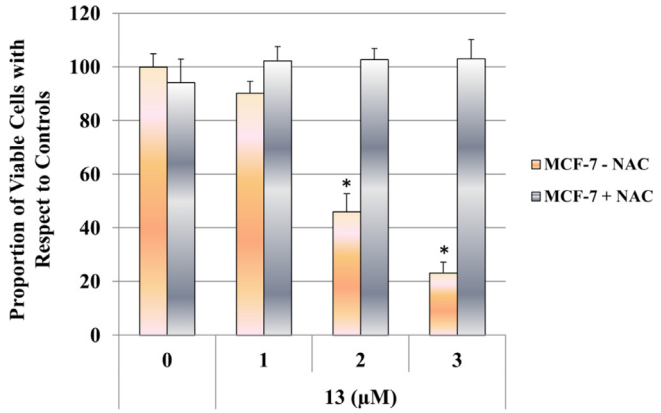


Fig. 3. Effect of ***13*** on the viability of MCF-7 cells in the presence (+) or absence (−) of the antioxidant NAC for 3 days. The histogram depicts means ± SD of four determinations.

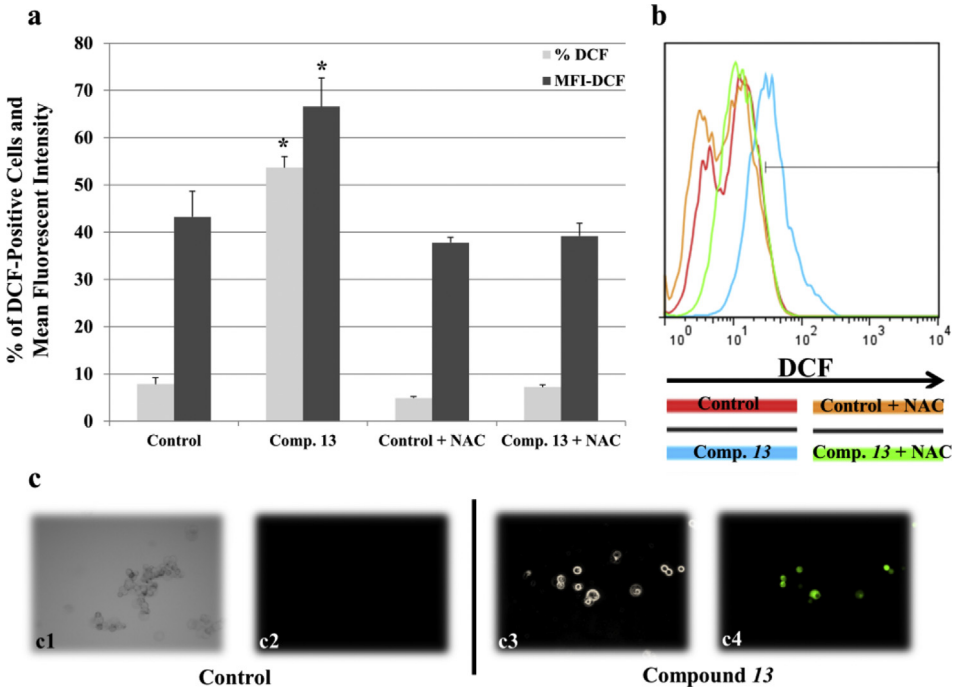


Fig. 4. ROS production in MCF-7 cultures induced with 2 μM of compound ***13*** for 12 h in the presence or absence of NAC. (a) Percentage of DCF-positive cells and mean fluorescent intensity. The histogram depicts means ± SD of four determinations. (b) Comparative cytogram of intracellular ROS production (c) Bright field micrographs (c1 and c3) and DCF-fluorescence (c2 and c4) of control (left) and ***13***-treated (right) MCF-7 cells.

mainly in initial stages. It has been suggested that foods with antioxidant capacity should not be consumed during treatment with certain drugs, but there is no clinical evidence to endorse this measure, and published clinical trials have been controversial [27–30]. It is clear that oxidative stress and ROS levels affect cancer development and treatments, and this issue warrants continued research to reach a consensus on the optimal recommendations for patients. In the present case, the effect of ***13***, a highly active compound with a promising TI value, was completely annulled in the presence of an antioxidant, as in the aforementioned cases of elesclomol and paclitaxel.

2.3. Cell cycle analysis

The cell cycle is one of the most important chemotherapeutic targets. In order to determine the effect of compound ***13*** on this process, we first synchronized MCF-7 cell cultures, maintaining them in a serum-free medium for 24 h. At that time, as shown in Fig. 3 (control 0), most of the cells were in phase G_0–G_1 (81.00%), while 12.86% were in phase S, and 6.15% in phase G_2-M. After 24 h in the presence of serum, culture cells begin to enter the cell cycle, with 42.34% of the cells in phase G_0–G_1, 49.46% in phase S, and 8.20% in phase G_2-M (control 24 h). However, the induction of the synchronized cultures with compound ***13*** at 1, 2, and 3 μM for 24 h produced dose-dependent cell cycle arrest in phase G_0–G_1, with an increase of cells in this stage to 51.40%, 66.61%, and 75.67%, respectively, in comparison to 42.34% in the control group. This increase in G_0–G_1 is associated with a corresponding reduction in the proportion of cells in phases S and G_2-M (Fig. 5).

