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ORIGINAL ARTICLE

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# Biological properties of jatrophane polyesters, new microtubule-interacting agents

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**Abstract** *Purpose*: The biological activities of macrocyclic jatrophane polyesters 1–3 from the Sardinian endemism *Euphorbia semiperfoliata* Viv. have not been evaluated in depth. We investigated the microtubule-interacting and antiproliferative activities of these drugs and the molecular mechanisms underlying their effects. *Methods*: We tested jatrophanes for their interaction with purified bovine brain tubulin by an in vitro polymerization assay and by electron microscopy. At a cellular level, the effects of jatrophanes on microtubular architecture, nuclear morphology, cell viability, cell cycle perturbations, and p53 and Raf-1/Bcl-2 involvement were investigated. *Results*: Jatrophanes exhibited microtubule-interacting activity. They stimulated purified tubulin assembly in vitro, and induced paclitaxel-like microtubules, as revealed by electron microscopy. In the cells, rearrangement of microtubule architecture was in contrast to the bundling produced by paclitaxel. Jatrophanes inhibited the growth of some human cancer cell lines without inducing cell cycle arrest in the G2/M phase. Moreover, they influenced p53 expression and Raf-1/Bcl-2 activation. *Conclusions*: Despite their structural difference from paclitaxel and other microtubule-interacting agents, jatrophanes may represent a new type of tubulin binder.

**Keywords** Jatrophanes · Paclitaxel · Microtubules · Proliferation · Cancer cells · Signal transduction

**Abbreviations**: *DAPI:* 4,6-diamidino-2-phenylindole · *DMEM:* Dulbecco's modified Eagle's medium · *ECL:* enhanced chemiluminescence · *EGTA:* ethylene-bis(oxyethylenenitrilo)tetraacetic acid · *FBS:* fetal bovine serum · *FITC:* fluorescein isothiocyanate · *GTP:* guanosine 5′-triphosphate · *MES:* 2-(*N*-morpholino)ethane sulfonic acid · *MTP:* microtubule protein · *PAGE:* polyacrylamide gel electrophoresis · *PBS:* phosphate-buffered saline · *SDS:* sodium dodecyl sulphate

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## Introduction

The archetypal microtubule promoter paclitaxel (Taxol) [1] played an important role in the discovery of new anticancer drugs. It induces tubulin polymerization into microtubules in vitro [2], and produces microtubule stability and bundling in cultured cells [3]. Paclitaxel dramatically alters microtubule dynamics, ultimately leading to inhibition of cell growth and promotion of apoptosis [4] through the arrest of cells in the G2/M phase of the cell cycle [5]. p53, an upstream gene of apoptosis generally induced by DNA-damaging agents, participates in the apoptotic process triggered by paclitaxel [6]. Raf-1 kinase activation has been proposed as a step following microtubule disruption leading to paclitaxel-induced apoptosis, as well as Bcl-2 phosphorylation [7].

The taxoids, paclitaxel and docetaxel, have been used as anticancer drugs in the treatment of a variety of solid tumours [8, 9], but their poor water solubility and side effects limit their clinical usefulness and leave space for improvement. This, coupled with low isolation yields and a complex chemical synthesis, has fostered research aimed at the discovery of biological analogues that share the mechanism of action of paclitaxel. Screening of natural products from various sources has revealed a host of agents targeting tubulin and mimicking the activity of paclitaxel.

Thus, structurally diverse natural products have been proposed as new antimitotic drugs. Among them, the antiproliferative sponge-derived lactone discodermolide potently induces the formation of stable tubulin polymers in vitro and of microtubule bundles in the cells [10, 11].

Eleutherobin, isolated from a marine soft coral, stimulates tubulin polymerization in vitro and is cytotoxic for cancer cells, inducing mitotic arrest and microtubule bundling [12]. Epothilones A and B, nontaxoid natural products of bacterial origin, induce tubulin polymerization in vitro, produce microtubule stability and bundling in cultured cells [13], and retain antiproliferative activity in multidrug-resistant cells [14]. Other biological paclitaxel-like agents have been identified, including rhazinilam [15], laulimaulide [16] and some polyisoprenylated benzophenones [17]. The macrolides epothilones [13] and the glycosylated diterpenoids eleutherosides [12, 18] are among the best-known examples of microtubule-interacting agents. These compounds are made up of a polyoxygenated macrocyclic core bearing an aromatic ester group, a feature also occurring in various classes of secondary metabolites of both marine and terrestrial origin.

Guided by these observations, our aim was to establish whether macrocyclic compounds may be identified as new paclitaxel-like agents. We studied the bioactivity of jatrophane polyesters 1–3 from the Sardinian endemism *Euphorbia semiperfoliata* Viv. (Fig. 1), and compared their effects with those of paclitaxel. We focused on their action on tubulin function, both in the assembly of purified tubulin and in living cells. In addition, we investigated their effects on cancer cell proliferation and cell cycle distribution, and some molecular mechanisms underlying these effects.

## Materials and methods

### Materials

Jatrophanes 1–3 were isolated from *E. semiperfoliata* as reported previously [19]. For all assay purposes, the compounds were dissolved in dimethyl sulfoxide, and the control mixtures contained solvent equivalent to the drug-treated mixtures.

