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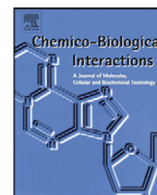

Brassinosteroids cause cell cycle arrest and apoptosis of human breast cancer cells

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ABSTRACT

Brassinosteroids (BRs) are plant hormones that appear to be ubiquitous in both lower and higher plants. Recently, we published the first evidence that some natural BRs induce cell growth inhibitory responses in several human cancer cell lines without affecting normal non-tumor cell growth (BJ fibroblasts). The aim of the study presented here was to examine the mechanism of the antiproliferative activity of the natural BRs 28-homocastasterone (28-homoCS) and 24-epibrassinolide (24-epiBL) in human hormone-sensitive and -insensitive (MCF-7 and MDA-MB-468, respectively) breast cancer cell lines. The effects of 6, 12 and 24 h treatments with 28-homoCS and 24-epiBL on cancer cells were surveyed using flow cytometry, Western blotting, TUNEL assays and immunofluorescence analyses. The studied BRs inhibited cell growth and induced blocks in the G_1 cell cycle phase. ER-α immunoreactivity was uniformly present in the nuclei of control MCF-7 cells, while cytoplasmic speckles of ER-α immunofluorescence appeared in BR-treated cells (IC_{50}, 24 h). ER-β was relocated to the nuclei following 28-homoCS treatment and found predominantly at the periphery of the nuclei in 24-epiBL-treated cells after 24 h of treatment. These changes were also accompanied by down-regulation of the ERs following BR treatment. In addition, BR application to breast cancer cells resulted in G_1 phase arrest. Furthermore, TUNEL staining and double staining with propidium iodide and acridine orange demonstrated the BR-mediated induction of apoptosis in both cell lines, although changes in the expression of apoptosis-related proteins were modulated differently by the BRs in each cell line. The studied BRs seem to exert potent growth inhibitory effects via interactions with the cell cycle machinery, and they could be highly valuable leads for agents for managing breast cancer.

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1. Introduction

Breast cancer is the most prevalent form of cancer among non-smoking women globally, although there are variations in its incidence, prevalence, and mortality among countries [1]. Approximately 70% of all primary breast cancers express estrogen receptor α (ER-α) and it is widely accepted that formation of estrogen with ER-α complexes plays a significant role in the initiation of tumor development and progression [2]. However, the biological effects of estrogen in both normal and cancer cells are mediated by two distinct estrogen receptors (ERs), ER-α and estrogen receptor β (ER-β), which are encoded by independent genes [3]. ER-α and ER-β have similar, but not identical, structure. Although they are the product of independent genes, they share homology at the DNA and ligand binding domains (96% and 58%, respectively) [4]. Both receptor subtypes bind estrogens with a similar affinity and activate the expression of reporter genes containing estrogen response elements in an estrogen-dependent manner [5]. However, ER-α is the main regulator of the aggressiveness of breast cancer tumors, and it is expressed much more strongly than ER-β in such tumors [6]. It is currently thought that ER-β represses growth by inhibiting ER-α-mediated transcriptional activity [7], and the balance between the levels of ER subtypes appears to be an important regulator of estrogen-mediated mitogenesis [8]. Hence, ER-α has been clinically exploited as a molecular

Abbreviations: BRs, brassinosteroids; 28-homoCS, 28-homocastasterone; 24-epiBL, 24-epibrassinolide; ER-α, estrogen receptor α; ER-β, estrogen receptor β; SERMs, selective estrogen receptor modulators; CDK, cyclin-dependent kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated UTP nick end labeling; DAPI, 4'-6-diamidino-2-phenylindole.

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target for the treatment of breast cancer, leading to the development of antiestrogen drugs such as tamoxifen and raloxifen, which are known to be selective estrogen receptor modulators (SERMs) [9]. Unfortunately, however, long-term treatment with SERMs is not effective in many breast cancer patients, since ER expression (and hence sensitivity to endocrine therapy) is lost in up to a third of initially ER-α-positive breast cancers [10]. Thus, there is a need for alternative therapies to treat cancers in this resistant state.

Agents obtained from plants have recently attracted considerable attention for their potential (and in some cases demonstrated and routinely applied) utility for preventing and/or treating certain diseases, including cancer. They also encompass greater chemical diversity than typical chemical libraries [11], and many have powerful biological effects on plants, eukaryotes in general and/or potential pathogens due to activities that have evolved in response to selective pressures over extremely long times [12]. Hence, almost 60% of the drugs approved for cancer treatment are of natural origin; vincristine, irinotecan, taxanes and camptothecines all being examples of plant-derived compounds [12]. However, until recently there have been few investigations of the potential for natural plant hormones to act as anticancer drugs. An important breakthrough in this context was the discovery of cytokinins' inhibitory effects on several human protein kinases, including cyclin-dependent kinases (CDKs); highly conserved regulators of the eukaryotic cell cycle, various members of which control specific phases of the cell cycle [13]. These cytokinins, such as olomoucine [13] and roscovitine [14], have been found to have strong ability to arrest cells at specific points of the cell cycle and to induce apoptosis. Furthermore, they are especially potent against cancer cell lines, in which cell cycle regulators are frequently mutated [15].