At 24 h after induction with compound ***13***, no major differences were found in the fraction of cells in sub-G_1; therefore, the cytograms in Fig. 3 only represent the cells found in any cycle phase. However, at 48 h post-induction, there was a dose-dependent increase in the sub-G_1 population from 8.02% in the control group, to 15.56%, 31.09%, and 39.91% after induction with 1, 2, and 3 μM of ***13***, respectively (Fig. 6), suggesting that the effect of compound ***13*** is mediated by apoptosis induction.

2.4. Apoptosis assays with annexin-V

Cultures of MCF-7 cells were induced with compound ***13*** for 48 h, and flow cytometry was used in a viability study with annexin V-FITC to determine whether the increased fraction of cells in Sub-G_1 was due to apoptosis promotion. Treatment with compound ***13*** reduced the cell viability from 93.0% in the control group to 80.5, 58.4, and 32.6% in the cultures treated with 1, 2, and 3 μM, respectively. This viability reduction was correlated with an increase in the fraction of cells in both early and late apoptosis, with total apoptosis increasing from 3.44% in the control group to 11.07, 29.13, and 57.8% in the cultures treated with 1, 2, and 3 μM, respectively (Fig. 7). These results indicate that the compound exerts its action by apoptosis induction.

Taken together, the above findings indicate that the action of compound ***13*** on MCF-7 cells is mediated by the induction of oxidative stress, cell cycle arrest in G_0–G_1, and induction of apoptosis. An increasing number of studies report the involvement of oxidative stress in the action mechanism of a wide range of antitumor compounds. The manner in which compound ***13*** exerts its action is similar to that reported for various other compounds, including: natural extracts of *Garcina epunctata* against promyelocytic leukemia [31], chromenopyrazolediones against hormone-sensitive prostate tumor cells [32], acetylsalicylic acid against hepatocellular carcinoma cells [33], and the synergic action of quercetin and 2-methoxyestradiol against human hepatocellular carcinoma lines [34].

2.5. Western blot analysis

After confirmation that the antitumor effect of compound ***13*** is mediated by cell cycle arrest and apoptosis activation, the