Paclitaxel, mouse monoclonal anti-$\beta$-tubulin antibody and FITC-conjugated sheep anti-mouse antibody were purchased from Sigma Chemical Co. (St. Louis, Mo.). MCF-7 cells and Caco-2 cells were a gift from Prof. A. Corsini, University of Milano, Italy. HL-60 and SK-OV-3 cells were a gift from Prof. G. Barrera and Prof. Di Renzo, respectively, University of Torino, Italy. Balb/c 3T3 fibroblasts were a gift from Prof. G. Bonelli, University of Torino, Italy. Mouse monoclonal anti-p53 antibody (Zymed Laboratories, San Francisco, Calif.), rabbit polyclonal anti-Raf-1 antibody, mouse monoclonal anti-Bcl-2 antibody, and anti-mouse and anti-rabbit horseradish peroxidase-conjugated antibody used for Western blotting were from Santa Cruz Biotechnology (Santa Cruz, Calif.). The ECL detection system was from Amersham Pharmacia Biotech (Uppsala, Sweden). Culture media and FBS were obtained from Gibco-BRL/Life Technologies (Paisley, UK). MOWIOL 4-88 was from Calbiochem (INALCO Spa, Italy).

### Turbidity assay

Electrophoretically homogeneous MTP was purified from bovine brain by two cycles of polymerization and depolymerization [20] and stored in liquid nitrogen until use. Polymerization was monitored turbidimetrically at 350 nm in a spectrophotometer equipped with a temperature controller. GTP (1 m*M*), test compounds, or an equivalent amount of dimethyl sulfoxide (1% v/v) were added and mixed with the tubulin solutions containing a final MTP concentration of 1–2 mg/ml. All polymerization studies contained assembly buffer (0.1 *M* MES, 1 m*M* EGTA, 0.5 m*M* $MgCl_2$) and the instrument was zeroed with this solution at 37°C. Purified tubulin in the absence of drugs did not assemble under these test conditions.

To evaluate calcium-destabilizing effects, the tubulin solution was adjusted to 4 m*M* $CaCl_2$ and the test compound was then added. Alternatively, 4 m*M* $CaCl_2$ was added to the drug-containing reaction mixture after 20 min of polymerization.

### Electron microscopy

Samples for electron microscopy were taken from the reaction mixtures after 30 min of polymerization. They were applied to 150 mesh Formavar-treated, copper grids, fixed into 0.25% glutaraldehyde and negatively stained with 1% uranyl acetate. The grids were viewed in a Philips 300 electron microscope. Tubulin incubated in the absence of added compound served as a negative control (not shown).

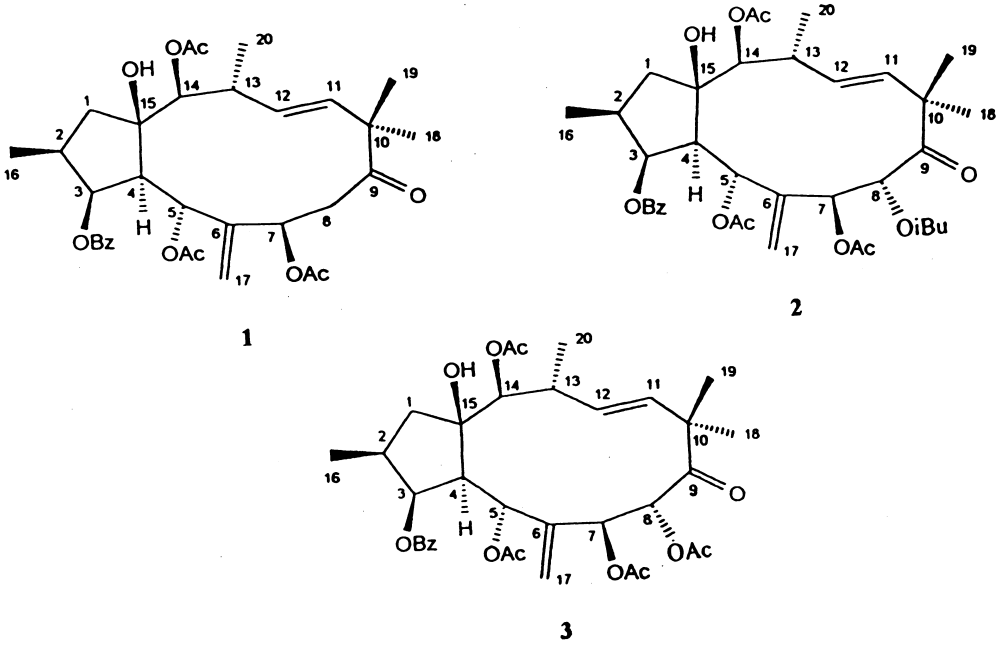


**Fig. 1** Chemical structures of jatrophanes

### Cell culture

Balb/c 3T3 fibroblasts, MCF-7 breast cancer cells and Caco-2 colon carcinoma cells were routinely grown as monolayers in DMEM containing 10% FBS for Balb/c 3T3 and MCF-7 cells, respectively, and 20% FBS for Caco-2 cells. SK-OV-3 ovarian carcinoma cells were grown as monolayers and HL-60 lymphoblastic cells were grown in suspension cultures in RPMI medium containing 10% FBS. All cell lines were cultured in plastic tissue culture flasks and kept in an incubator at 37°C in humidified air containing 5% $CO_2$. Balb/c 3T3, HL-60 and SK-OV-3 cells were subcultured 1:9 every third day. MCF-7 and Caco-2 cells were subcultured 1:3 every third day, using a 0.5% trypsin, 0.53 m*M* EDTA solution. All cell lines were used within 20 passages of the initial stock culture.

