Effects of natural prenylated flavones in the phenotypical ER (+) MCF-7 and ER (-) MDA-MB-231 human breast cancer cells

ARTICLE *in* TOXICOLOGY LETTERS · JULY 2006

Impact Factor: 3.26 · DOI: 10.1016/j.toxlet.2005.11.007 · Source: PubMed

Available online at www.sciencedirect.com
SCIENCE DIRECT
Toxicology Letters 164 (2006) 24–36
Toxicology Letters
www.elsevier.com/locate/toxlet

Effects of natural prenylated flavones in the phenotypical ER (+) MCF-7 and ER (−) MDA-MB-231 human breast cancer cells

Madalena Pedro [a], Cátia F. Lourenço [a], Honorina Cidade [a,b], Anake Kijjoa [c,d], Madalena Pinto [a,b], Maria São José Nascimento [a,e,*]

[a] *CEQOFFUP-Centro de Estudos de Química Orgânica, Fitoquímica and Farmacologia da Universidade do Porto, Portugal*
[b] *Serviço de Química Orgânica, Portugal da Faculdade de Farmácia da Universidade do Porto, Portugal*
[c] *ICBAS-Instituto de Ciências Biomédicas de Abel Salazar, Portugal*
[d] *CIIMAR-Centro Interdisciplinar de Investigação Marítima e Ambiental, Portugal*
[e] *Serviço de Microbiologia da Faculdade de Farmácia da Universidade do Porto, Portugal da Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal*

Received 20 September 2005; received in revised form 7 November 2005; accepted 9 November 2005
Available online 20 December 2005

Abstract

The effect of seven natural prenylated flavones in DNA synthesis of two human breast cancer cell lines, the estrogen-dependent ER (+) MCF-7 and the estrogen-independent ER (−) MDA-MB-231 cells, was evaluated. Flavones with an isopentenyl group at C-8 and a ring linking C-3 and C-2′ presented a biphasic effect in DNA synthesis of ER (+) MCF-7 and displayed a stimulation at low concentrations (0.02–0.78 μM) whilst at higher concentrations (>3.12 μM) inhibition was observed. No stimulation was observed in ER (−) MDA-MB-231. In contrast, all the flavones exhibited an antiproliferative effect in both ER (−) and ER (+). Curiously, the inhibition of DNA synthesis was accompanied by a high capacity of these cells to reduce MTT, which was concurrent with the appearance of an intense intracytoplasmic vacuolization. The accumulation of the formazan product in these vacuoles could justify the enhancements of MTT reduction. The characterization of these vacuoles with the autophagic marker monodansylcadaverine (MDC) is consistent with autophagic vacuoles, which led to the suggestion that these flavones could induce autophagy in both ER (+) and ER (−) breast cancer cell lines.

Keywords: Prenylated flavones; *Artocarpus elasticus*; Estrogen receptors; Biphasic effect; Cytoplasmic vacuolization

1. Introduction

Flavonoids compose a large group of phenolic compounds, found in fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers, as well as tea and red wine. These compounds display a remarkable spectrum of biological activities affecting various cellular systems (Middleton et al., 2000). The antiproliferative activity of flavonoids is well documented and has been extensively studied in several human tumor cell lines (Kuntz et al., 1999; Harborne and Williams, 2000; Middleton et al., 2000). However, studies on estrogen-dependent human breast cancer cell lines showed that some flavonoids, depending on the concentration used, exhibited a proliferative effect in addition to antiproliferative activity (Wang and Kurzer, 1997). This growth promoting effect has been attributed to an estrogen activity. The most well-known

* Corresponding author. Tel.: +351 222078920; fax: +351 222003977.
E-mail address: saojose@ff.up.pt (M.S.J. Nascimento).

0378-4274/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.toxlet.2005.11.007

naturally occurring flavonoid with estrogen activity is the isoflavone genistein which has been shown to compete with estradiol for the estrogen receptor (Wang et al., 1996). This kind of similarity between flavonoids and estrogens explains the extensive research undertaken in recent years on this group of compounds for estrogen–antiestrogen activities.

Most studies involving phytoestrogens use the estrogen-dependent ER (+) MCF-7 human cancer cell line (Schmitt et al., 2001; Kinjo et al., 2004; Murata et al., 2004) in parallel with the estrogen-independent ER (−) MDA-MB-231 cells to prove the dependence of the stimulatory effect of these compounds on the estrogen receptors (Wang and Kurzer, 1997; Schmitt et al., 2001; Rowlands et al., 2002). In the ER (+) MCF-7 cells phytoestrogens normally exhibited a biphasic effect, which is expressed by a stimulatory effect on cell growth at low concentrations and an inhibitory effect at high concentrations (Wang and Kurzer, 1997). While the stimulatory effect of phytoestrogens seems to be mediated via estrogen receptors, the antiproliferative effect appears to involve an ER-independent cellular mechanisms (Collins-Burow et al., 2000). Most studies on the evaluation of the proliferative and antiproliferative properties of phytoestrogens use DNA synthesis or MTT assays (Wang and Kurzer, 1998; Wang and Lou, 2004). However, the MTT assay may represent a pitfall in measuring the antiproliferative effect because increases in cellular MTT-reducing activity have been reported in the presence of cell growth inhibition (Bernhard et al., 2003). The mechanism of MTT reduction is far from being clear. Although the reduction in MTT is generally attributed to mitochondrial respiratory chain activity, nonmitochondrial enzymes have also been implicated (Berridge and Tan, 1993). Moreover, MTT reduction in the intracellular vesicles, identified as endosomes and lisossomes formed during the autophagic process, was reported (Liu et al., 1997).

In previous work, while studying the cytotoxic effect of artelastin, a prenylated flavone isolated from *Artocarpus elasticus,* on MCF-7 cells (Kijjoa et al., 1996) it was noticed that this compound exhibited a biphasic effect in DNA synthesis of this ER (+) breast cancer cell line (Pedro et al., 2005) which was stimulatory at low concentrations and inhibitory at high concentrations, resembling the effect of phytoestrogens. Curiously, we found that concentrations of artelastin responsible for an antiproliferative effect were associated with an increased capacity of cells to reduce MTT, at the same time causing massive cytoplasmic vacuolization. These previous results prompted us to undertake a more thorough study of some of these findings. Consequently the aims of the present work were: (i) to evaluate if the biphasic effect exhibited by artelastin in DNA synthesis was equally present in a ER (−) breast cancer cell line; (ii) to extend the investigation of the effect in DNA synthesis to other prenylated flavones (Fig. 1), also isolated from *A. elasticus* (Kijjoa et al., 1996, 1998; Cidade et al., 2001) and structurally related to artelastin; (iii) to evaluate if the cytoplasmic vacuolization induced by flavone treatment was responsible for the increases detected in the cellular MTT reduction capacity; (iv) to evaluate if the cytoplasmic vacuoles could be related with the autophagic process.

2. Material and methods

2.1. Reagents

RPMI-1640, fetal bovine serum (FBS), L-glutamine, gentamicin and trypsin were supplied from Gibco Invitrogen Co. (Scotland, UK). Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and monodansylcadaverine (MDC) were from Sigma–Aldrich Co. (Saint Louis, U.S.A.). Sodium dodecyl sulfate (SDS), Entellan, methanol, paraformaldehyde and Hemacolor® were sourced from Merck (Darmstadt, Germany), [^{3}H]-thymidine from Amersham (Illinois, U.S.A.), dimethylformamide (DMF) from Romil Chemicals (England) and scintillation liquid from Perkin-Elmer (Boston, U.S.A.).