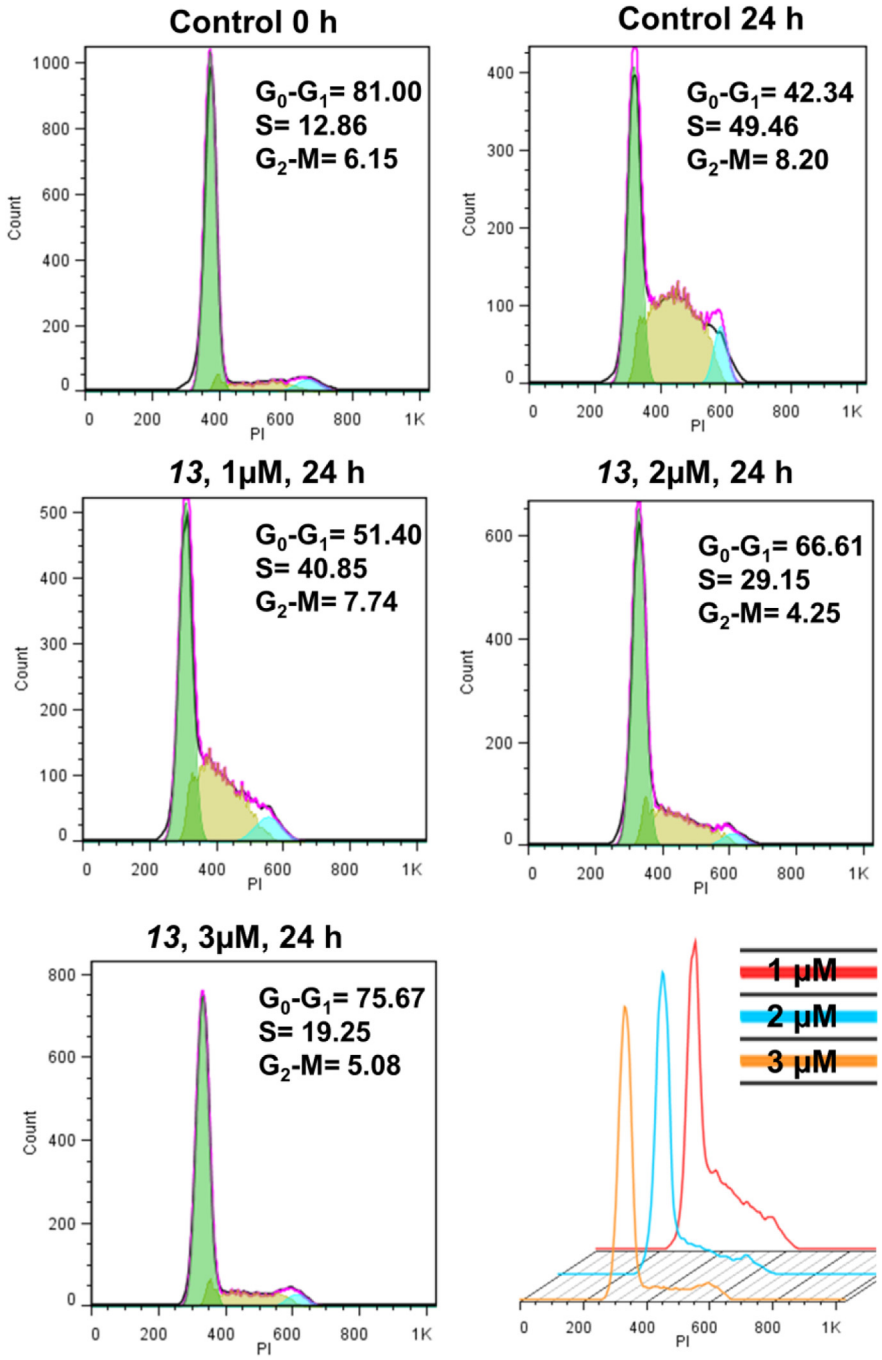


Fig. 5. Effect of compound ***13*** on MCF-7 cell cycle. Cultures were synchronized for 24 h with serum-free medium (control 0), and were subsequently incubated with complete medium for 24 h without (control 24 h) or with compound ***13*** at 1, 2, and 3 μM. The experiment was repeated independently three times yielding similar results.

expression of proteins involved in these processes was studied by western blot and band densitometry (Fig. 8).

Cell cycle is mainly regulated by cyclin-dependent kinase (CDK) proteins. Their activation depends on their binding to cyclins, which are expressed throughout the cycle and regulate the transition between phases [35]. The expression of cyclin B1, which is essential for the passage from phase G_2 to M [36], was markedly reduced after treatment with compound ***13*** and was almost absent (6%) at 16 h. This result is in agreement with the cell cycle study findings, given that the induction by ***13*** of arrest in phase G_0–G_1 means that the proteins involved in subsequent phases are no longer expressed. Likewise, the expression of cyclin D1, which is involved in the transition between phases G_1 and S, remained low during phase G_0 and then gradually increased during G_1 and was maintained during the remainder of the cycle [37]. Researchers have demonstrated that the degradation of cyclin D1 is sufficient for cycle arrest in G_0–G_1 [43]; therefore, its decreased expression is evidence of cycle arrest in phase G_0–G_1. In the present study, 4 h of treatment of MCF-7 cells with compound ***13*** produced an 80% decrease in its expression in comparison to baseline values.

pRb is the product of the retinoblastoma tumor suppressor gene, an essential protein for the transition between phases G_1 and S. It forms a complex with transcription factor E2F, inhibiting its activity and progression throughout the cell cycle. When there are

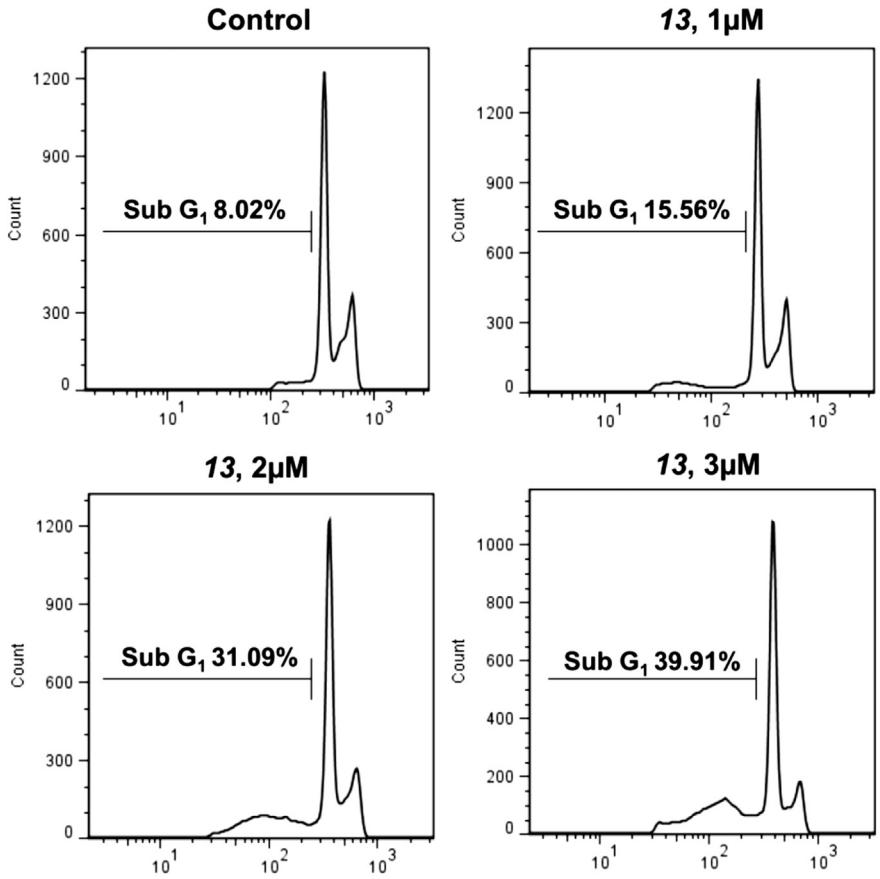


Fig. 6. Effect of compound ***13*** on the fraction of MCF-7 cells in Sub-G_1. Cultures were induced in complete medium for 48 h without (control) or with compound ***13*** at 1, 2, and 3 μM. The experiment was repeated independently three times yielding similar results.