### Cell viability

The viability of the cells was assessed by morphological analysis using the trypan blue exclusion assay and an inverted phase-contrast microscope (Leitz, Wetzlar, Germany). Cells in the exponential growth phase were plated at $4\times10^4$ cells/well in 24-well plates. After attachment overnight, the medium was changed and the cells were exposed to drugs or an equivalent amount of dimethyl sulfoxide (0.1% v/v). After treatment, attached cells were trypsinized and incubated in a 0.5% trypan blue solution for 10 min at room temperature. Blue-labelled cells were considered as nonviable and unlabelled cells were regarded as viable.

### Fluorescence microscopy

To stain microtubules, 3T3 fibroblasts were plated onto sterile glass coverslips and allowed to attach and grow for 24 h. The medium was removed, and attached cells were treated with test compounds or an equivalent amount of dimethyl sulfoxide (0.1% v/v). After 24 h, the cells were fixed by treatment in ice-cold methanol, and permeabilized in ice-cold acetone. Following three rinses in PBS, the cells were incubated with mouse anti-$\beta$-tubulin antibody, successively rinsed in PBS and incubated with FITC-conjugated goat anti-mouse antibody.

To stain chromatin, MCF-7 cells were plated onto sterile glass coverslips and allowed to attach and grow for 24 h. Following treatment, the cells were rinsed in PBS, fixed in ice-cold ethanol and incubated with DAPI.

The coverslips were mounted with MOWIOL on glass slides. Fluorescence patterns of stained microtubules or chromatin were visualized and photographed using a Leitz Dialux 20 fluorescence microscope with optics for fluorescein and DAPI.

### Flow cytometry

MCF-7 cells were grown in DMEM under standard conditions of humidity and a $CO_2$-containing atmosphere. Cells were plated in 75-$cm^2$ dishes overnight and the following day, the cells were treated with test compounds. After 24 h, the cells were harvested, fixed in ice-cold ethanol, and stained with propidium iodide at a concentration of 1 ml/$10^6$ cells. The cell suspensions were analysed on a Becton Dickinson fluorescence-activated cell sorter (FACS; Mountain View, Calif.) with excitation at 488 nm and emission at 690 nm. The proportions of cells in the different phases were calculated with the use of the CellFIT DNA Analysis System, version 2.0 (Becton-Dickinson).

### Western blotting

Subconfluent MCF-7 cells were untreated or treated for 16 h with test compounds and then lysed for 1 h on ice in buffer containing protease inhibitors (20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 5 m*M* EDTA, 0.05% aprotinin, 1 m*M* phenylmethanesulphonyl fluoride, 1 m*M* $Na_3VO_4$, 1% IGEPAL). An aliquot of 5–10 µl was used to determine the protein concentration using Hartree's method [21], using bovine serum albumin as a standard. Samples were than adjusted with an appropriate volume of buffer, then 50 µg of protein per lane and 10 µl of proper coloured marker (Rainbow coloured protein molecular weight marker; Amersham Pharmacia Biotech) were subjected to electrophoresis on a minigel (Biorad Mini Protean II apparatus) with 10%, 7.5% and 9.5% SDS-PAGE running gel for p53, Raf-1 and Bcl-2, respectively, at 100 V. The gels were equilibrated in transfer buffer (25 m*M* Tris base, 192 m*M* glycine and 10% methanol) and then transferred to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) at 4°C for 2 h at 100 V. The membranes were blocked for 1 h with 5% milk in TBS-Tween (10 m*M* Tris, pH 7.4, 150 m*M* NaCl, 0.05% Tween 20) and then probed with antibodies for p53 (diluted 1:800), Raf-1 (diluted 1:2000) and Bcl-2 (diluted 1:500). Blots were developed by proper horseradish peroxidase-conjugated antibody (diluted 1:10,000) and the ECL chemoluminescence system (Amersham Pharmacia Biotech) with impressed Hyperfilm ECL (Amersham Pharmacia Biotech), according to the manufacturer's recommendations. Bands were quantified by computerized densitometry using image analysis software (version 1.1; Biorad Multi Analyst).

### Statistical analysis

Differences between means were analysed for significance using the one-way ANOVA test and with the Bonferroni post test to assess the differences between independent groups.

## Results

### Effects of jatrophanes on tubulin polymerization

Jatrophanes 1–3 were first examined for their effect on GTP-induced tubulin assembly (Fig. 2), but they did not significantly influence the polymerization of tubulin under these test conditions.

