Ingenol derivatives inhibit proliferation and induce apoptosis in breast cancer cell lines

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Summary

We present an analysis of the antitumour effects of a library of ingenol derivatives synthesized in our laboratory and published elsewhere. Fluoro-ingenol (1), ingenol-20-deoxy-20-phtalimido (2), ingenol-3-benzoate-20-deoxy-20-benzamide (3), ingenol-3-benzoate (4), ingenol-3,5-dibenzoate (5), ingenol-3,20-dibenzoate (6), 20-deoxy-20-benylureidoingenol-3-benzoate (7), ingenol-20-deoxy-20-fluoro-3-benzoate (8), ingenol-20-deoxy-20-fluoro-3,5-dibenzoate (9), ingenol-20-phenylcarbamate (10), ingenol-20-benzoate (11), ingenol-3-benzoate-20-phenylcarbamate (12) were tested *in vitro* on two well characterized breast cancer cell (BCC) lines, namely T47D and MDA-MB-231, as representative of two opposite types of hormone-sensitiveness and differentiation stage. These experiments led us to identify ingenol-20-benzoate (11) as a promising antitumour compound characterized by a relevant inhibition of cell growth and apoptotic cell death involving a p53-mediated pathway.

Key words: Apoptosis; Breast cancer; Chemotherapy; Ingenol; PKC.

Introduction

Ingenol is a diterpenoid polyol first isolated from *Euphorbia ingens* by Zeichmeister and co-workers in the 70s during studies aimed at identifying irritant and carcinogenic constituents of the spurge family (*Euphorbiaceae*) [1]. Along with phorbol esters, the natural ingenol monoesters in C-3 are the most potent tumour promoters known to date. At a molecular level, ingenol esters mimic the function of 1,2-diacylglycerol, the endogenous activator of protein kinase C (PKC), a key enzyme involved in cell signal transduction [2, 3].

Notwithstanding their tumour promoting activity, extracts of Euphorbiaceae plants have also been shown to have anti-leukemic and anti-HIV activity [4-6]. Dual co-cancerogenic and antitumour activity is not unprecedented in the field of ingenols and phorbols and it is recognised that the biological activity of these classes of natural products can be significantly altered by subtle chemical modifications. For example, it is well known that tumor promoting activities are mainly due to the presence of a free hydroxyl group at the C-20 position of ingenol. This hydroxyl group appears to be fundamental in promoting the activation of PKC acting both as a donor and as an acceptor of hydrogen bonding. On the other end esterification of this alcohol abolishes PKC activation retaining the anti-tumour activity [7].

Based on these scientific facts and with the aim to dissect anti-tumour from tumour promoting activities of ingenol derivatives, we recently investigated the possibility of replacing the 20-ester group of ingenol-3,20-dibenzoate, the first ingenoid for which anti-cancer activity was reported [5], with more stable bonds in hydrolytic terms (amide, carbamate and urea). These modifications were still able to render the resulting ingenoids incapable of functioning as PKC activators, retaining their antitumour activity [8].

As some reports from other groups have shown that ingenol derivatives share antitumour activity against a wide panel of tumour cell lines as sarcoma 180, Walker 256 carcinosarcoma, Lewis lung carcinoma and P-388 lymphocytic leukemia [5], we decided to evaluate the biological effects of some ingenol derivatives on breast cancer, a main field of interest of our group.

Breast cancer is the first cause of death in women between 40 and 50 years, with an annual world incidence of 500,000 new cases [9]. Breast cancer cell lines represent a widely used model for the study of new approaches and mechanistic information for the development of anti-cancer drugs [10, 11]. In this paper we describe the results obtained during the evaluation of the antitumour activity of a library of ingenol derivatives on two breast carcinoma cell lines, T-47D and MB-MDA-231. These cancer cell lines were chosen as representative of the two opposite clinical situations: well differentiated and hormone responsive cancer cells (T-47D) or poorly differentiated and hormone unresponsive cancer cells (MB-MDA-231).

Materials and Methods

Cells and reagents

Human BCC lines T-47D (well differentiated, estrogen-responsive) and MDA-MB-231 (poorly-differentiated, estrogen-unresponsive) were obtained from ATCC. Cells were grown in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Seromed, Berlin, Germany), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) (Sigma).