2.2. Plant material

A detailed description of the isolation, purification and identification of the seven prenylated flavones from the wood of *A. elasticus*, are published elsewhere (Kijjoa et al., 1996, 1998; Cidade et al., 2001). The purity of compounds was assessed to de ≥99% (HPLC). A stock solution of each flavone was prepared in DMSO and kept at −20 °C. Appropriate dilutions of flavones were freshly prepared just prior the different assays.

2.3. Cell cultures

Two human breast cancer cell lines were used, the ER (+) MCF-7 and the ER (−) MDA-MB-231. The MCF-7 cell line was provided by the National Cancer Institute (NCI, Bethesda, U.S.A.) and MDA-MB-231 was obtained from the American Type Culture Collection (ATCC, Manassas, U.S.A.). Cells were grown as monolayers and maintained in RPMI-1640 medium supplemented with 5% or 10% heat-inactivated FBS for MCF-7 and MDA-MB-231 respectively, 2 mM glutamine and 50 μg/ml of gentamicin, at 37 °C in a humidified atmosphere with 5% CO_2.

The exponential growing MCF-7 and MDA-MB-231 cells were obtained by plating 1.5×10^5cells/ml followed by 24 h incubation. The effect of the vehicle solvent (DMSO) was eval-

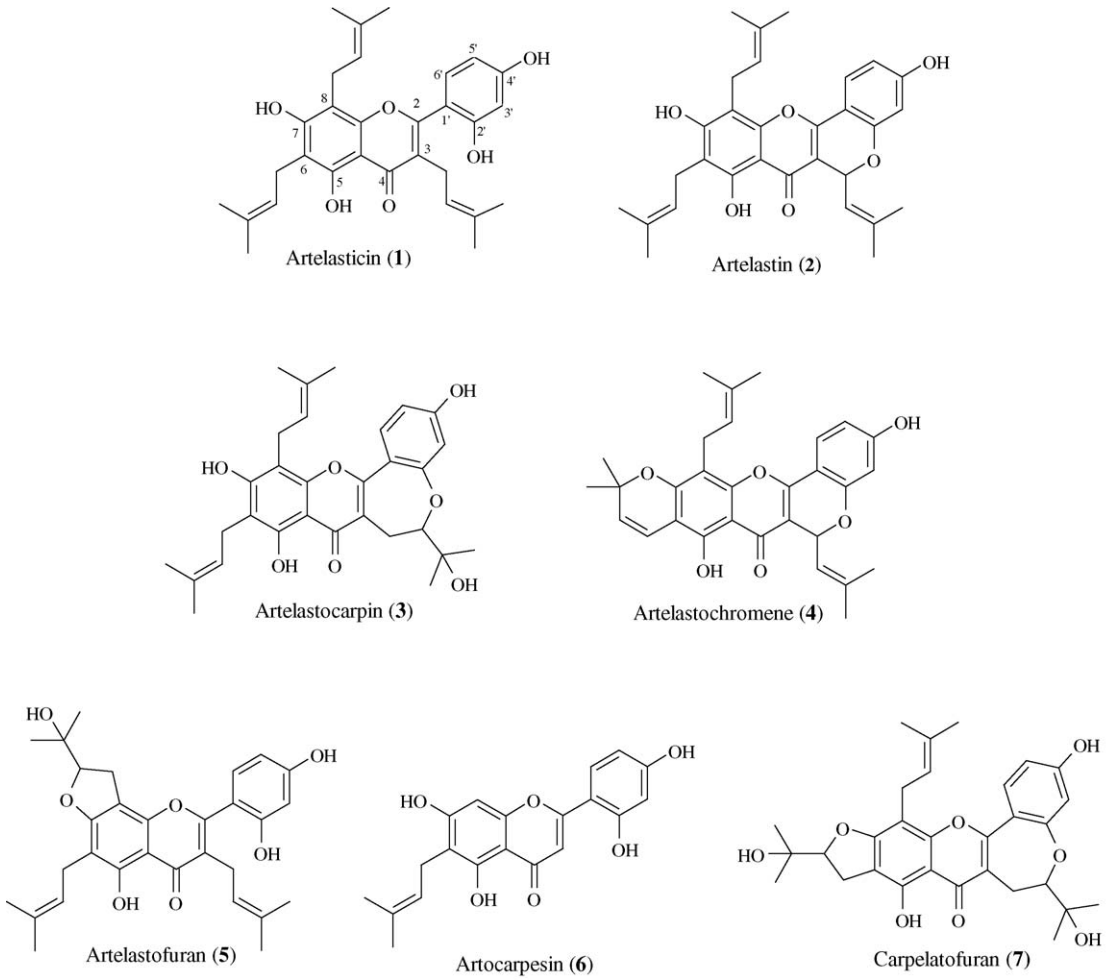

Fig. 1. Natural prenylated flavones studied.

uated in all experiments by exposing untreated control cells to the maximum concentration (0.3%) of DMSO used in each assay.

2.4. DNA synthesis assay

MCF-7 and MDA-MB-231 cells growing exponentially in 96-well microplates were treated with a range of concentrations 0.02–50 μM or 0.04–50 μM, respectively, of each prenylated flavone for 6, 12 and 24 h. After treatment, 1 μCi of [^{3}H] thymidine was added and cells were incubated for a further 4 h. Pulsed cells were then harvested on a glass filter 102 × 256 mm (Skatron, Norway) using a semiautomatic cell harvester (Skatron Instruments, Norway) and allowed to dry. Incorporation of radioactive thymidine was determined by liquid scintillation in a scintillation counter (LS 6500, Beckman Instruments, CA, U.S.A.) and defined by comparing the arithmetic mean of counts per minute (cpm) of treated cells with that of the untreated control cells.

2.5. MTT assay

MCF-7 and MDA-MB-231 cells growing exponentially in 96-well microplates were treated with a range of concentrations (0.02-50 μM) of each prenylated flavone for 6, 12 and 24 h. After treatment, MTT was added (0.5 mg/ml) and cells were incubated for a further 4 h. Formazan products were solubilized with SDS/DMF solution (20% SDS in 50% DMF, pH 4.7) overnight at 37 °C. Absorbance was measured at 550 nm in a plate reader (EAR 400, STL-Labinstruments). The percentage of MTT reduction was calculated comparing the absorbance of treated cells to that of untreated control cells.

2.6. Trypan blue exclusion assay

MCF-7 cells growing exponentially in 6-well plates, were treated with 25 μM for 6 h of each prenylated flavone. MDA-MB-231 cells were only treated with 25 and 50 μM artelastin for 6 h. After treatment, attached cells were released by

trypsinization and mixed with non-adherent cells. Cells were then stained with 0.2% trypan blue and viable and non-viable cells were counted in a hemocytometer. Results were expressed in terms of percentage of viable cells in the total cell number.

2.7. Cell morphologic analysis

MCF-7 and MDA-MB-231 cells growing exponentially in coverslips were treated respectively, with 25 and 50 μM of each flavone for 6 h. After treatment cells were fixed with methanol and stained with Hemacolor® according to the supplier's recommended protocol. Slides were mounted in Entellan and observed under a light microscope.

2.8. MDC staining of cytoplasmatic vacuoles

Cytoplasmic vacuoles were stained with MDC according to the method described elsewhere (Biederbick et al., 1995). MCF-7 and MDA-MB-231 cells growing exponentially in coverslips were treated with 25 μM or 50 μM of artelastin for 6 h, respectively. Cells were then exposed to 50 μM of MDC for 1 h at 37 °C and fixed with 4% paraformaldehyde. Slides were observed under a fluorescence microscope (Eclipse E400 Nikon, Japan).