Other important phytohormones that can function as growth regulators are the brassinosteroids (BRs); steroid substances that play important physiological roles in various plant processes, including growth, differentiation, root and stem elongation, disease resistance, stress tolerance and senescence [16]. BRs have been detected in and isolated from seeds, fruits, leaves, galls and pollen [17]. Furthermore, like their animal counterparts, BRs regulate the expression of numerous plant genes, affect the activity of complex metabolic pathways, and contribute to the regulation of cell division and differentiation [18]. We also recently discovered that some natural BRs can inhibit the growth of several cancer cell lines at micromolar concentrations, and provided the first evidence that natural BRs can induce cell growth inhibitory responses, arrest cells in the G_1 phase of the cell cycle and induce apoptosis in both hormone-sensitive and -insensitive breast cancer cell lines [19].

Compounds capable of affecting and overcoming the apoptosis deficiency of cancer cells are of high medical significance [20], and various natural products have particularly high potential as leads in this context since they play highly specific roles in relevant cellular processes [21]. Notably, plants have evolved very distinctive ways to regulate, induce, and execute cell death [22]. This may explain the impressive ability of various plant-based compounds to induce apoptosis in mammalian cells. Therefore, plant hormones such as BRs may also be good leads for potential anticancer drugs [19].

BRs are a relatively new group of anticancer agents, and the molecular mechanisms underlying their activities are not fully understood. Hence, the study presented here focuses on the effects, and the mechanisms whereby they are exerted, of two natural BRs, 28-homocastasterone (28-homoCS) and 24-epibrassinolide (24-epiBL), on hormone-sensitive and -insensitive breast cancer cells. The results show (for the first time, to our knowledge) that BRs can affect specific components of the cell cycling machinery with profound consequent effects on cell cycle regulation and also on the induction of apoptosis in cancer cells.

2. Materials and methods

2.1. Chemicals and antibodies

28-homoCS and 24-epiBL were obtained from either SciTech or Olchemim Ltd., (Czech Republic) then stock solutions (10 mM) were prepared in dimethylsulfoxide (DMSO) obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, F-12 medium, fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were also purchased from Sigma. For Western blot analysis, we obtained the primary antibodies against: Bax (clone B-9), CDK2 (clone D-12), CDK4 (clone DCS-35), cyclin A (clone H-432), cyclin D_1 (clone 72-13G), ER-β (H-150), mcm-7 (clone DCS 141.2), mdm-2 (clone SMP14) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Bcl-2 (clone 100) from Biogenex (San Ramon, CA, USA); Bcl-X_L (clone H-62), cyclin B_1 (clone 7A9), cyclin E (clone 13A3), ER-α (clone 6F11), and pRb (13A10) from Novocastra (Newcastle upon Tyne, UK); Bid and caspase-3 (clone Asp175), p21 (clone DCS-60), p53, p-p53 (SER15), and p-p53 (SER392) from Cell Signaling Technology (Danvers, MA, USA); p27 (clone SX53G8) from Dako (Glostrup, Denmark), and PARP (clone C-2-10) from Zymed (San Francisco, CA, USA). Other chemicals included Mowiol medium obtained from Calbiochem (Fremont, CA, USA) and Rainbow™ colored markers from Amersham Biosciences (Vienna, Austria). The goat anti-mouse-fluroescein, goat anti-rabbit-fluroescein and Texas Red fluorescently-conjugated secondary antibodies were purchased from Jackson Immunoresearch (Malvern, PA, USA). The secondary goat anti-mouse and goat anti-rabbit IgG-horseradish peroxidase-conjugated antibodies were supplied by Santa Cruz Biotechnology and Dako, respectively.

2.2. Cell cultures

The human breast cancer cell lines MCF-7 (wild-type p53) and MDA-MB-468 (which carries a mutation at codon 273 of the p53 gene; and is pRb-negative) were obtained from the American Type Culture Collection. The MCF-7 cells were cultured in F-12 medium and the MDA-MB-468 cells in DMEM. All media were supplemented with 10% FBS, L-glutamine (250 mg/l), penicillin (100 U/ml) and streptomycin (100 mg/l). All cultures were maintained under standard conditions at 37 °C and 5% CO_2 in a humid environment.