appropriate mitogenic signals, pRb is phosphorylated by the cyclin D1/CDK4 complex, permitting the release of E2F and consequent activation of the genes required for progression to phase S [38]. Total and phosphorylated forms of pRb expression both decreased in the ***13***-treated cells, reaching a reduction of around 90% at 16 h in both cases (Fig. 8). One of the target genes of E2F is proto-oncogene c-myc, a transcription factor that participates in numerous cell processes, including the transition from G_0–G_1 to S. Overexpression and amplification of this gene is frequent in many tumors, including breast cancer, and novel strategies are under development to achieve its inhibition [39]. In the present study, treatment with compound ***13*** reduced c-myc expression by 50% at 16 h and by 80% at 24 h (Fig. 8), supporting the results reported above for cell cycle arrest markers.

Besides activators such as cyclins, Cip/Kip protein family members are also of major importance in CDK expression and therefore in cell cycle regulation, leading to inhibition of the kinase activity of CDK/cyclin complexes and cell cycle arrest [40]. One of the family members, p27, is used as a prognostic marker in breast cancer, because its decreased expression is associated with tumor development and progression [41]. Treatment of the MCF-7 line with compound ***13*** produced a significant increase in p27 expression (Fig. 8). In previous studies, arrest in G_0–G_1 induced by lapatinib and euphol compounds was also associated with the overexpression of p27 in breast tumor cells; euphol compounds also produced reductions in cyclins B1 and D1 and in pRb hypophosphorylation [42,43].

In many cases, the response to cell stress is mediated by cell cycle arrest, DNA repair and, when damage is not repaired, apoptosis. Tumor suppressor gene p53 is one of the main genes involved in regulating this response. The baseline expression of p53 under normal conditions increases in response to stress and undergoes a series of posttranslational modifications, including phosphorylation, which lead to its stabilization and activation. This permits its action as a transcription factor at nuclear level, regulating the expression of genes involved in the cell cycle and apoptosis, among others [44–46]. Given that the MCF-7 line is wild-type for p53, we assessed the effect of ***13*** on its expression, finding that it increases the levels of total p53 and its active phosphorylated form (Fig. 8). This may indicate that ***13***-induced apoptosis is dependent on p53. It may also support the effect of ***13*** on the cell cycle, because it has been reported that there may be an increase in p53 expression in phase G_1 arrest as well as a decrease in cyclin D1 [47].

Poly (ADP-ribose) Polymerase (PARP) is the protein responsible for the synthesis of poly (ADP-ribose) chains, which are essential for DNA repair, among other processes. However, PARP cleavage by caspases is an unequivocal sign of apoptosis [48,49]. In the present study, we found an increase in the cleaved fraction of the protein, confirming the induction of apoptosis (Fig. 8).

Finally, the expression of phospho-p44/42 mitogen-activated protein kinases (MAPKs) was analyzed, because these proteins are involved in cell proliferation and survival signaling pathways [50]. The p44/42 MAPK signaling pathway is known to be activated in human breast tumor cells in response to sexual steroid hormones and growth factors, including estrogens, progesterone, and epidermal growth factor (EGF) [51]. It has also been verified that a reduction in phospho-p44/42 MAPK levels in breast cancer patients