3. Results

3.1. Effect of prenylated flavones in DNA synthesis of ER (+) MCF-7 cells

The effects of increasing concentrations of the seven prenylated flavones in DNA synthesis of the estrogen sensitive ER (+) MCF-7 cell line were evaluated. For this purpose, exponentially growing cells were treated with a broad concentration range (0.02–50 μM) of the different flavones, for 6, 12 and 24 h and DNA synthesis quantified by thymidine incorporation. The dose-response profile obtained (Fig. 2) showed that artelastin (2), artelastocarpin (3), artelastochromene (4) and carpelastofuran (7) exerted a biphasic effect in DNA synthesis of MCF-7 cells, expressed by a stimulatory effect at low concentrations and an inhibitory effect at high concentrations. This stimulatory effect was mainly observed for concentrations between 0.02 and 0.78 μM and exposure periods of 6 and 12 h. Maximum stimulations were obtained with artelastocarpin (3) and carpelastofuran (7) after 6 h treatment, reaching values of 150% and 200% after exposure to 0.09 and 0.05 μM, respectively. Artelastochromene (4) and artelastin (2) also induced increases of 140% and 130% after 12 h treatment with 0.39 and 0.78 μM, respectively. Twenty-four hours treatments were never associated with a stimulatory effect. This cell growth promoting effect was, however, abruptly lost and replaced by a growth inhibitory effect when treatments were performed with concentrations greater than 3.12 μM, independently of the exposure time used. At concentrations above 25 μM all compounds, except artocarpesin (6), caused a complete stop in DNA synthesis. Curiously, flavones 2, 3, 4 and 7 that exhibited the biphasic effect have two structural features in common, an isopentenyl at C-8 and an additional ring linking C-3 and C-2′on their skeleton. These features seem to be important for the biphasic effect since they are absent in flavones 1, 5 and 6, which did not show this effect.

3.2. Effect of prenylated flavones in DNA synthesis of ER (−) MDA-MB-231 cells

To determine if the biphasic effect detected in DNA synthesis of ER (+) MCF-7 cells was equally present in a ER (−) breast cancer cell line, the MDA-MB-231 cell line was exposed to the same serial range concentrations of the seven flavones for 6, 12 and 24 h. The dose-response profiles obtained with this estrogen-independent cell line (Fig. 3) showed that treatments with low concentrations (0.04–0.78 μM) and short exposure times (6 and 12 h) did not stimulate DNA synthesis. Only the inhibitory effect associated with high concentrations (>10 μM) was observed with this cell line.

Since the stimulation of DNA synthesis was only observed in the ER (+) MCF-7 cells, this could suggest that the growth promoting effect (proliferation) exhibited by these flavones could be related with the presence of estrogen receptors. In contrast the antiproliferative effect that was observed in both ER (+) and ER (−) cell lines seems not to be related with their presence.

3.3. Effect of prenylated flavones in MTT reducing capacity of ER (+) MCF-7 and ER (−) MDA-MB-231 cells

The proliferative and antiproliferative effects of these prenylated flavones were also investigated by the widely used MTT cell proliferation assay (Mosmann, 1983). The effects of increasing concentrations of these flavones on the MTT reducing capacity of ER (+) MCF-7 and ER (−) MDA-MB-231 cells after exposures for 6, 12 and 24 h are shown in Figs. 4 and 5, respectively.

No enhancements in MTT reducing activity were observed in MCF-7 cells after treatments with low concentrations (0.02–0.78 μM) and short exposure times (6 and 12 h). However, this did not agree with the proliferative effect detected previous by the thymidine incorporation assay. Furthermore, high concentrations associated with the inhibition of DNA synthesis (>3.12 μM) were also associated with an increased capacity of cells to

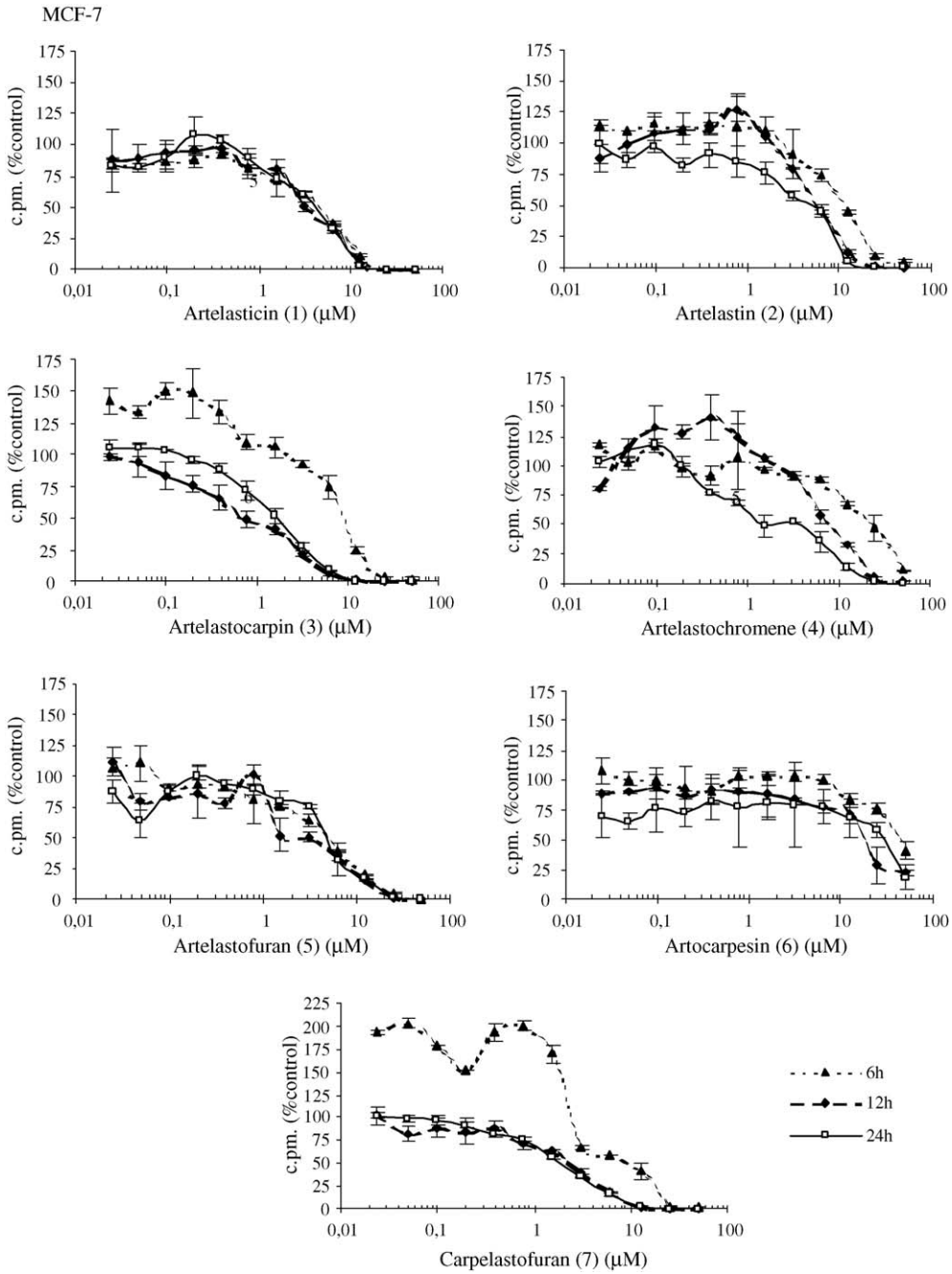

Fig. 2. Time and dose-dependent curves of the seven prenylated flavones in DNA synthesis of MCF-7 cells, determined by the [^{3}H]-thymidine incorporation assay. Cells were treated with each flavone at 0.02, 0.05, 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 μM for 6, 12 and 24 h. Results are the mean ± SEM of three replicates from a representative experiment of two carried out independently.

reduce MTT. In MCF-7 cells, this capacity reached maximum values of 145% and 142% respectively, after 6 h treatment with 12.5 μM artelastin (2) and 50 μM artelastochromene (4), concentrations that were responsible for drastic decreases in DNA synthesis. In MDA-MB-231 cells, this increase in MTT reducing capacity was also observed with growth inhibitory concentrations. Maximum values of 148% were registered for treatments with 25 and 50 μM for artelastin (2) and carpelastofuran (7) for 6 h.