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Ingenol derivatives

A panel of ingenol esters (Scheme 1), namely: fluoro-ingenol (1), ingenol-20-deoxy-20-phtalimido (2), ingenol-3-benzoate-20-deoxy-20-benzamide (3), ingenol-3-benzoate (4), ingenol-3,5-dibenzoate (5), ingenol-3,20-dibenzoate (6), 20-deoxy-20phenylureidoingenol-3-benzoate (7), ingenol-20-deoxy-20-fluoro-3-benzoate (8), ingenol-20-deoxy-20-fluoro-3,5-dibenzoate (9), ingenol-20-phenylcarbamate (10), ingenol-20-benzoate (11), ingenol-3-benzoate-20-phenylcarbamate (12) were synthesized in our laboratory as previously described [8].

Ingenol derivatives were dissolved in DMSO (Sigma); 10 mM aliquots were immediately frozen and stored protected from light at -20°C.

Cell proliferation assay

The day before the beginning of the experiments, exponentially-growing cells (1 x 10^5) were seeded in 75 cm^2 flasks. On day 0, 0.1, 1 and 10 μM of the indicated ingenol compounds were added. Control cells were incubated in the presence of the same amounts of vehicle alone. After 72 hours of culture, cells were detached with trypsin/EDTA and washed twice in culture medium. Viable cells were counted by a haemocytometer after trypan blue exclusion. Trypan blue is a dye that stains only dead or late apoptotic cells, thus discriminating between vital cells and necrotic or late apoptotic cells. Experiments were performed at least three times.

Apoptosis assays

After 72 hours of culture with ingenol esters at different concentrations, cells were collected, fixed in 70% ethanol at -20°C overnight, washed twice and stained with 50 μg/ml propidium iodide (PI) (Sigma) supplemented with RNAse (10 mg/ml), according to the method described by Darzynkiewicz [12]. Nuclear morphology was analysed by fluorescence microscopy and DNA content by flow cytometry using a FACScalibur flow cytometer and CELLQUEST software (Becton Dickinson).

The microscopic observation of nuclear condensation, nuclear fragmentation and the formation of apoptotic bodies were assumed to be a marker of genuine apoptosis.

Similarly, the presence of a sub-G1 population was assumed as indicative of apoptosis, quantified with CELLFIT software and represented as percent of the whole cell population. For each cell line, at least three independent experiments were performed.

Determination of IC_{50} by means of Alamar-blue assay

The Alamar-blue assay (Biosource International, CA) incorporates a fluorometric growth indicator based on the detection of metabolic activity [13, 14]. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that fluoresces in response to chemical reduction of the growth medium resulting from cell growth. Cells were seeded in a 96-flat-bottom plate at the initial density of 2x10^3 cells/well. The day after seeding, ingenol esters, in triplicate, were added at the indicated concentration. After 72 hours 10% Alamar-blue solution was added to each well and incubation continued for a further eight hours, after which the plate was analysed on a FluoroCount-fluorometer (Canberra-Packard, CA). Negative controls included the measure of base level fluorescence performed in cell-free wells (fully oxidized), while positive controls included the detection of the maximum fluorescence level acquired on experimentally reduced reagent (fully reduced by autoclaving). In this range, fluorescence was shown to be correlated with cell number in a nearly linear fashion. By means of standard curves it was feasible to obtain the IC_{50} of the compound under evaluation.

Western blot analysis

T-47D and MB-MDA-231 were treated in presence or absence of one of the following compounds 5, 7, 3, 11, 12, 9 at 1 μM for 72 hours. Western blot analysis was performed according to previously published protocols [15]. Briefly cells were lysed in buffer (1% NP-40, 50 mM Tris-HCI pH 8, 0.15 M NaCI, 5 mM EDTA, 10 μg/ml leupeptin, 4 μg/ml pepstatin, 0.1 U/ml aprotinin), kept on ice 20 min and centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method (Bio-Rad, CA). Lysates (30 μg) were separated on an 12% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose (Amersham Italia, Milan, Italy) and the membrane was cut into two at the ~30 kDa mark: the upper part was probed with anti-p53 mAb and the lower with anti-bcl-2 mAb. After incubation with horseradish peroxidase-conjugated goat-anti-mouse antibody (Sigma), detection was carried out by chemiluminescence with ECL reagents (Amersham). Three independent experiments were performed.