2.3. TdT-mediated dUTP nick end labeling (TUNEL) assay

The terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) technique was used to detect apoptotic cells. Using the appropriate media, cells at densities of either 1.4×10^4 cells/cm^2 (MCF-7) or 1.6×10^4 cells/cm^2 (MDA-MB-468) were seeded in 60-mm culture dishes with coverslips. Cells were then grown 24 h and treated with either 28-homoCS or 24-epiBL (IC_{50}) for 6, 12, and 24 h. After the selected treatment periods, the cells were washed with phosphate-buffered saline (PBS) and fixed on the coverslips with cold acetone–methanol (1:1, v/v) for 10 min. Apoptosis-induced nuclear DNA fragmentation was then detected by the TUNEL technique using an In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's recommended protocol. Finally, the coverslips were washed in three changes of PBS, incubated with 4′-6-diamidino-2-phenylindole (DAPI, 50 μg/ml; Sigma) for 10 min in the dark, washed in deionized water, and then mounted on glass slides with the hydrophilic Mowiol medium in glycerol–PBS (1:3, v/v) to measure their fluorescence. The cells were then visualized through a BX50F fluorescence microscope (Olympus, Japan), and the treated and control cells were compared.

2.4. Acridine orange (AO)–propidium iodide (PI) double staining cell morphological analysis

Apoptotic morphology was investigated by double staining with propidium iodide (PI) and acridine orange (AO). The cells were plated at densities of either 1.4×10^4 cells/cm^2 (MCF-7) or 1.6×10^4 cells/cm^2 (MDA-MB-468) in 6-well plates. They were allowed to grow at 37 °C in a humidified CO_2 incubator until they were 70–80% confluent. Then the cells were treated with BRs (IC_{50}) for 24, 48 h. After incubation, cells were detached with trypsin and washed twice with PBS. Ten microliters of fluorescent dyes containing AO (10 μg/ml) and PI (10 μg/ml) were added into the cellular pellet at equal volumes of each. Freshly stained cell suspension was dropped on to a glass slide and covered by coverslip. Slides were observed under UV-fluorescence microscope (Olympus, Japan) within 30 min before the fluorescence color starts to fade. Viable cells had green fluorescent nuclei with organized structure, early apoptotic cells had yellow chromatin in nuclei that were highly condensed or fragmented; apoptotic cells also exhibited membrane blebbing. Late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented; necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, nonbiased manner.

2.5. Immunofluorescence labeling methods

MCF-7 and MDA-MB-468 cells were seeded, cultured, treated and fixed as described for the TUNEL assay. The cells on the coverslips were then labeled with antibodies against ER-α and ER-β for 90 min at room temperature in the dark, and then washed with three changes of PBS prior to incubation with the appropriate fluorescently-conjugated secondary antibodies (goat anti-mouse-fluorescein, goat anti-rabbit-fluorescein, or Texas Red). Finally, the coverslips were washed in three changes of PBS, incubated with DAPI (50 μg/ml) for 10 min in the dark, washed in deionized water, and then mounted on glass slides with the hydrophilic Mowiol medium in glycerol–PBS (1:3, v/v) to measure their fluorescence. The cells were then visualized through a BX50F fluorescence microscope (Olympus, Japan), and the treated and control cells were compared.

2.6. Preparation of cells and Western blot analysis

The cells were seeded at densities of 1.4×10^4 cells/cm^2 (MCF-7) and 1.6×10^4 cells/cm^2 (MDA-MB-468) using appropriate culture media in 100-mm culture dishes. After 24 h incubation, the cultures (which had reached ca. 70% confluence) were treated with either 28-homoCS or 24-epiBL (IC_{50}) for 6, 12 and 24 h. Following these treatments, the cells were washed with three changes of cold PBS and scraped into an ice-cold protein extraction buffer containing HEPES (50 mM, pH 7.5), NaCl (150 mM), EDTA (1 mM), EGTA (2.5 mM), 10% glycerol and 0.1% Tween 20 with protease and phosphatase inhibitors (25 μg/ml phenylmethanesulphonyl fluoride, 1 mM NaF, 2.5 μg/ml leupeptin, 0.1 mM Na_3VO_4, 2.5 μg/ml aprotinin, 10 mM β-glycerol-phosphate and 1 mM dithiothreitol). The resultant lysates were collected into microfuge tubes, incubated on ice for 1 h and then cleared by centrifugation at 45,000 × *g* for 30 min at 4 °C. Finally, the supernatant was collected, aliquoted, and stored at −80 °C until further analysis. The lysate protein content was measured using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol.