Data from the MTT assay were inconsistent with that obtained from the DNA synthesis assay. It was surprising

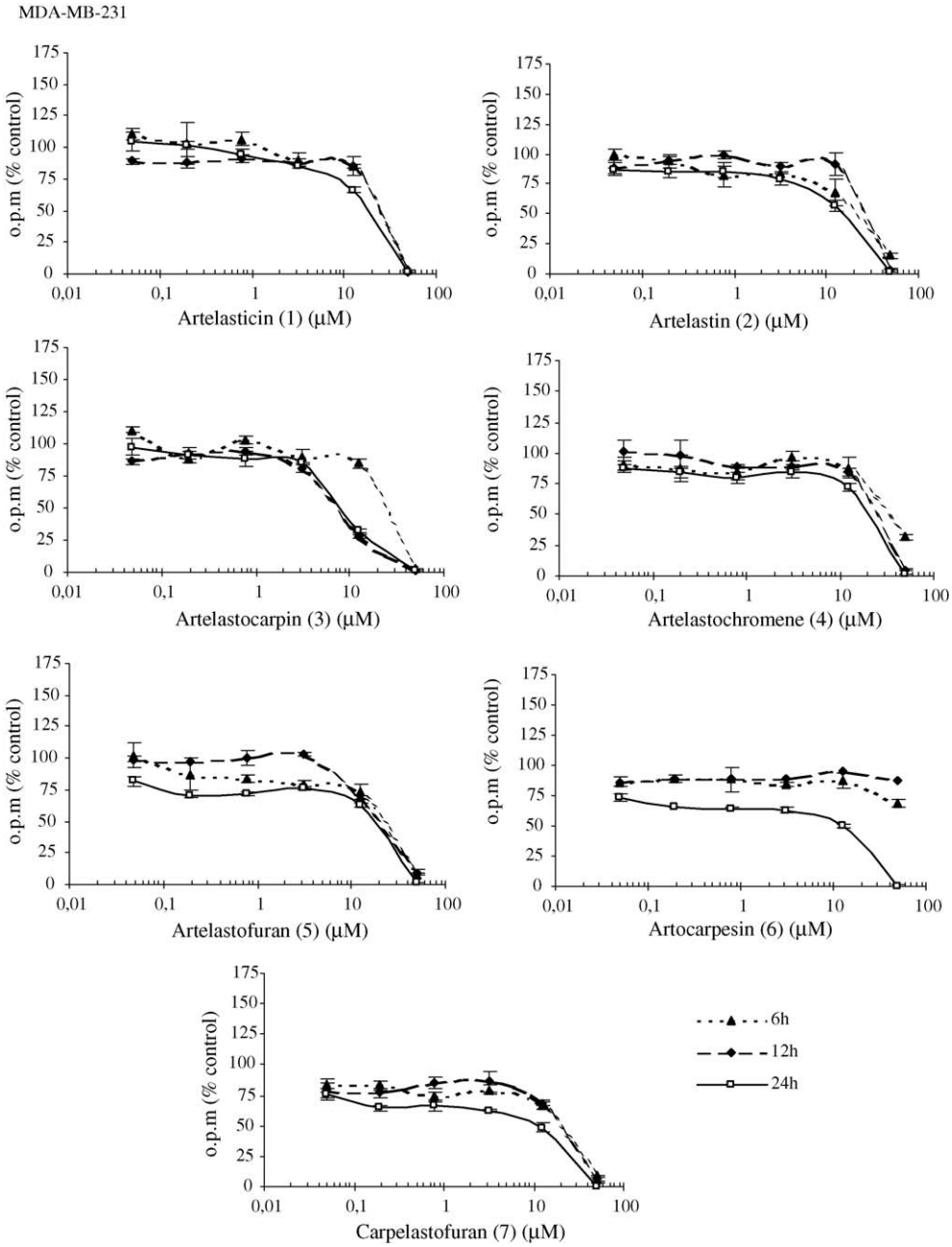

Fig. 3. Time and dose-dependent curves of the seven prenylated flavones in DNA synthesis of MDA-MB-231 cells, determined by the [^{3}H]-thymidine incorporation assay. Cells were treated with each flavone at 0.05, 0.19, 0.78, 3.12, 12.5 and 50 μM for 6, 12 and 24 h. Results are the mean ± SEM of three replicates from a representative experiment of two carried out independently.

that the increase in DNA synthesis observed in MCF-7 cells was not accompanied by an enhancement in the cellular MTT reduction capacity indicative of cellular proliferation. Moreover, the decline in DNA synthesis caused by high concentrations was associated with an increase in MTT reduction levels instead of a decrease, as expected. These discrepancies led us to investigate the cause of this intense formazan production in cells that had their DNA synthesis dramatically affected. A direct reduction of MTT by the prenylated flavones was excluded as the cause of this enhanced formazan production since these compounds showed no capacity to reduce this tetrazolium salt at any concentration used (data not shown). However, microscopic observation of