Results

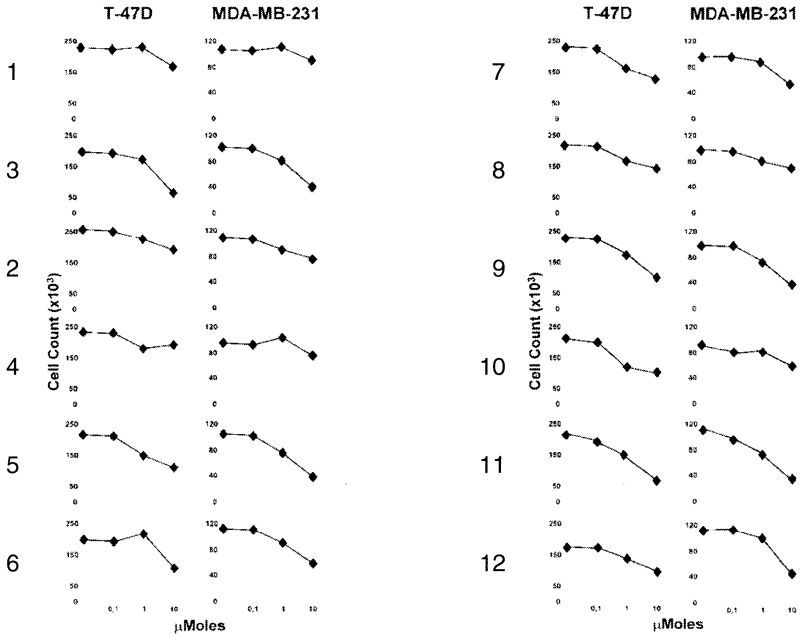
Ingenol derivatives inhibit the growth of breast cancer cells in vitro

To evaluate the effects of ingenol esters on the *in vitro* growth of BCC, T-47 and MDA-MB-231 cells were cultured in 0.1, 1 and 10 μM of the selected compounds, and viable cells counted after 72 hours (Figure 1). At 0.1 μM 11 only gave a mild reduction of cell count on both cell lines varying from 10 to 30% of the control, while the other compounds did not influence cell growth significantly. At 1 μM and more relevantly at 10 μM all 3, 7, 12, 11, 10, 9 and 5 compounds produced a relevant decrease in cell number of both T-47D and MDA-MB-231 (Figure 1). The other ingenoids tested did not show such relevant effects on BCC proliferation notwithstanding that a mild reduction in cell growth could be observed at higher concentrations.

Ingenol derivatives induce apoptosis in BCC lines

To evaluate whether apoptotic cell death was involved in the mechanisms ruling growth inhibition T-47D and MDA-MB-231 cell lines were cultured in 0.1, 1 and 10 μM of the selected compounds and successively analysed for chromatin content. The apoptotic effects, if any, were evaluated after 72 hours of treatment as the number of nuclei with a sub-G1 DNA content determined by PI staining by FACS analysis. PI binds stoichiometrically to DNA becoming fluorescent, so that the staining intensity is proportional to the DNA content of the cell. Thus, reduced PI-staining is suggestive of a reduced DNA content, a main feature of apoptosis. Apoptosis was also confirmed by the microscopic observation of nuclear fragmentation and the presence of apoptotic bodies *(not shown).*

The results obtained showed relevant and dose-dependent apoptotic effects with 11: the percentage of apoptosis after 72 hours was 12% and 27% at 0.1 and 1 μM,



Scheme 1: Library of ingenol derivatives tested.

Figure 1. — Effects of ingenol esters on the growth of T-47D and MDA-MB-231 breast cancer cells.
Cells were incubated for 72 h with 0.1, 1, 10 µM of the indicated compounds or control medium. Cells were then harvested and counted with trypan blue exclusion. One representative experiment out of three is presented.

respectively, on T-47D and 20% at 1 µM on MDA-MB-231. Apoptosis exceeded 70% at 10 µM on both cell lines (Figure 2) compound: three treatments induced a relevant dose-dependent increase in apoptosis only for doses as high as 10 µM, with 20ù apoptosis on T-47D and 15% on MDA-MB-231. Cells treated with compounds 4, 6, 1, 2, 8, 12 and 10 did not show a significant increase in apoptotic cell number if compared to the untreated cells on both T-47D and MDA-MB-231 also at the highest concentration (Figure 2). A weak but significant increase in apoptotic cell number was observed with 7 and 9 at 10.

Determination of IC_{50} by means of Alamar-blue assay

The data derived from the experiments of cell growth and apoptosis prompted us to further characterise the most interesting compounds, namely 3, 11, 7 and 12 in terms of IC_{50}. The Alamar-blue assay (Biosource), performed on T47D according to the instruction of the manufacturer, led us to characterise the growth curve of cells exposed to medium alone or scaling concentrations of the selected compounds over a 72-hour incubation. For each compound IC_{50} was calculated: it was near to µM 4.2 for 3, 5 µM for 11, 7.5 µM for 7 and 10 µM for 12 (Figure 3).