The procedure for Western blot analysis involved electrophoretically separating the proteins in portions of the lysates (15–30 μg/well) in 10% or 12% SDS-PAGE gels, then transferring them onto nitrocellulose membranes (Amersham Biosciences, Vienna, Austria) by semi-dry electrophoretic transfer. The Rainbow™ colored markers were used as protein molecular weight standards. Non-specific binding sites were blocked by incubating the blots for 2 h at room temperature with 5% (w/v) non-fat dry milk in PBS. The blots were then incubated overnight at 4 °C with the appropriate primary antibody for detecting the protein epitope of interest and washed in PBS with 0.1% Tween 20 for 1 h. The blots were then incubated for 45 min at 4 °C with either the secondary goat anti-mouse (dilution 1:6000) or the goat anti-rabbit (dilution 1:2000) IgG-horseradish peroxidase-conjugated antibodies as appropriate, then once again washed in PBS with 0.1% Tween 20 for 1 h. Proteins were then detected with a chemiluminescence detection system (Amersham Biosciences) according to the manufacturer's protocol. The equality of the protein loading was confirmed by Ponceau S membrane staining (Sigma) and immunostaining of the MCM-7 protein in each lane. The experiments were repeated three times and expression levels of the proteins of interest were compared between treated and untreated control cells.

2.7. Flow cytometry analysis

Flow cytometry was used to evaluate the number of cells in specific phases of the cell cycle, and the proportions with subG_1 DNA levels. The cells were seeded at densities of 1.4×10^4 cells/cm^2 (MCF-7) and 1.6×10^4 cells/cm^2 (MDA-MB-468) using appropriate culture media in 60-mm culture dishes. After 24 h incubation, the cells, which had reached approximately 70% confluence, were treated with 28-homoCS or 24-epiBL (IC_{50}) for 6, 12 and, 24 h. Controls were treated with BR-free DMSO. After treatment, samples of the cells (1×10^{-6}) were washed twice with cold PBS, pelleted, and fixed with chilled ethanol (70%, v/v) by mild vortexing. Low molecular weight apoptotic DNA was then extracted in citrate buffer (3.9 mM), and RNA was cleaved by RNAse (50 μg/ml) (Sigma). Propidium iodide was used to stain the cells' DNA, which was then quantified with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.8. Statistics

Data are reported as means ± standard deviations (SD) obtained from at least three independent experiments. Differences between means were evaluated by Student's *t*-test and regarded as being significant if $p < 0.05$.

3. Results

We recently published preliminary analyses showing that: the BRs 28-homoCS and 24-epiBL have dose-dependent effects on the viability of MCF-7 and MDA-MB-468 cells; MCF-7 cells are significantly more sensitive to 28-homoCS than MDA-MB-468 cells (IC_{50} 40 ± 1.5 and 65 ± 2.8 μM, respectively); and 24-epiBL has weaker effects than 28-homoCS on both the MCF-7 and MDA-MB-468 cells (IC_{50} 60 ± 1.8 and 68 ± 2.5 μM, respectively). The IC_{50} BR concentrations determined in the cited study were used for the further in vitro molecular studies presented in this paper [19].

3.1. Expression and localization of estrogen receptors (ERs)

To examine the influence of the BRs on ER expression and distribution in breast cancer cell lines, cell cultures were treated with 28-homoCS or 24-epiBL (IC_{50}), fixed, and immunolabeled with specific antibodies. Both ER-α and ER-β are expressed in MCF-7 cells, whereas MDA-MB-468 cells lack ER-α expression due to epigenetic silencing, and only express ER-β. Changes in ER-α and ER-β localization patterns were observed in MCF-7 cells after 24 h of BR treatment (Fig. 1A and B). Strong, uniform ER-α immuno-nuclear