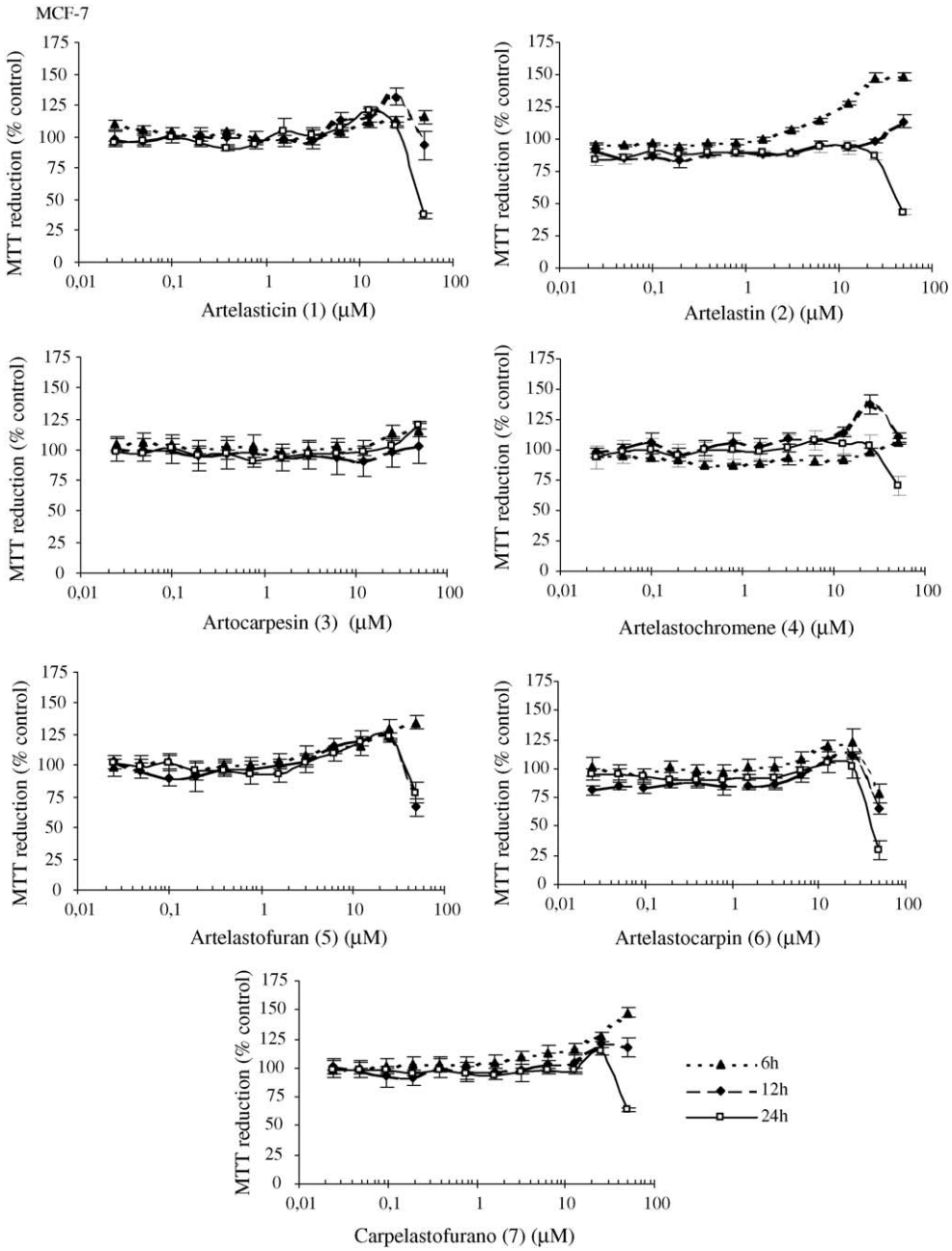

Fig. 4. Time and dose-dependent curves of the seven prenylated flavones in MTT reduction capacity of MCF-7 cells. Cells were treated with each flavone at 0.02, 0.05, 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 μM for 6, 12 and 24 h. Results are the mean ± SEM of three replicates from a representative experiment of two carried out independently.

cells showed an intense vacuolization mainly in cells treated with 25 μM for 6 h, which was not detected in untreated control cells (Fig. 6). Large vacuoles that occupied almost all the cytoplasm and remained uncoloured after staining with Hemacolor® were visualized in cells treated with artelasticin (1), artelastin (2), artelastocarpin (3), artelastochromene (4) and carpelastofuran (7). Curiously, these flavones have in common an isopentenyl group at C-8. This type of morphologic alteration was not observed in cells treated with artelastofuran (5) and artocarpesin (6), flavones without the prenyl group at C-8. This intracytoplasmic vacuolization was also detected in MDA-MB-231 cells, but higher concentrations (50 M) of flavones were needed to induce them (Fig. 6). A 6 h

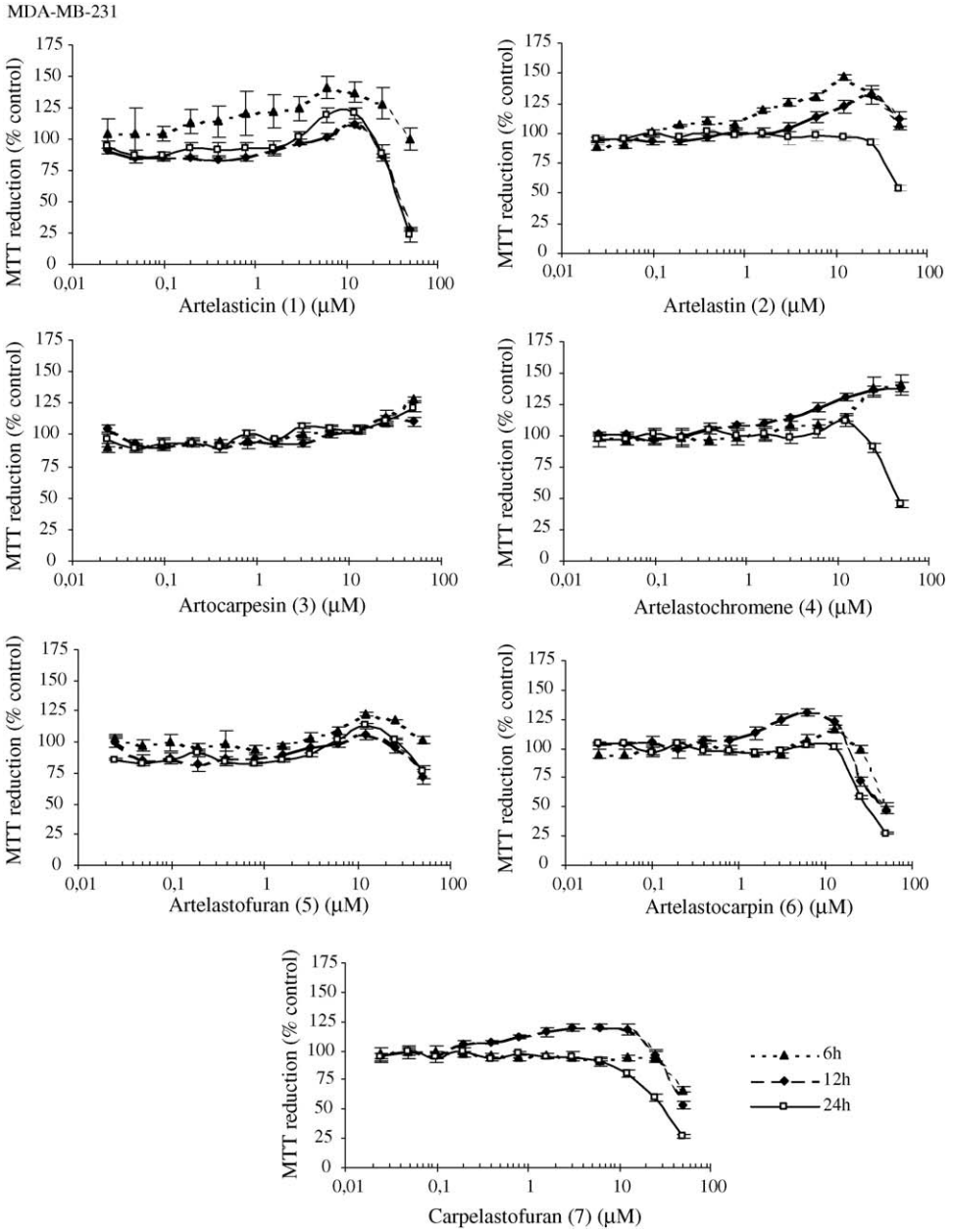

Fig. 5. Time and dose-dependent curves of the seven prenylated flavones in MTT reduction capacity of MDA-MB-231 cells. Cells were treated with each flavone at 0.02, 0.05, 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 μM for 6, 12 and 24 h. Results are the mean ± SEM of three replicates from a representative experiment of two carried out independently.

treatment with 25 μM artelastin was unable to induce the appearance of vacuoles. As with the MCF-7 cell line the vacuolization of MDA-MB-231 cells was only observed after treatments with flavones possessing an isopentenyl group at C-8. In spite of the intense intracellular vacuolization, the viability of MCF-7 and MDA-MB-231 remained very high (>75%) as determined by trypan blue exclusion dye.