Apoptosis correlates with p53 expression but not with bcl-2

Intrigued by the varying sensitivity to ester ingenols shown by our BCC lines and based on our previous experience in apoptosis studies [16], we investigated the possible correlation between ingenol-induced cell death and the expression of two well characterized proteins involved in apoptosis, namely p53 and bcl-2.

Expression of p53 and bcl-2 was analysed by Western blot using cell extracts from untreated and ingenols-treated BCC lines (1 µM for 72 h). Compounds 5, 7, 3, 11, 12 and 9, identified by the previous experiments as the most effective in cell growth inhibition and apoptosis induction, were further investigated. Only 3 and 11 were found to up-modulate p53 protein expression on both T-47D and MDA-MB-231, while bcl-2 levels were unaffected (Figure 4).

Discussion

The dual activity of ingenol derivatives namely tumour promoter and anticancer can be significantly altered by subtle, yet little understood structural modifications.

This target seems, nonetheless, to be accessible by eliminating the free hydroxyl at the C-20 position, a group required for binding phorboids to PKC by a twofold hydrogen bonding interaction [17]. Simple esterification of the 20-hydroxyl has been suggested to abolish PKC activation while keeping the anticancer activity. In this paper we report the biological activity of a library of ingenol esters, that we have previously synthesized and characterized in their PKC activation properties and expected to exert antitumour activity [8] on breast cancer cell lines.

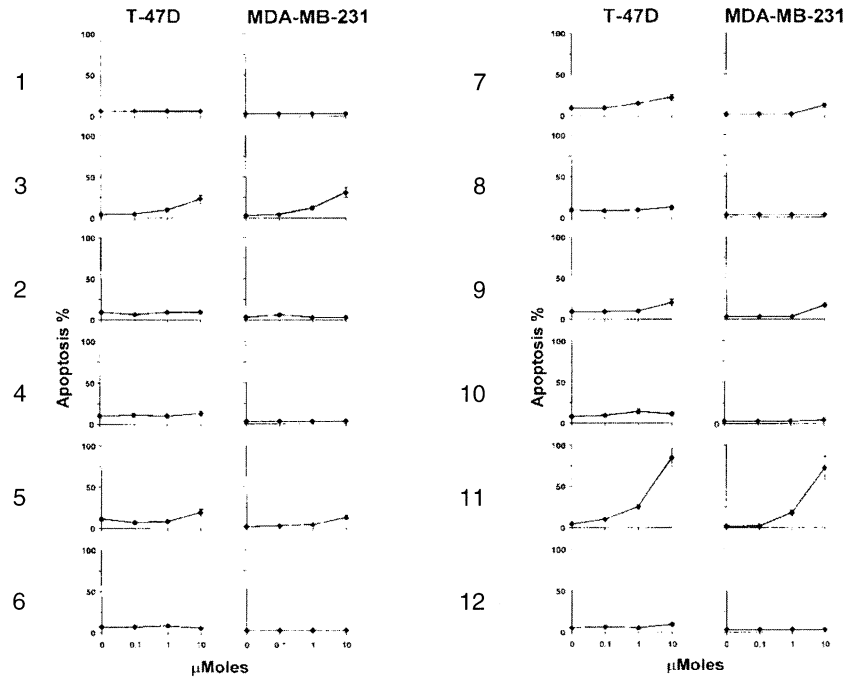


Figure 2. — Effects of selected ingenol compounds or controls on DNA cell content of breast cancer cells in vitro. Cells were incubated for 72 h with 0.1, 1, 10 µM of the indicated compounds or control medium. Cells were then harvested, fixed in 70% cold ethanol and stained with propidium iodide. Cells were then analysed on a FACScalibur flow cytometer with Cellquest software and data are presented as % of sub-G1 population in treated cells or controls.