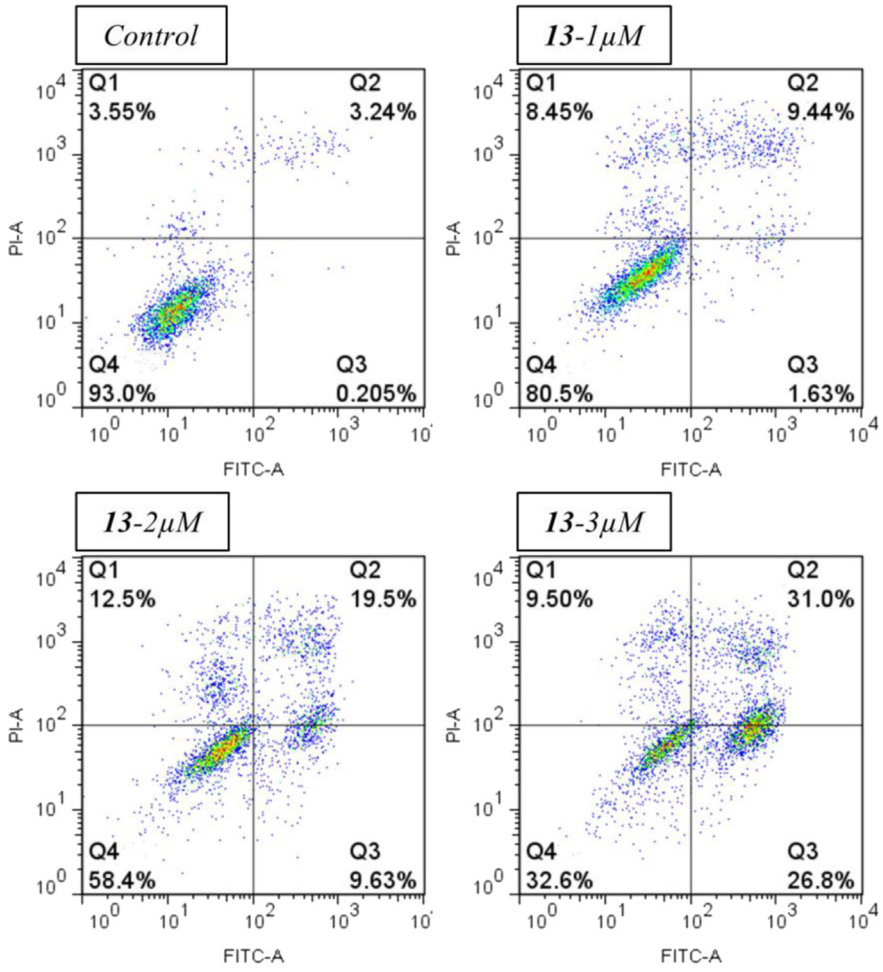


Fig. 7. Apoptosis assay with annexin-V by flow cytometry of MCF-7 cells induced with 1, 2, and 3 μM compound ***13*** for 48 h. Viable (Q4), necrotic (Q1), early (Q3), and late (Q2) apoptotic cells can be distinguished. The experiment was repeated independently three times yielding similar results.

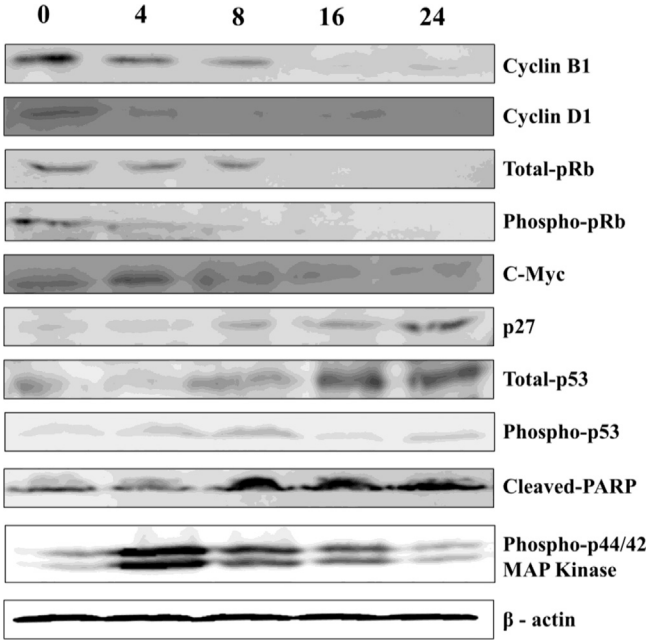


Fig. 8. Western blot analysis of cell cycle and apoptosis-related markers in MCF-7 human breast cancer cells induced by 2 μM compound ***13*** for 0, 4, 8, 16, and 24 h.

is associated with good clinical response and higher global survival after treatment with neoadjuvant chemotherapy [52]. In our study, a major increase in the expression of phospho-p44/42 MAPKs was observed at 4 h after treatment with compound ***13*** (Fig. 8), likely because the compound acts downstream from these proteins in the mitogenic signaling pathway. From 4 h onwards, a progressive reduction was observed in the expression of proteins downstream from p44/42 MAPK, especially that of cyclins and pRb, as well as in phospho-p44/42 MAPK levels, which were lower than control levels at 24 h of treatment. This may be explained by the reduced expression of pRb, which is responsible for the expression of steroidal hormones, especially the estrogen receptor [53]. Consequently, pRb reduction may downregulate the estrogen receptor and reduce the emission of mitogenic signals towards p44/42 MAPK, which would produce a decrease in its phosphorylated active forms.

2.6. Expression of epithelial–mesenchymal transition (EMT) markers

Epithelial–mesenchymal transition (EMT) is an essential process during development, when embryonic cells change their characteristics and acquire a mesenchymal phenotype. However, EMT also plays a key role in the acquisition of metastatic capacity by cancer cells, which involves cytoskeleton remodeling, polarity and intercellular contact loss and the consequent acquisition of high

motility and invasiveness [54,55]. At molecular level, this process is associated with a decrease in the expression of cell adhesion markers, such as E-cadherin and its intracellular association molecule, β-catenin [56]. E-cadherin is a transmembrane glycoprotein that binds to the cytoskeleton of actin *via* β-catenin. Its extracellular region, stabilized by Ca^{2+}, binds to itself and forms homodimers that interact with those of adjacent cells [57]. β-catenin is also a highly important protein in the regulation of the Wnt pathway, which is crucial for correct cell development and functioning [58].