Based on the knowledge that MTT is also reduced in intracellular vesicles and that these vesicles accumulate the reduced formazan product (Liu et al., 1997) we hypothesized that the enhancements of formazan detected after flavone treatments, could be due to an accumulation of the MTT reduced product in the large vacuoles induced by these compounds. To prove this, we selected one of the treatments that caused the greatest

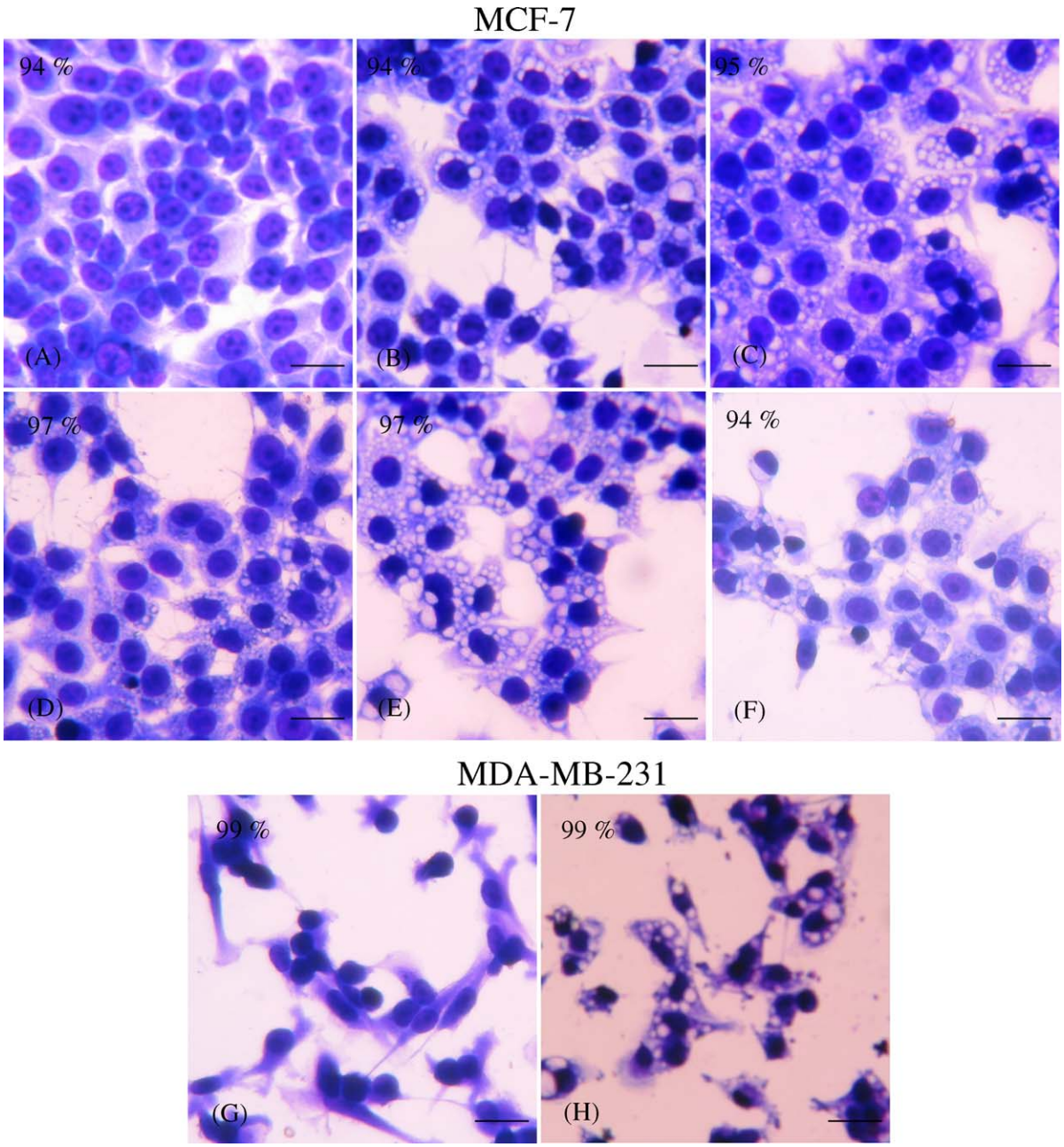

Fig. 6. Morphological alterations induced by the seven prenylated flavones in MCF-7 cells (A–F) and MDA-MB-231 cells (G, H). Light microscopy of stained untreated control MCF-7 cells (A) and treated with 25 μM for 6 h of artelasticin (B), artelastin (C), artelastocarpin (D), artelastochromene (E), carpelastofuran (F), untreated control MDA-MB-231 cells (G) and treated with 50 μM for 6 h of artelastin (H). Untreated control cells were exposed to the equivalent concentration (0.15% or 0.3%) of DMSO for 6 h. Scale bar = 30 μm. The percentage inserted expresses the viability of cells measured by trypan blue exclusion assay.

enhancements in MTT reduction and the most intense vacuolization of MCF-7 cells (treatment with 25 μM artelastin for 6 h). After this treatment, cells were incubated with MTT for 4 h and immediately observed by light microscopy (Fig. 7). As expected, an intense accumulation of violet-coloured formazan confined to the large cytoplasmic vacuoles was observed, which could explain the enhancements in MTT reduction detected.

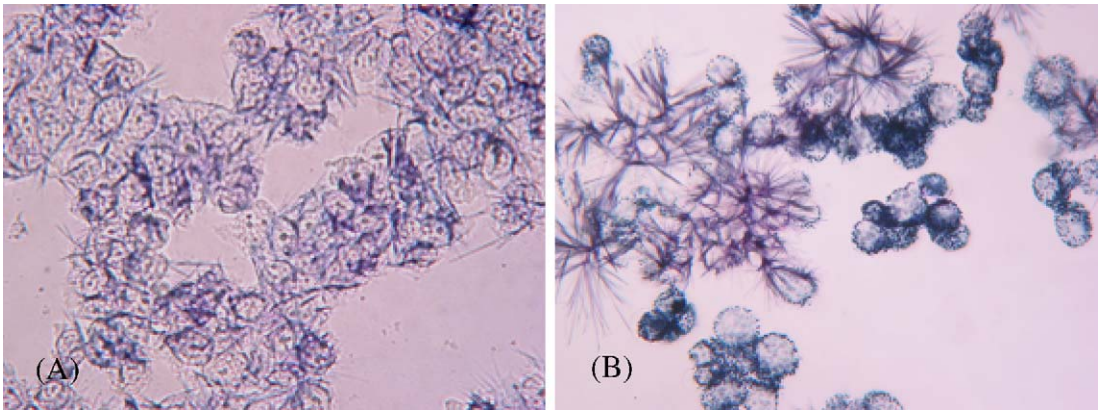

Fig. 7. Accumulation of MTT formazan product in the cytoplasmatic vacoules of MCF-7 cells induced by artelastin (2) treatment. Light microscopy (400×) of untreated control cells (A) and cells treated with 25 μM artelastin for 6 h (B). Control cells were exposed to 0.15% of vehicle solvent (DMSO) for 6 h.

3.4. Characterization of the cytoplasmic vacuoles with the autophagic marker monodansylcadaverine (MDC)

The above results prompted us to undertake a more detailed characterization of the cytoplasmic vacuoles detected. Since, similar vacuoles have been described in the autophagic process, we characterized the vacuoles induced by artelastin (2) in MCF-7 and MDA-MB-231 cells with the autophagic marker MDC (Biederbick et al., 1995). For this purpose, after artelastin treatment, cells were stained with MDC and immediately analyzed by fluorescent microscopy (Fig. 8). Fluorescent dot-like structures, characteristic of MDC-positive cells, were visualized in artelastin treated cells, suggesting that these vacuoles could have an autophagic origin.