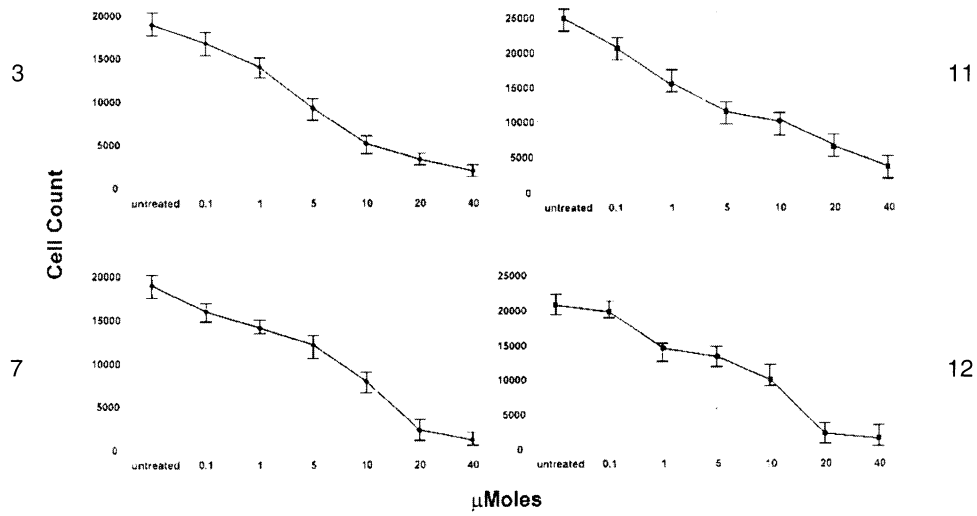


Figure 3. — Evaluation of the IC_{50} by means of the Alamar blue assay. To evaluate the IC_{50} of the indicated ingenol esters, cells were seeded in a 96-well-flat-bottom plate at an initial density of $2x10^3$ cells/well and incubated for 72 h with the indicated concentrations of the selected compounds. During the last 8 hr before the analysis cells were added to 10% alamar blue reagent and then analysed on a FluoroCount-fluorometer (Canberra-Packard, USA) following a protocol reported in the materials and methods section. By means of standard curves it was feasible to obtain the IC_{50} of the compound under evaluation.

Interestingly six of the 12 compounds tested, that is to say 3, 7, 9, 10, 11 and 6, inhibited cell growth of T-47D and MDA-MB-231 cells in a dose-dependent manner. This biological effect is reached by some compounds (3 and 11) through a prevalent apoptotic pathway, as indicated by the presence of a microscopic and cytoflurographic profile suggestive of genuine apoptosis, and by others mainly through a cytotoxic mechanism. Interestingly estrogen receptor status, a main prognostic factor in breast cancer therapy, did not seem to affect the antiproliferative effect of ingenol esters since it was observed equivalently either in estrogen-responsive or estrogen-unresponsive cells.

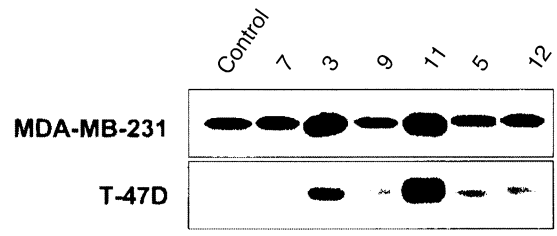


Figure 4. — Western blot analysis of the modulation of p53 protein by selected ingenol esters. Cells were incubated for 72 h with 0.1, 1, 10 μM of the indicated compounds or control medium. Cells were then harvested and lysed. Lysates (30 μg) separated on a 12% SDS-polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose (Amersham Italia, Milan, Italy) and the membrane was probed with anti-p53 mAb. After incubation with horseradish peroxidase-conjugated goat-anti-mouse antibody (Sigma), detection was carried out by chemiluminescence with ECL reagents (Amersham). One of three independent experiments is shown.

The compounds that gave the best results in these experiments were further characterised in terms of IC_{50}; our results showed IC_{50} values near 5 μM for 3, 2.5 μM for 11, 7.5 μM for 7 and 9.5 μM for 12.

Finally our interest was focused on compounds 3 and 11 mainly due to their relevant apoptotic effect. Differently from necrosis that is undergone by the cell as a passive process, apoptosis requires the active participation of cell metabolism and a specific mechanism of action involving, usually, a cascade of strictly coordinated and finely regulated enzymatic activities. p53 and bcl-2 are two of the main actors playing in these machineries as they are involved in apoptotic cell death induced by many antitumour agents, such as paclitaxel and arsenic trioxide.

Our experiments indicate that incubation with 11, identified as the leading apoptotic agent among those tested, was followed by a relevant upmodulation of p53 protein in breast cancer cells. This data suggests an active role for p53 in 11-induced apoptosis that will be further investigated employing p53-defective cell systems still available in our laboratory.

This body of evidence should encourage further studies on ingenol derivatives and candidate ingenol-20-benzoate [11] as interesting leads for the development of new antitumoural agents.

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