One characteristic of most microtubule-stabilizing agents is their ability to initiate polymerization in the absence of other promoters. Thus we further investigated jatrophanes for their effects on tubulin polymerization in the absence of GTP. Jatrophanes 1–3 were tested at relatively high (10 µ*M*) and low (10 n*M*) concentrations, and the tubulin polymerization profiles were plotted (Fig. 3). As a group, jatrophanes were able to induce tubulin polymerization, and the assembly reactions with these three analogues appeared similar to

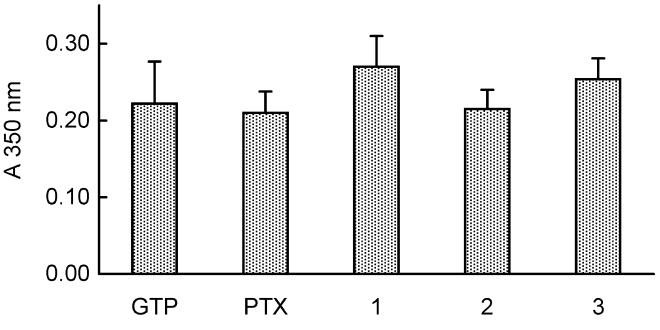


**Fig. 2** Effects of jatrophanes 1–3 and paclitaxel (*PTX*) on GTP-induced tubulin polymerization. Vehicle (DMSO, 1% v/v) or drug at 10 µ*M* were added to tubulin solutions (2 mg/ml) in assembly buffer, and the polymerization reaction was initiated by adding 1 m*M* GTP. Data are presented as absorbance values recorded after a 30-min incubation and represent the mean of three independent experiments (*bars* SD)

that with paclitaxel. The maximum assembly was yielded at an approximately equimolar concentration of drug (10 μ*M*) and tubulin, and little polymerization occurred at the lowest concentrations, except with jatrophane 1.

## Effects on calcium-induced destabilization

To study the stabilizing activity of jatrophanes, tubulin polymerization was monitored in the presence of calcium ions. Unlike paclitaxel, these compounds were not able to stimulate tubulin assembly in the presence of calcium ions (Fig. 4), and jatrophane-induced polymers were disassembled by calcium (Fig. 5).

## Microtubule morphology

We used electron microscopy to determine whether the increase in turbidity observed during the polymerization experiments really corresponded to the formation of microtubules. As shown in Fig. 6A, ultrastructurally paclitaxel-induced microtubules showed well-arrayed long fibres. Polymers formed by jatrophanes and those induced by paclitaxel were quite indistinguishable at all magnifications. In Fig. 6B are shown the polymers formed with jatrophane 1, and similar results were obtained with jatrophanes 2 and 3. These polymers displayed the morphology of microtubules, and were organized in regular arrays of long fibres.

## Effects on cellular microtubules

3T3 fibroblasts were used to evaluate the effects of jatrophanes on microtubule architecture. Microtubules of control cells were organized in arrays of long fibres radiating to the cell periphery (Fig. 7A). Cells treated with 100 n*M* paclitaxel exhibited an extensive network of bundled microtubules (Fig. 7B). Otherwise, treatments with an equivalent concentration of jatrophanes induced fewer morphological changes, although some altered cytoskeletal arrangement was observed, as shown for jatrophane 1 (Fig. 7C).

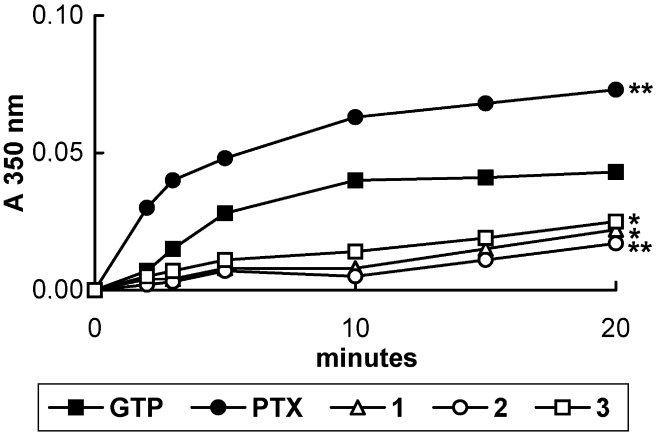


**Fig. 4** Comparison of the effects of calcium on tubulin polymerization induced by jatrophanes and paclitaxel (*PTX*). To each reaction mixture containing assembly buffer and 1 mg/ml tubulin was added 1 m*M* GTP, 10 μ*M* paclitaxel, or 10 μ*M* of each jatrophane. Changes in turbidity were recorded for 20 min as described above. All points represent the mean of three different experiments; standard deviations are not shown because they were < 10%. $^{**}P < 0.01$, $^{*}P < 0.05$ vs values obtained with GTP

## Effects on nuclear morphology

Nuclei of MCF-7 cells are compact and rounded (Fig. 8A), whereas 10 n*M* paclitaxel induced breakdown of nuclei into micronuclei (Fig. 8B), a typical hallmark of paclitaxel-treated cells [22]. Otherwise, cells treated with 100 n*M* jatrophane 1 exhibited a swollen nucleus containing vesicle-like areas (Fig. 8C).