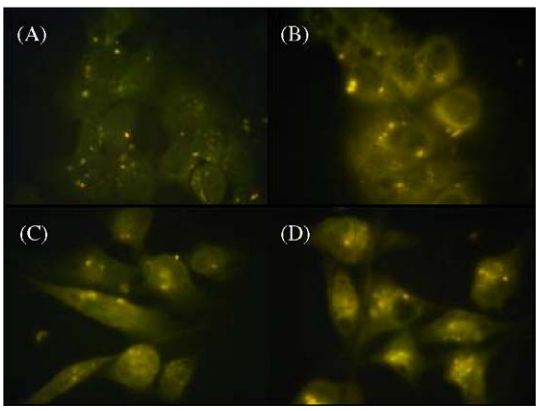

Fig. 8. MDC staining of cytoplasmic vacuoles induced by artelastin (2) treatment in MCF-7 and MDA-MB-231 cells. Fluorescence microscopy (1000×) of untreated control MCF-7 cells (A) and treated with 25 μM artelastin for 6 h (B). Untreated control MDA-MB-231 cells (C) and treated with 50 μM of artelastin for 6 h (D). Control cells were exposed to 0.15% and 0.3% of vehicle solvent (DMSO) for 6 h.

4. Discussion

In the present study we started to characterize the nature of the biphasic effect in DNA synthesis, detected in previous work, in the human breast cancer cell line MCF-7 after treatment with artelastin, a natural prenylated flavone isolated from *A. elasticus* (Pedro et al., 2005). This biphasic effect was indicated by enhancements of DNA synthesis at low concentrations and an inhibitory effect at high concentrations, resembling the behavior described for phytoestrogens on ER (+) breast cancer cells (Wang and Kurzer, 1997, 1998). This led us to hypothesize that the effect of artelastin on the ER (+) MCF-7 breast cell line could be related to the presence of estrogen receptors and motivated us to study the effect of a range of low and high concentrations of artelastin together with the influence of exposure time in DNA synthesis of MCF-7 cells. The effect of artelastin was also studied on the ER (−) MDA-MB-231 breast cancer cell line in order to evaluate if this biphasic effect was equally present in this cell line. This investigation was extended to a group of six more natural prenylated flavones, structurally related to artelastin and also isolated from *A. elasticus*. Results showed that besides artelastin (2) the compounds, artelastocarpin (3), artelastochromene (4) and carpelastofuran (7) induced a biphasic effect in DNA synthesis of ER (+) MCF-7 cells. Curiously, no stimulation in DNA synthesis was observed with artelasticin (1), artelastofuran (5) and artocarpesin (6), which led us to conclude that some structural feature might be related with the growth promoting effect of

the former prenylated flavones. Although it is difficult to draw a structure activity relationship from this small group of compounds, from our data it was possible to observe that the presence of an isopentenyl group at C-8 and an additional ring linking C-3 and C-2′ in compounds 2, 3, 4 and 7 were associated with the appearance of the growth-promoting effect. Estrogenic properties of some prenylflavonoids have been ascribed to the isopentenyl group at position 8, which has been shown to be crucial for their estrogenic activity, namely for 8-prenylnaringenin (Kitaoka et al., 1998; Miyamoto et al., 1998) which is the most potent phytoestrogen described to date (Schaefer et al., 2003; Zierau et al., 2004; Zierau et al., 2005). The presence and position of 8-prenyl group was shown by others to participate in hydrophobic interactions with the estrogen receptor, since a change of this group to another position results in a loss of estrogenic binding activity (Kitaoka et al., 1998; Harborne and Williams, 2000). The 8-prenylnaringenin represents one of the best examples of the importance of the isopentenyl group at C-8 in a key position on the flavonoid framework and is in agreement with our data.

But if a growth stimulation effect was detected on ER (+) MCF-7 no stimulation in DNA synthesis was observed in the ER (−) MDA-MB-231 cells. This absence of growth promoting effect in this estrogen-independent breast cancer cell line together with the proliferative effect detected on the ER (+) MCF-7 cells could suggest an involvement of the estrogen receptors in the proliferative effect of the flavones under study, which is consistent with the known estrogenic activity of the phytoestrogens (Peterson and Barnes, 1991; Wang and Kurzer, 1997; Zava and Duwe, 1997). However, more studies are needed to assess the estrogenic activity of these flavones that would imply the use of E-screen assay and of other more specific tests such as the reporter gene assay and the ER binding assay.

In contrast to the growth-promoting effect associated with low concentrations, higher concentrations of the flavones studied (>3.12 μM) were shown to inhibit the proliferation of both ER (+) MCF-7 and ER (−) MDA-MB-231 breast cancer cells. The antiproliferative effect of these flavones (1–7) has also been demonstrated in several non-breast tumor cell lines and on human lymphocytes (Cidade et al., 2001; Cerqueira et al., 2003), which point out that cell growth inhibition induced by these flavones occurs through different molecular mechanisms not involving estrogen receptors. The mechanisms underlying the growth inhibitory effect of these prenylated flavones are unknown, but artelastin has been shown to interfere with DNA replication, causing an accumulation in S phase and hampering cell cycle progression, while disturbing the microtubule network in MCF-7 cells (Pedro et al., 2005).

The effects of the prenylated flavones (1–7) in MCF-7 and MDA-MB-231 cells were also evaluated by the MTT assay, a valid alternative method to the [^{3}H] thymidine uptake method for analyzing cell proliferation (Mosmann, 1983). It was surprising that treatments with high concentrations that have markedly affected DNA synthesis were associated with enhancements in cellular MTT reduction capacity in both cell lines. A direct reduction of MTT described for some flavonoids (Bruggisser et al., 2002), could explain the increases detected. Since the prenylated flavones under study (1–7) showed no capacity to reduce this tetrazolium salt this hypothesis was excluded. Increases of the cellular MTT-reducing activity in the presence of growth inhibition have also been reported by others (Pagliacci et al., 1993; Bernhard et al., 2003) and represent a pitfall in the MTT proliferation assay. This false negative result would have lead to the conclusion that our prenylated flavones did not affect cell growth. The antiproliferative effect of these compounds would have been missed if only the MTT assay was used. Despite the great use of MTT assay, the cellular mechanism of MTT reduction is poorly understood. The exclusive role of mitochondria in cellular MTT reduction has been questioned (Berridge and Tan, 1993) and the exact cellular site and enzymes involved is far from being clarified. It was demonstrated that MTT can be reduced in intracellular vesicles, many of which were identified as endosomes and lisossomes, with autophagy being suggested as a possible mechanism for the accumulation of the MTT formazan product in these vesicles (Liu et al., 1997). Autophagy is the process by which a cell sequesters part of its cytosol and intracellular organelles and then delivers them to the lisossome for degradation (Dunn, 1990a, 1990b). We showed that the enhancements in MTT reduction observed in flavones treated cells were accompanied by an intense vacuolization of the cytoplasm and that the MTT formazan product was accumulated in these cytoplasmic vacuoles. This accumulation could explain the observed increases in MTT reduction. This vacuolization was observed in both ER (+) MCF-7 and ER (−) MDA-MB-231 cells. The appearance of vacuoles seems to be associated with the presence of the isopentenyl group at C-8 since they were only detected in cells treated with flavones containing this feature. In spite of the intense intracellular vacuolization MCF-7 and MDA-MB-231 cells displayed a high viability. Given the similar appearance between the flavones induced vesicles and autophagic vacuoles added to the high viability of cells, led us to think that we might have encountered compounds with the capac-

ity to induce autophagy. The characterization of these vacuoles with the autophagic marker MDC showed a granular pattern of fluorescence that is consistent with autophagossomes. However, more studies are needed to prove if these prenylated flavones are autophagic inducers. If confirmed this will give to these compounds new perspective as candidates for breast cancer treatment.