Given the importance of EMT in the development of metastasis, flow cytometry was used to assess the effect of compound ***13*** on the expression of E-cadherin and its associated protein, β-catenin, after 48 h of induction. As observed in Fig. 9, ***13*** promoted the expression of both markers, suggesting that merosesquiterpene acts by inhibiting EMT development, which is of major translational interest given the importance of this process.

2.7. In vivo assay

The *in vitro* assay results prompted us to assess the effect of compound ***13*** on tumor development *in vivo*. C57BL/6 mice were inoculated with cells from the breast cancer tumor line E0771, established from a C57BL/6 mouse tumor. This model of murine breast cancer syngeneic allograft generates highly proliferative tumors in immunocompetent animals of the same species, offering a scenario that is closer to the clinical situation from a translational perspective. It also allows assays to be developed in a more convenient manner and at a lower cost in comparison to immunodepressed models, enhancing the histological reproducibility and growth rate of the tumor and simplifying the statistical analysis for data validation [59]. Crucially, E0771 cells are of the luminal A subtype, as are the MCF-7 cells used in the *in vitro* assays, allowing the study of compound ***13*** in cells with the same breast cancer subtype [60].

Forty C57BL/6 mice were subcutaneously injected with 1 million E0771 cells. At 9 days, when the tumor volume reached 75 mm^3, four experimental groups of 10 randomly assigned animals each were orally administered with the vehicle (1% methylcellulose solution) on six occasions or with compound ***13*** at a concentration of 5, 10, or 15 mg/kg Fig. 10 depicts the mean tumor volumes in each experimental group, indicating the days on which ***13*** was administered (arrows). The volume of tumors started to increase faster in control *versus* treated animals from day 27 onwards, and the difference reached significance by day 33. At the end of the assay on

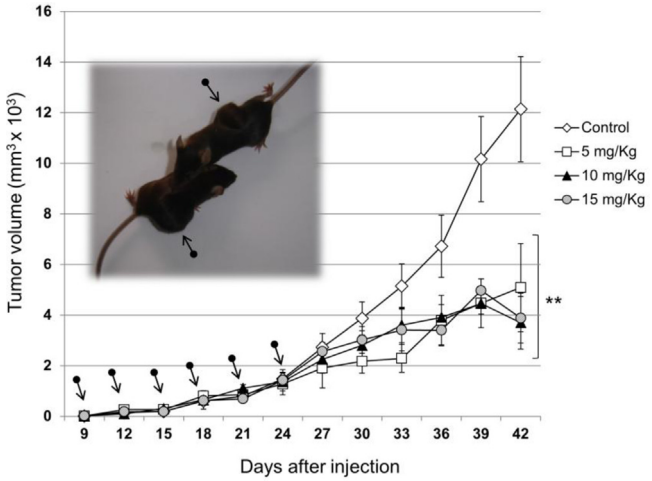


Fig. 10. Effect of compound ***13*** on tumor growth in C57BL/6 mice with E0771 mouse breast cancer cell allografts; the tumor volume is plotted against the days elapsed since tumor cell inoculation. Arrows indicate days on which the vehicle (control) or compound ***13*** (5, 10, or 15 mg/kg) were orally administered. **Significant difference with control group, $p < 0.01$. In the photograph, a control animal (below) can be distinguished from an animal treated with 15 mg/kg of compound ***13*** (above).

day 42, the mean volume of control tumors was 2.4- to 3.3-fold that of treated tumors ($p < 0.01$), confirming the *in vivo* antitumor activity of compound **13**. Paradoxically, no significant differences were found on day 42 among the animals treated with 5, 10, or 15 mg/kg Fig. 10 includes a photograph that permits differentiation between a control animal (below) and an animal treated with 15 mg/kg ***13*** (above), due to the major difference in tumor volumes (arrow).

There were no significant differences in animal survival among the study groups. No signs of toxicity, e.g., hair loss or diarrhea, were observed in the animals treated with ***13***, despite its oral administration. These findings suggest a very good tolerance to compound ***13***. The absence of significant differences among groups treated at 5, 10, or 15 mg/kg indicates that the dosage could be increased to optimize treatment outcomes.

3. Conclusion

We screened the antitumor activity of seven new merosesquiterpenes, whose synthesis and properties are under the

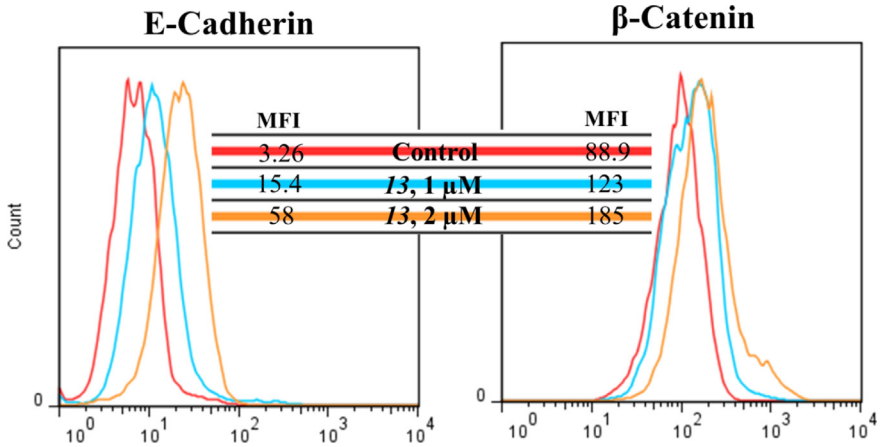


Fig. 9. E-cadherin and β-catenin expression in MCF-7 cells after treatment for 48 h with 1 and 2 μM compound ***13***. The figures depict the superposition of the cytograms and express the mean fluorescence intensity (MFI) values. The experiment was repeated independently three times yielding similar results.