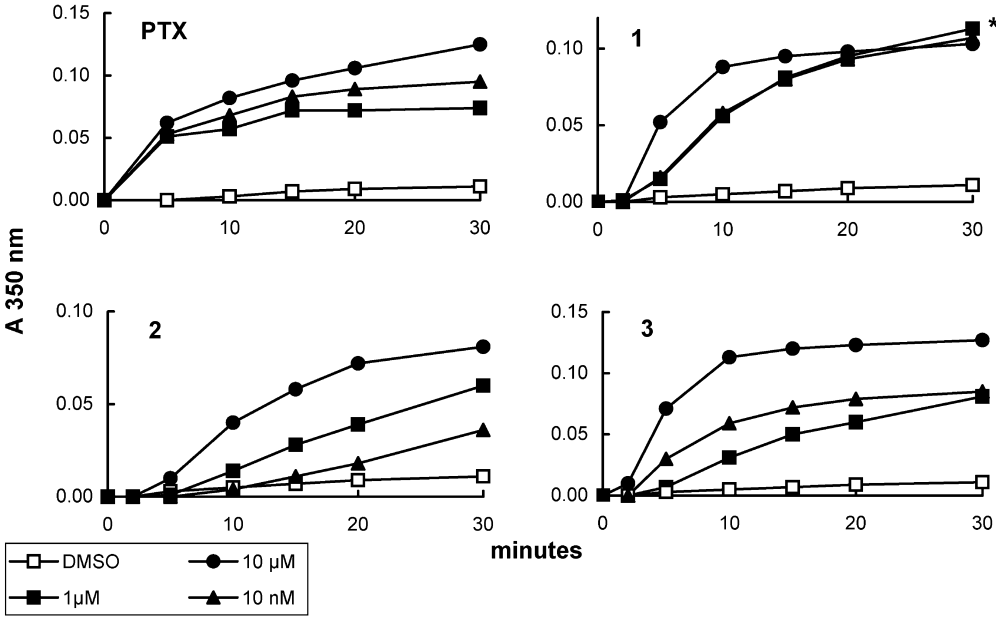


**Fig. 3** Stimulation of tubulin assembly by jatrophanes as compared with paclitaxel. Each reaction mixture contained assembly buffer and 1 mg/ml tubulin. At time zero, vehicle, paclitaxel (*PTX*), or jatrophanes 1–3 were added at the indicated concentrations. The polymerization reaction was monitored turbidimetrically at 350 nm for 30 min. Three experiments were carried out for each stimulant. Each point represents the mean of the different experiments; standard deviations are not shown because they were < 10%. $^{*}P < 0.05$ vs values obtained with paclitaxel

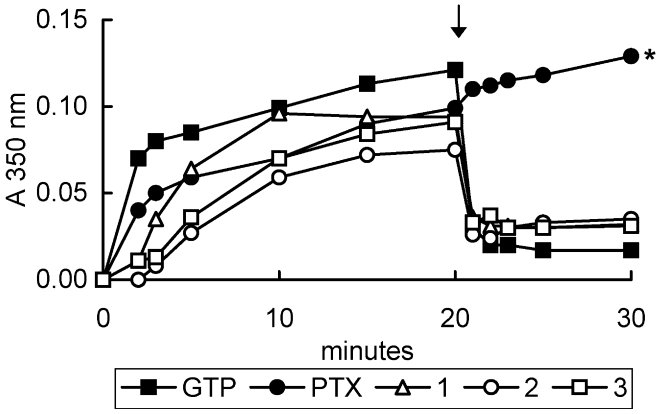


**Fig. 5** Calcium-induced effects on drug-assembled microtubules. To each reaction mixture containing assembly buffer and 1 mg/ml tubulin was added 1 m*M* GTP, 10 μ*M* paclitaxel, or 10 μ*M* of each jatrophane, and the mixture polymerized at 37°C. At the time indicated (*arrow*), 4 m*M* $CaCl_2$ was added and turbidity was monitored for a further 10 min. All points represent the mean of three different experiments, standard deviations are not shown because they were < 10%. *$P < 0.001$ vs values obtained with GTP

## Effects on cell viability

To compare the effects on cell viability of jatrophanes and paclitaxel, we chose breast (MCF-7), leukaemia (HL-60), colon (Caco-2), and ovarian (SK-OV-3) human cancer cell lines. These cell cultures were exposed to different concentrations of test compounds for 24 h. Trypan blue staining was used to assess cell viability, and the results obtained varied with respect to the cell lines. Profiles of cell growth were evaluated in terms of viable cell number, and presented as surviving cell fraction plotted versus control (Fig. 9).

SK-OV-3 cells were the most sensitive to jatrophanes, as viable cell numbers were significantly reduced up to 55%. Interestingly, this effect is comparable to that of paclitaxel, and could be reported as cytostatic activity, the fraction of dead cells being quite negligible. A dose-dependent inhibition of cell growth was observed for HL-60 and Caco2 cells, with lower inhibitory effects compared with paclitaxel. MCF-7 cells showed minor drug sensitivity, and the numbers of surviving cells were twofold greater than those observed with paclitaxel.

## Effects on cell cycle progression

There was a substantial increase in the G2/M population of MCF-7 cells treated with paclitaxel at 10 and 100 n*M*. In contrast, no perturbation of the distribution of cells in the cell cycle was observed after treatment with jatrophane 1 at 10 n*M*, and at 100 n*M* jatrophane 1 induced an increase in cells arrested in G0/G1 (Table 1).

## Effects on p53 induction and Raf-1/Bcl-2 activation

Treatment of MCF-7 cells with 250 n*M* jatrophanes for 16 h markedly increased the expression of p53, compared to p53 levels in control and paclitaxel-treated cells. Jatrophanes even induced Raf-1 activation more effectively than paclitaxel (Fig. 10), as indicated by hyperphosphorylation of Raf-1 protein [6]. Similar results were obtained for Bcl-2, as detectable levels of this antiapoptotic protein were higher in lysates from jatrophane-treated cells than in those from paclitaxel-treated cells.