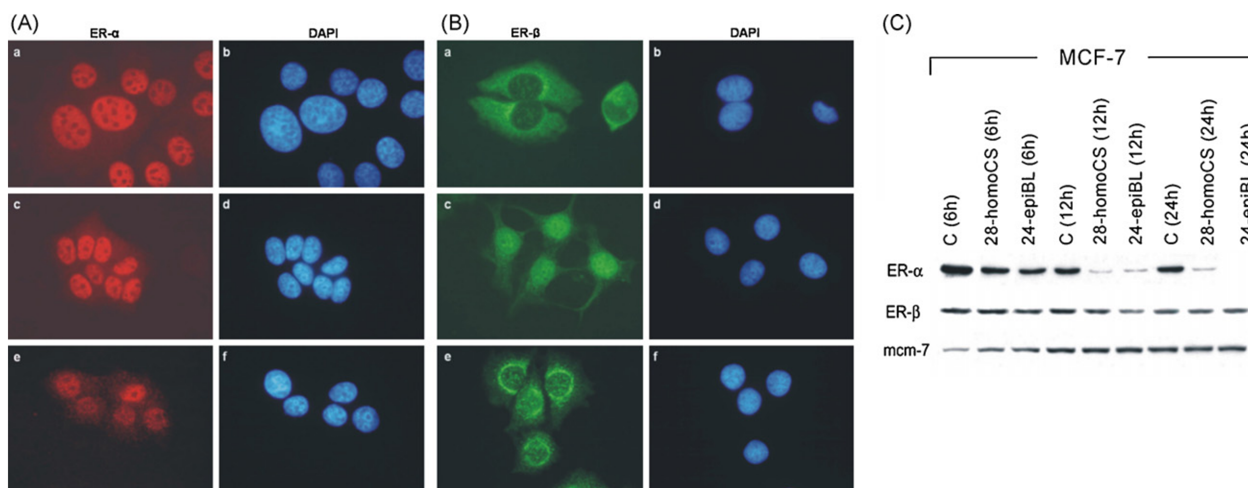

Fig. 1. Expression and localization of ER-α and ER-β in MCF-7 cells as determined by immunofluorescence and Western blot analyses (representative fluorescence results shown at 1000× magnification). (A) Cells were fixed and immunolabeled with an antibody against ER-α (red) then counterstained with DAPI to visualize all nuclei (blue). (a andb) Control cells showing ER-α labeling restricted to nuclei. (c and d) Cells treated with 28-homoCS (IC_{50}) for 24 h. Note the appearance of bright cytoplasmic speckles of ER-α immunofluorescence. (e and f) Cells treated with 24-wpiBL (IC_{50}) for 24 h. Note the variable labeling of nuclei, the homogenous cytoplasmic labeling throughout the cells, and the diminished intensity of the overall nuclear labeling. (B) Cells were fixed and immunolabeled with an antibody against ER-β (green) then counterstained with DAPI to visualize all nuclei (blue). (a and b) Control cells. Note the specific cytoplasmic labeling of ER-β immunofluorescence. (c and d) Cells treated with 28-homoCS (IC_{50}) for 24 h. Note the markedly uniform labeling of the nuclei with ER-β antibody and the homogeneous labeling of the cytoplasm. (e and f) Cells treated with 24-epiBL (IC_{50}) for 24 h. Note the bright perinuclear localization of ER-β. (C) The effects of BRs on ER-α and ER-β protein expression in MCF-7 cells. Cells treated with IC_{50} concentrations of 28-homoCS or 24-epiBL for 6, 12 and 24 h were compared with untreated cells. MCM-7 was used as a protein loading marker, the experiment was repeated three times with similar results, and the presented data are from a single representative run. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

labeling was detected in the control MCF-7 cells, while cytoplasmic speckles of ER-α immunofluorescence appeared in corresponding cells treated with 28-homoCS or 24-epiBL. Furthermore, the intensity of nuclear labeling was weaker in the 24-epiBL-treated cells (Fig. 1A). In contrast to ER-α, ER-β was predominantly found in the cytoplasm of control MCF-7 cells. However, ER-β was notably relocalized to the nuclei after 28-homoCS treatment, whereas it was predominantly present at the periphery of the nuclei in 24-epiBL-treated cells (Fig. 1B). Uniform nuclear and cytoplasmic ER-β immunolabeling was detected in the control and treated MDA-MB-468 cells (Fig. 2). No significant changes in the localization of ER-β were found following BR treatment in MDA-MB-468 cells.

The effects of each BR on ER expression in MCF-7 and MDA-MB-468 cells were also assessed and compared by Western blot analysis. The control MCF-7 cells showed relatively high expression of both ER-α and ER-β, while MDA-MB-468 cells expressed relatively low levels of ER-β and, as expected, no ER-α (data not shown). However, treatment with either BR led to down-regulation of ER-α expression in MCF-7 cells in a time-dependent manner, while ER-β expression was decreased after 6 and 12 h of 24-epiBL treatment, and relatively slightly decreased after 12 h treatment with 28-homoCS (Fig. 1C). In contrast, no significant changes in ER-β expression were detected in the MDA-MB-468 cells (data not shown).