In conclusion, in this work it was compared the proliferative and the antiproliferative effects of natural prenylated flavones in ER (+) and ER (−) breast cancer cell lines with a specific focus on the variable results obtained by two different methods of accessing cell proliferation, [^{3}H] thymidine and MTT assay. It was demonstrated that among the prenylated flavones under study (1–7) only those possessing both an isopentenyl group at C-8 and an additional ring linking C-3 and C-2′ on their skeleton (2, 3, 4 and 7) exhibited a proliferative effect at low concentrations while at high concentrations they all presented an antiproliferative effect which was independent of these two structural features and detectable in both ER (+) and ER (−) breast cancer cell line. This antiproliferative effect was accompanied by an intense cytoplasmic vacuolization that could be associated with autophagy.

Acknowledgements

This work was supported by FCT (project grant POCTI/37591/FCB/2001, I&D No. 226/94; M.P. PhD grant SFRH/BD/1456/2000) QCA III and FEDER funding. The authors thank to NCI (U.S.A.) for the generous gift of the MCF-7 tumor cell line.

References

Bernhard, D., Schwaiger, W., Crazzolara, R., Tinhofer, I., Kofler, R., Csordas, A., 2003. Enhanced MTT-reducing activity under growth inhibition by resveratrol in CEM-C7H2 lymphocytic leukemia cells. Cancer Lett. 195, 193–199.

Berridge, M.V., Tan, A.S., 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence and involvement of mitochondrial electron transport in MTT reduction. Arch. Biochem. Biophys. 303, 474–482.

Biederbick, A., Kern, H.F., Elsasser, H.P., 1995. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur. J. Cell Biol. 66, 3–14.

Bruggisser, R., Daeniken, K.V., Jundt, G., Schaffner, W., Tullberg-Reinert, H., 2002. Interference of plant extracts, phytoestrogens and antioxidants with the MTT tetrazolium assay. Planta Med. 68, 445–448.

Cerqueira, F., Cordeiro-da-Silva, A., Araujo, N., Cidade, H., Kijjoa, A., Nascimento, M.S., 2003. Inhibition of lymphocyte proliferation by prenylated flavones: artelastin as a potent inhibitor. Life Sci. 73, 2321–2334.

Cidade, H.M., Nacimento, M.S., Pinto, M.M., Kijjoa, A., Silva, A.M., Herz, W., 2001. Artelastocarpin and carpelastofuran, two new flavones and cytotoxicities of prenyl flavonoids from *Artocarpus elasticus* against three cancer cell lines. Planta Med. 67, 867–870.

Collins-Burow, B.M., Burow, M.E., Duong, B.N., McLachlan, J.A., 2000. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. Nutr. Cancer 38, 229–244.

Dunn Jr., W.A., 1990a. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. J. Cell Biol. 110, 1923–1933.

Dunn Jr., W.A., 1990b. Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. J. Cell Biol. 110, 1935–1945.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481–504.

Kijjoa, A., Cidade, H., Pinto, M., Gonzalez, M.J., Anantachoke, C., Gedris, T., Herz, W., 1996. Prenylflavonoids from *Artocarpus elasticus*. Phytochemistry 43, 691–694.

Kijjoa, A., Cidade, H., Gonzalez, M.J., Afonso, C., Silva, A., Herz, W., 1998. Further prenylflavonoids from *Artocarpus elasticus*. Phytochemistry 47, 875–878.

Kinjo, J., Tsuchihashi, R., Morito, K., Hirose, T., Aomori, T., Nagao, T., Okabe, H., Nohara, T., Masamune, Y., 2004. Interactions of phytoestrogens with estrogen receptors alpha and beta (III). Estrogenic activities of soy isoflavone aglycones and their metabolites isolated from human urine. Biol. Pharm. Bull. 27, 185–188.

Kitaoka, M., Kadokawa, H., Sugano, M., Ichikawa, K., Taki, M., Takaishi, S., Iijima, Y., Tsutsumi, S., Boriboon, M., Akiyama, T., 1998. Prenylflavonoids: a new class of non-steroidal phytoestrogen (Part 1). Isolation of 8-isopentenylnaringenin and an initial study on its structure-activity relationship. Planta Med. 64, 511–515.

Kuntz, S., Wenzel, U., Daniel, H., 1999. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity and apoptosis in human colon cancer cell lines. Eur. J. Nutr. 38, 133–142.

Liu, Y., Peterson, D.A., Kimura, H., Schubert, D., 1997. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT) reduction. J. Neurochem. 69, 581–593.

Middleton, E., Kandaswami Jr., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. Pharmacol. Rev. 52, 673–751.

Miyamoto, M., Matsushita, Y., Kiyokawa, A., Fukuda, C., Iijima, Y., Sugano, M., Akiyama, T., 1998. Prenylflavonoids: a new class of non-steroidal phytoestrogen (Part 2). Estrogenic effects of 8-isopentenylnaringenin on bone metabolism. Planta Med. 64, 516–519.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Murata, M., Midorikawa, K., Koh, M., Umezawa, K., Kawanishi, S., 2004. Genistein and daidzein induce cell proliferation and their metabolites cause oxidative DNA damage in relation to isoflavone-induced cancer of estrogen-sensitive organs. Biochemistry 43, 2569–2577.

Pagliacci, M.C., Spinozzi, F., Migliorati, G., Fumi, G., Smacchia, M., Grignani, F., Riccardi, C., Nicoletti, I., 1993. Genistein inhibits tumour cell growth in vitro, but enhances mitochondrial reduction of tetrazolium salts: a further pitfall in the use of the MTT assay for evaluating cell growth and survival. Eur. J. Cancer 29A, 1573–1577.

Pedro, M., Ferreira, M.M., Cidade, H., Kijjoa, A., Bronze-da-Rocha, E., Nascimento, M.S., 2005. Artelastin is a cytotoxic prenylated

flavone that disturbs microtubules and interferes with DNA replication in MCF-7 human breast cancer cells. Life Sci. 77, 293–311.

Peterson, G., Barnes, S., 1991. Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. Biochem. Biophy. Res. Commun. 179, 661–667.

Rowlands, J.C., Berhow, M.A., Badger, T.M., 2002. Estrogenic and antiproliferative properties of soy sapogenols in human breast cancer cells in vitro. Food Chem. Toxicol. 40, 1767–1774.

Schaefer, O., Humpel, M., Fritzemeier, K.H., Bohlmann, R., Schleuning, W.D., 2003. 8-Prenyl naringenin is a potent ERalpha selective phytoestrogen present in hops and beer. J. Steroid Biochem. Mol. Biol. 84, 359–360.

Schmitt, E., Dekant, W., Stopper, H., 2001. Assaying the estrogenicity of phytoestrogens in cells of different estrogen sensitive tissues. Toxicol. In Vitro 15, 433–439.

Wang, C., Kurzer, M.S., 1997. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr. Cancer 28, 236–247.

Wang, C., Kurzer, M.S., 1998. Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. Nutr. Cancer 31, 90–100.

Wang, T.T., Sathyamoorthy, N., Phang, J.M., 1996. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis 17, 271–275.

Wang, Z.Q., Lou, Y.J., 2004. Proliferation-stimulating effects of icaritin and desmethylicaritin in MCF-7 cells. Eur. J. Pharmacol. 504, 147–153.

Zava, D.T., Duwe, G., 1997. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. Nutr. Cancer 27, 31–40.

Zierau, O., Hamann, J., Tischer, S., Schwab, P., Metz, P., Vollmer, G., Gutzeit, H.O., Scholz, S., 2005. Naringenin-type flavonoids show different estrogenic effects in mammalian and teleost test systems. Biochem. Biophys. Res. Commun. 326, 909–916.

Zierau, O., Hauswald, S., Schwab, P., Metz, P., Vollmer, G., 2004. Two major metabolites of 8-prenylnaringenin are estrogenic in vitro. J. Steroid Biochem. Mol. Biol. 92, 107–110.