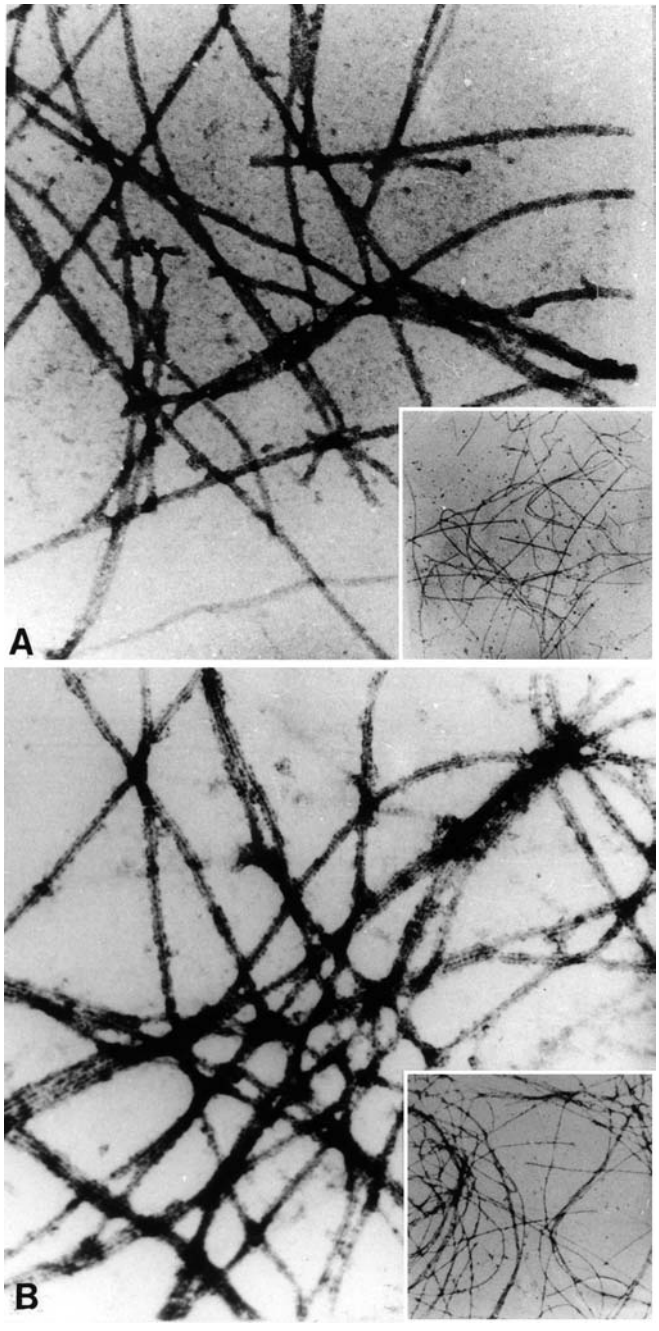


**Fig. 6A, B** Electron micrographs of tubulin polymers induced by jatrophane 1, as compared with paclitaxel. Solutions containing 1 mg/ml tubulin were induced to polymerize by adding 1 μ*M* paclitaxel (**A**) or 1 μ*M* jatrophane 1 (**B**). Following 30 min of assembly, an aliquot of each reaction mixture was processed for electronic evaluation. Figures are representative of at least three experiments (×72,000, *insets* ×39,000)

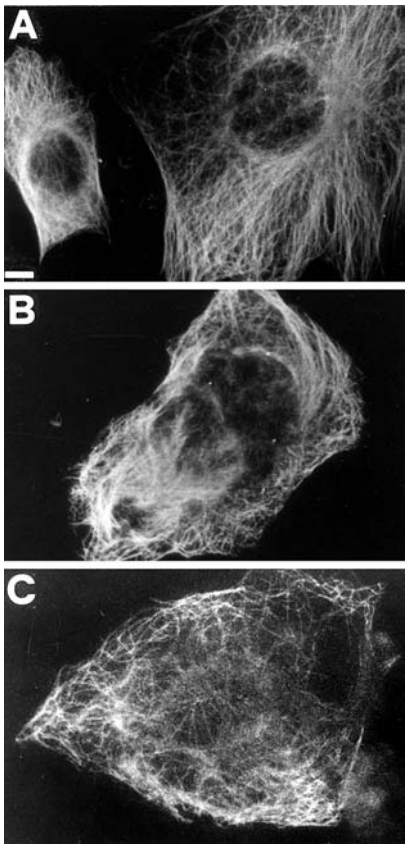


**Fig. 7A–C** Immunofluorescence images of 3T3 fibroblasts stained with anti-$\beta$-tubulin antibody. Cells were untreated (**A**) or treated for 24 h with 100 n*M* paclitaxel (**B**) or 100 n*M* jatrophane 1 (**C**), prior to fixing and staining. Figures are representative of at least three experiments (*bar* 10 μm)