3.2. *Effects of 28-homoCS and 24-epiBL on cell cycle regulators*

As previously reported [19], both 28-homoCS and 24-epiBL induced blocks in the G_1 phase of the MCF-7 and MDA-MB-468 cell cycles, with concomitant reductions in the percentages of cells in the S phase (Fig. 3A). Hence, we used Western blot analysis to examine whether the antiproliferative effects of 28-homoCS and 24-epiBL are mediated via interactions with cell cycle regulators (Fig. 3B and C). One of the key regulators is the retinoblastoma protein (pRb), which prevents entry into the S phase during the cell cycle, and is thought to be inactivated by CDK- and cyclin-mediated phosphorylation during the late G_1 phase [23]. Therefore, we examined the effects of 28-homoCS and 24-epiBL treatment on the expression of total and phosphorylated pRb in MCF-7 and MDA-MB-468 cells, but no expression occurred in the latter line, which is pRb-negative. Time-dependent reductions in the expression of both phosphorylated and dephosphorylated forms of pRb were observed in MCF-7 cells following application of either 28-homoCS or 24-epiBL, in accordance with the G_1 blocks. We then investigated molecular events upstream of pRb expression to examine mechanisms that could be involved in the observed changes in cell cycle profiles, initially focusing on the effects of 28-homoCS and 24-epiBL on two key regulators of cell cycle progression, the cyclin-dependent kinase inhibitors (CKIs) $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, which inhibit cyclin/CDK complexes [24]. There were increases in $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ expression in MCF-7 cells after 6 h of 28-homoCS treatment (Fig. 3B). However, $p21^{Waf1/Cip1}$ expression returned to control levels after 12 h, and $p27^{Kip1}$ expression decreased after 12 h. On the other hand, 24-epiBL treatment of MCF-7 resulted in reductions in $p27^{Kip1}$ expression after 6 and 12 h treatment, while $p21^{Waf1/Cip1}$ expression was unaffected. In the MDA-MB-468 cells, significant increases in $p21^{Waf1/Cip1}$ expression were observed after 12 and 24 h treatment with 24-epiBL, while $p27^{Kip1}$ expression was decreased at all time points. In contrast, treatment with 28-homoCS resulted in no significant changes in expression of the studied CKIs in the MDA-MB-468 cells (Fig. 3B).

Many studies have also shown that certain exogenous stimuli may result in either p53-dependent or p53-independent induction of $p21^{Waf1/Cip1}$, which in turn may trigger a series of events that ultimately result in cell cycle arrest and/or apoptosis. The MCF-7 cells showed a slight increase in p53 protein expression accompanied by decreased expression of its negative regulator MDM-2 after 6 h exposure to either BRs. In contrast, expression of the p53 and MDM-2 proteins remained unchanged in the MDA-MB-468 cells, irrespective of BR treatment (Fig. 3B). Hence, the studied BRs appear to exert their growth inhibitory and cell cycle dysregulatory effects regardless of p53 status. Data were supplemented by a detection of phosphorylated forms of p53 (SER15; SER392) but no alterations were found in either cell line after BR treatment (Fig. 3B).