protection of international patents. These compounds demonstrated elevated activity against human breast, colon, and lung tumor cells. Compound ***13*** evidenced the highest activity and specificity against MCF-7 breast cancer cells, which offer a good model for the study of the most prevalent molecular subtype of breast cancer, luminal A. Compound ***13*** acts against the cells by inducing oxidative stress, arresting the cell cycle in phase G_0–G_1, and activating apoptosis. Epithelial–mesenchymal transition, associated with metastasis promotion and a worse disease prognosis, also appears to be inhibited by compound ***13***. This compound strongly inhibits tumor development in C57BL/6 immunocompetent mice with allografts of E0771 mouse breast tumor cells, which are also luminal A subtype. These findings indicate that this new family of compounds, especially compound ***13***, may be highly useful to treat breast cancer in humans. Studies are under way to further explore their mechanism of action and to gather key data for structure-based drug design.

4. Materials and methods

4.1. Compounds

We assessed the antitumor effect of seven merosesquiterpene compounds, ***11a***, ***12***, ***13***, ***14***, ***15***, ***16***, and ***18*** (Fig. 2), which were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. For each experiment, the stock solutions were further diluted in medium to obtain the desired concentrations. The final solvent concentration in cell culture was <0.1% v/v DMSO.

4.2. Cell lines and culture

Human breast tumor lines MCF-7, T-47D, and MDA-MB-231, mouse breast tumor line E0771, human colon tumor lines RKO, HT-29, SW-480, and T-84, human lung tumor line A-549, and non-tumor lines of breast MCF-10A and colon CCD-18Co were supplied by the Department of Cell Cultures of the Granada University Scientific Instrumentation Center. All lines were cultured at 37 °C in 5% CO_2 and 90% humidity and, except for MCF-10A, with Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat inactivated fetal bovine serum, 10 ml/L penicillin–streptomycin 100×, and 2 mM L- glutamine. MCF-10A line was cultured in DMEM with Ham F-12 nutrient mixture (DMEM/F-12; 1:1) supplemented with 10 ml/L penicillin–streptomycin 100×, 20 ng/ml EGF, 100 ng/ml cholera toxin, 10 μg/ml bovine insulin, 0.5 μg/ml hydrocortisone, and 5% inactivated horse serum. Culture media and respective supplements were supplied by Sigma–Aldrich (**St. Louis, MO**).

4.3. In vitro cytotoxicity assay

In order to calculate the IC_{50} of compounds, 5×10^3 cells/cm^2 were seeded by quadruplicate. At 24 h, cells were induced with increasing compound concentrations for 3 days. Subsequently, cells were fixed with 10% cold trichloroacetic acid (4 °C) and stained with 0.4% sulforhodamine in 1% acetic acid. The colorant was solubilized with 10 mM Tris-base pH 10.5, and optical density values were determined by colorimeter at 492 nm (Multiskan EX, Thermo Electron Corporation, Milford, MA, USA). IC_{50} values were calculated from the semi-logarithmic dose–response curve by linear interpolation. The TI was calculated as the Normal line IC_{50}/Tumor line IC_{50}, with a higher TI value indicating greater specificity of the compound towards tumor *versus* normal cells.

The IC_{50} of cultures established at high cell density (5×10^4 cells/cm^2) was also calculated, because the oxidative stress, cell cycle, apoptosis, and gene expression assays were conducted under these conditions to obtain sufficient cell biomass, taking the IC_{50} obtained at high cell density as reference for induction of these cultures.

4.4. Study of the induction of oxidative stress

MCF-7 cells were seeded at high density (5×10^4/cm^2) and, at 24 h, induced with different concentrations of compound ***13*** for 3 days in the presence or absence of 20 mM NAC. The cells in culture were counted by staining with sulforhodamine B (see above section). Intracellular ROS levels were also determined in these cultures, using a FACScan flow cytometer (Becton Dickinson, San José, CA, USA) to quantify the fluorescence intensity emitted after incubation with 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA, Sigma–Aldrich) for 30 min at 37 °C. The percentage of DCF-positive cells and mean fluorescent intensity were calculated by FlowJo software (v 7.6.5, Tree Star, Inc., Ashland, OR, USA). Samples of cultures were also analyzed by fluorescence microscopy (Leica DM5500B).