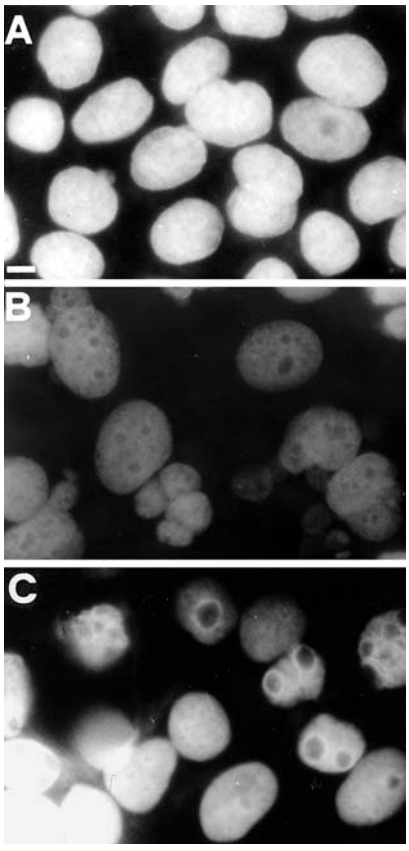


**Fig. 8A–C** DNA staining with DAPI. MCF-7 cells were incubated with vehicle (**A**) or with 10 n*M* (**B**) paclitaxel or 10 n*M* jatrophane 1 (**C**) for 24 h. Nuclei were visualized by fixation and staining for chromosomes. Figures are representative of at least three experiments (*bar* 10 μm)

## Discussion

Spurges (*Euphorbia* spp, Euphorbiaceae) are prolific producers of a wide range of structurally unique macrocyclic diterpenoids. Among these, compounds of the jatrophane type are prominent, but until now their biological activity has been unexplored.

As part of an investigation aimed at the discovery of taxoid mimics, we observed that jatrophane polyesters 1–3 are endowed with microtubule-interacting activity, despite having no structural relationship to other known spindle poisons and microtubule assemblers. They are polyoxygenated macrocyclic diterpenoids and can be isolated in relatively high yields [19]. Structural variations within these compounds derive from the oxygenation mode and the esterification moiety at C-8. Therefore, it is not surprising that their biological activity was similar.

Unlike other microtubule-interacting drugs, these jatrophanes did not interfere with GTP-induced tubulin assembly, but they rapidly induced the formation of tubulin polymers in the absence of other promoters. Jatrophanes were equal to paclitaxel in inducing tubulin polymerization, both in the polymerizing mode of action and in the morphology of microtubule polymers formed. Otherwise, jatrophane polymerization products were disassembled by calcium ions, which inhibited the jatrophane-induced tubulin assembly as well. Thus, the polymers induced by jatrophanes were not stable, whereas paclitaxel stabilizes microtubules under unfavourable conditions [3]. In addition, no aberrant tubulin polymerization products were formed, in sharp contrast to the products of other paclitaxel-like drugs [23]. These observations suggest that jatrophanes are likely to affect microtubule polymer formation differently from paclitaxel, and that their biological activity cannot be identified with suppression of microtubule dynamics, which is the target of many microtubule-interacting agents [24].

At a cellular level, jatrophanes reorganize microtubules without inducing microtubule bundling, a common trait of tubulin-polymerizing agents. These findings are in contrast with biochemical assays, indicating the formation of a massive number of polymers.

The differences in the polymerizing characteristics of jatrophanes in cell microtubules versus those formed by purified tubulin suggest that jatrophanes interact with the tubulin protein in a different fashion, presumably according to the presence of endogenous or exogenous factors. It is therefore difficult to establish the most plausible