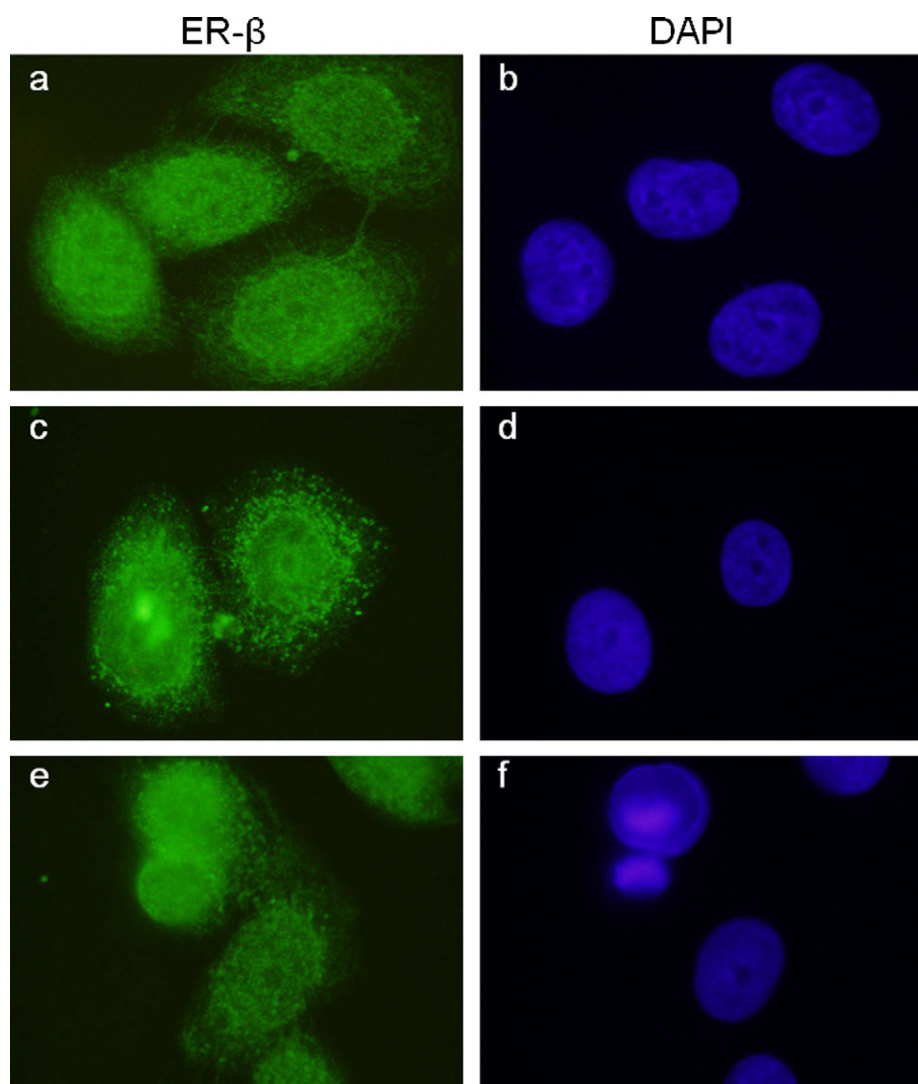

Fig. 2. Expression and localization of ER-β in MDA-MB-468 cells as determined by immunofluorescence (representative fluorescence results shown at 1000× magnification). Cells were fixed and immunolabeled with an antibody against ER-β (green) then counterstained with DAPI to visualize all nuclei (blue). (a and b) Control cells. Note the specific nuclei and cytoplasmic labeling of ER-β immunofluorescence. (c and d) Cells treated with 28-homoCS (IC_{50}) for 24 h. (e and f) Cells treated with 24-epiBL (IC_{50}) for 24 h. Note the similar nuclei and cytoplasmic labeling of ER-β immunofluorescence in BR-treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The expression of cyclins D_1 and E was decreased in both cell lines after 24-epiBL treatment for 6, 12 or 24 h. There was also reduction in CDK2 and CDK4 expression in both cell lines observed after 24 h of BR treatments (Fig. 3C). Pronounced decreases in CDK levels were found after the treatment with 24-epiBL (24 h). Expression levels of cyclin B_1 protein were only slightly decreased following BRs treatment in both cancer cell lines. These findings suggest that 28-homoCS and 24-epiBL may restore normal checkpoint control in the cell cycles of both studied human breast carcinoma cell lines.

3.3. Brassinosteroid induction of apoptotic pathways in breast cancer cells

The initial cell cycle analysis also showed that application of the selected BRs resulted in increases in the subG_1 fraction [19], which represents apoptotic cells, in MDA-MB-468 cultures, but not in MCF-7 cultures. Hence, we used TUNEL staining to test whether the studied BRs were really able to induce apoptosis in the breast cancer cell lines. The TUNEL staining showed that apoptosis occurred in both cell lines. Both BR treatments at IC_{50} concentrations resulted in increased numbers of TUNEL-positive cells at all tested timepoints (6, 12 and 24 h). There were substantial increases in the percentages of such cells in the 24-epiBL-treated MDA-MB-468 cultures (18.3%) after 24 h and in the 28-homoCS-treated MCF-7 cultures (13.7%) after 12 and 24 h (Table 1).

Table 1
Detection of DNA strand breaks in apoptotic nuclei of MCF-7 and MDA-MB-468 cells by TUNEL staining.

Control/BRs (IC50)	MCF-7	MDA-MB-468
Ctrl 6	0.0 ± 0.0	0.0 ± 0.0
28-homoCS 6	10.6 ± 1.5*	1.7 ± 0.6
24-epiBL 6	2.7 ± 1.2*	4.7 ± 0.6*
Ctrl 12	0.0 ± 0.0	0.0 ± 0.0
28-homoCS 12	13.7 ± 3.8*	2.3 ± 0.6
24-epiBL 12	8.3 ± 3.1*	16.7 ± 4.7*
Ctrl 24	0.3 ± 0.6	0.0 ± 0.0
28-homoCS 24	13.7 ± 2.6*	4.3 ± 0.6*
24-epiBL 24	11.7 ± 3.8*	18.3 ± 3.0*

Note: Cells were treated with 28-homoCS or 24-epiBL (IC_{50}) for 6/12/24 h. Data indicate mean (±SD) percentages of TUNEL-positive cells obtained from three independent experiments. Asterisks (*) denote values that are significantly different from the respective control values at $p < 0.05$.