4.5. Cell cycle analysis

MCF-7 cells were seeded (5×10^4/cm^2), and the culture medium was replaced with a serum-free medium at 24 h to synchronize their cell cycles. 24 h later, they were again placed in culture medium with serum and induced with different concentrations of ***13*** for 24 or 48 h, depending on the case. After this time, cultures were washed with PBS, fixed with 70% cold ethanol, and incubated with a DNA extraction solution (0.2 M Na_2HPO_4, 0.1 M Citric Acid, pH 7.8) for 15 min at 37 °C. Cells were then centrifuged, washed with PBS, and resuspended in 250 μl of a solution of propidium iodide (40 μg/ml) and RNAse (100 μg/ml) for 30 min at 37 °C in the dark. Finally, samples were analyzed in a FACScan flow cytometer, using a linear scale for the cell cycle and a logarithmic scale to determine the sub-G1 fraction. Results were analyzed with FlowJo software (v 7.6.5, Tree Star, Inc.).

4.6. Apoptosis assays with annexin-V

Cell viability was determined by flow cytometry using the Annexin V-FITC kit (Pharmingen, San Diego, CA, USA). MCF-7 cells were seeded (5×10^4/cm^2) and, after 24 h, were induced with different concentrations of ***13*** for 48 h. Cells were then detached with PBS-EDTA, washed twice with cold PBS, and collected by centrifugation at 500 g for 10 min. Cells were stained following the manufacturer's protocol, and samples were then analyzed in a FACScan flow cytometer, using FlowJo software (v 7.6.5, Tree Star, Inc.).

4.7. Western blot analysis

We seeded MCF-7 cells (5×10^4/cm^2) and, after 24 h, induced them with 2 μM ***13*** for different time periods. The medium was then removed, and cells were lysed with sample buffer (62.76 mM Tris– HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 0.5% bromophenol blue). Proteins were separated by electrophoresis in 12% acrylamide gels in a Mini Protean Tetracell (Bio-Rad, Hercules, CA, USA) and were then transferred to nitrocellulose membranes (Bio-rad, 162-0115), which were blocked for 1 h at room temperature with 5% nonfat milk powder in PBS. The membranes were incubated overnight at 4 °C with primary antibodies against cyclin B1(Cell Signaling, 4138), cyclin D1 (Cell Signaling, 2978), total pRb (Santa Cruz Biotechnology, Inc., sc-102), phospho-pRb (Cell Signaling, 9301), C-Myc (Santa Cruz Biotechnology, Inc., sc-70465), p27 (Cell Signaling, 2552), total p53 (Sigma, p5813), phospho-p53 (Cell Signaling, 9284), cleaved-PARP (Cell Signaling, 9541),

phospho-p44/22 MAPK (Cell Signaling, 4370), or β actin (Sigma, A3854). They were then washed and incubated for 1 h at room temperature with the corresponding secondary antibodies (Cell Signaling, 7074 or Sigma, A9044). Protein-antibody complexes were detected by chemiluminescence using the ECL system (Amersham Pharmacia Biotech, UK). The relative expression of the proteins was semi-quantified with Quantity One software (Bio-Rad Laboratories, Hercules, USA).

4.8. Expression of epithelial–mesenchymal transition (EMT) markers

MCF-7 cells ($5 \times 10^4/cm^2$) were seeded and, after 24 h, induced with different concentrations of ***13*** for 48 h. They were subsequently detached with PBS-EDTA, washed with PBS, and resuspended in 100 μl blocking buffer (PBS, 2% BSA, 0.07% EDTA) to which 10 μl of the corresponding antibody, E-cadherin (R&D Systems, FAB18381P) or β-catenin (R&D Systems, IC13292F), was added. Cells were incubated for 30 min at 4 °C in the dark, washed with PBS, and analyzed by flow cytometry (FACScan). Results were analyzed using FlowJo software (v 7.6.5, Tree Star, Inc.).

4.9. In vivo assay

We used 40 female C57BL/6 mice weighing 25–30 g (Scientific Instrumentation Center of Granada University), which were kept in a laminar flow cabinet in a room with a controlled environment (37 °C, 40–70% relative humidity, 12-h light/dark cycle, and pathogen-free). The *in vivo* study was approved by the ethics committee of the University of Granada. Tumors were induced by subcutaneous injection of 1×10^6 cells of the E0771 line in the right side of the mice. At 9 days, when the tumor volume reached 75 mm^3, mice were randomly distributed into four groups for oral treatment with vehicle alone (1% methylcellulose) or with compound ***13*** at concentrations of 5, 10, or 15 mg/kg. The compound was administered on days 9, 12, 15, 18, 21, and 24 post-injection, and periodical measurements were made during the *in vivo* assay of the largest (a) and the next largest (b) diameter using a digital caliper. Tumor volume was calculated as $V = ab^2\pi/6$.

4.10. Statistical analysis

SPSS 14 for Windows (SPSS, Chicago, IL, USA) was used for the statistical analysis. Results were compared with the Student's test, one- and two-way ANOVAs, and Spearman's test. $p < 0.05$ was considered significant. Data were graphically represented by using Microsoft Excel 2010 software (Microsoft Corporation).

Competing interests

All authors declare that they have no conflict of interest.

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