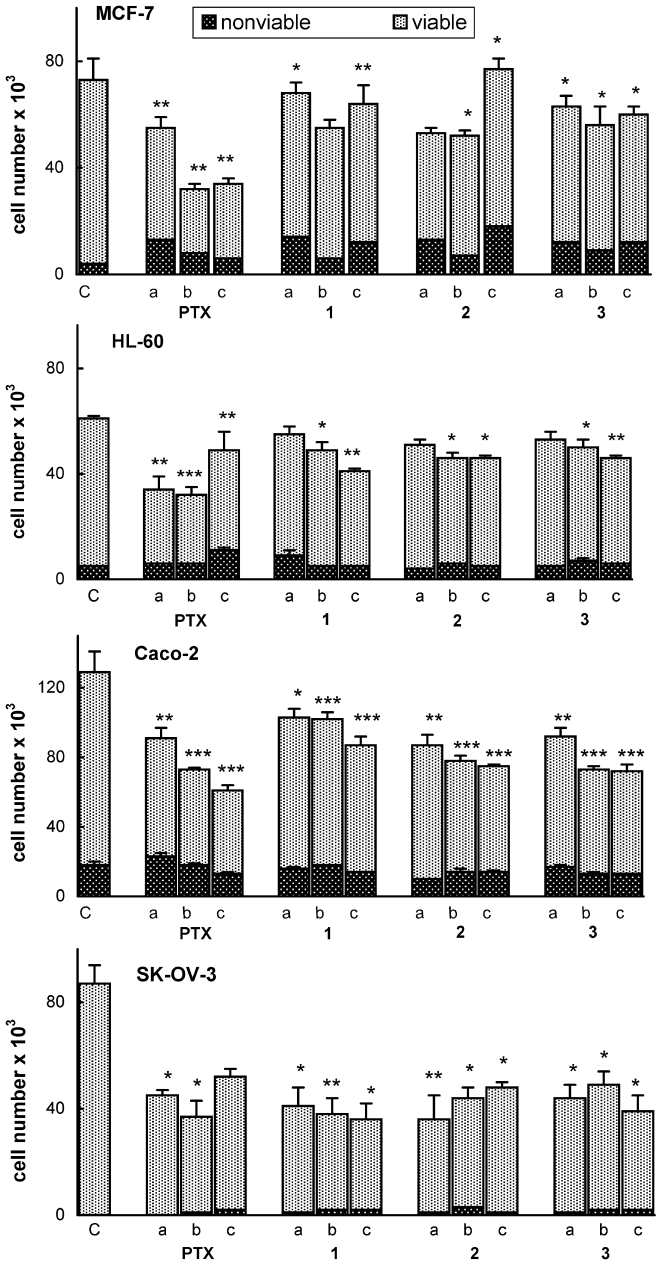


**Fig. 9** Effects on cell viability of jatrophanes 1–3 and paclitaxel (*PTX*). Four human cancer cell lines were treated with different concentrations (*a* 10 n*M*, *b* 100 n*M*, *c* 1 μ*M*) of paclitaxel or jatrophane for 24 h, stained with trypan blue, counted and scored as trypan blue-positive or trypan blue-negative as described in Materials and methods. Untreated cells were used as a control (*C*). The data are presented as means ± SD of three independent experiments, each performed in triplicate. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs untreated cells

mechanism of action for jatrophanes, that likely results in the stimulation or inhibition of tubulin assembly.

These differences in their interactions with tubulin and microtubules may play a role in the low antiproliferative activity of jatrophanes, which cannot be directly related to the ability of jatrophanes to induce the polymerization of tubulin. A partial separation of cytotoxic activity from

**Table 1** Effects of paclitaxel and jatrophane 1 on cell cycle distribution. MCF-7 cells were exposed for 24 h to 10 n*M* or 100 n*M* paclitaxel or jatrophane 1, and the DNA content was analysed by flow cytometry. The proportions of cells in the various phases of the cell cycle are shown as percentages of total cells. Data are the means of two independent experiments

| | G1 | S | G2/M |
|---|---|---|---|
| Control | 55 | 34 | 12 |
| Paclitaxel | | | |
| 10 n*M* | 25 | 46 | 28 |
| 100 n*M* | 29 | 37 | 32 |
| Jatrophane 1 | | | |
| 10 n*M* | 54 | 30 | 15 |
| 100 n*M* | 63 | 31 | 6 |

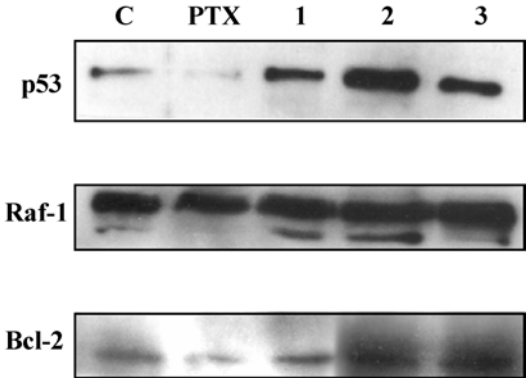


**Fig. 10** p53 induction and Raf-1/Bcl-2 activation mediated by paclitaxel (*PTX*) and jatrophanes 1–3. Extracts of MCF-7 cells, untreated (control, *C*) or treated with 250 n*M* drug for 16 h, were analysed for p53, Raf-1 and Bcl-2 protein levels by Western blotting. The results presented are representative of at least three experiments

tubulin interaction has been achieved with some aromatic taxoids derived from borneol [25], as well as with sarcodictyins [26], rhazinilam [27] and the benzophenones xanthochymol and guttiferone E, which all show poor cytotoxicity [17]. Anyway, despite their effects as antiproliferative compounds, jatrophanes apparently lacked of effects on the distribution of cells within the cell cycle, at the concentrations tested. Thus, jatrophanes did not appear to induce apoptosis, in contrast to paclitaxel and other stabilizing agents [28, 29].

Paclitaxel has been shown to induce apoptosis via peculiar cell cycle arrest at the G2/M transition [30] by a mechanism based on the disturbance of microtubule dynamics. In an attempt to explain the biochemical events downstream of microtubule stabilization leading to apoptosis, several reports have indicated that G2/M arrest of the cell cycle is not the only apoptotic mechanism for paclitaxel, but additional phosphoregulatory pathways may be involved. Raf-1 kinase is implicated in apoptosis [31] and can alternatively phosphorylate nuclear factors such as p53 protein [32], normally induced by DNA-damaging agents. Paclitaxel-mediated cytotoxicity occurs through the activation of Raf-1 kinase

[7]. Through a Raf-1-mediated signalling pathway, paclitaxel can also abrogate the antiapoptotic activity of Bcl-2 [33, 34], and cause accumulation of p53 [6]. The high levels of Raf-1 and Bcl-2 obtained following treatments with jatrophanes suggest that their effects may partly be mediated through Raf-1/Bcl-2 activation. Jatrophanes also induced relevant levels of p53, and these reports may indicate that p53 may also be involved in jatrophane-induced inhibition of cell growth.

In conclusion, the results presented here illustrate that jatrophane polyesters from *E. semiperfoliata* can interact with tubulin and may represent a new type of tubulin binder. These results could provide further information useful for improving our knowledge of the active tubulin-interacting pharmacophore and help to define its still-elusive structural elements.

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