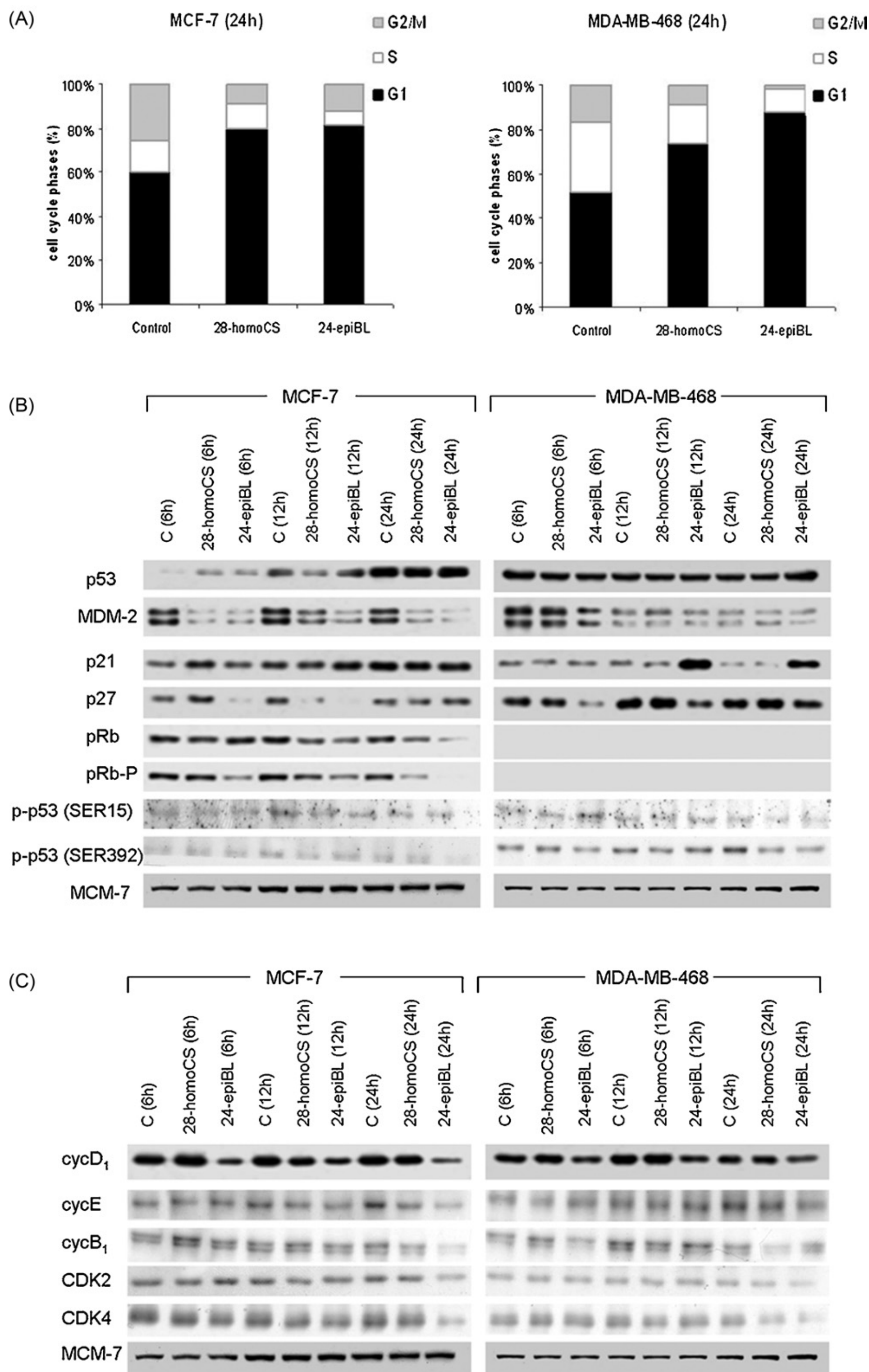

Fig. 3. Effect of BRs on cell cycle distribution. (A) MCF-7 and MDA-MB-468 cells were treated with 28-homoCS or 24-epiBL (IC_{50}) for 24 h and analysed by flow cytometry. The percentages of cells in each cell cycle phase (G_1, S and G_2/M) are indicated. Western blot comparison of levels in cell cycle-related proteins (B) p53, MDM-2, p21, p27, pRb, pRb-P, p-p53 (SER15), p-p53 (SER392) and (C) cyclin D_1, cyclin E, cyclin B_1, CDK2 and CDK4 in breast cancer cells treated with 28-homoCS or 24-epiBL for 6, 12, and 24 h, and untreated controls. MCM-7 was used as a protein loading marker, the experiment was repeated three times with similar results, and the presented data are from a single representative run.

Representative results obtained from the acridine orange–propidium iodide double staining are shown in (Fig. 4). Viable cells with intact DNA and nuclei show round and green nuclei. Early apoptotic cells will have fragmented DNA which gives several green colored nuclei. The DNA of late apoptotic and necrotic cells would be fragmented and stained orange and red. From the data it was clear that both types of BRs (IC_{50}) led to the decreased number of viable cells after 24 and 48 h incubation. Some cells exhibited typical characteristics of apoptotic cells like plasma membrane blebbing. However, the number of cells stained

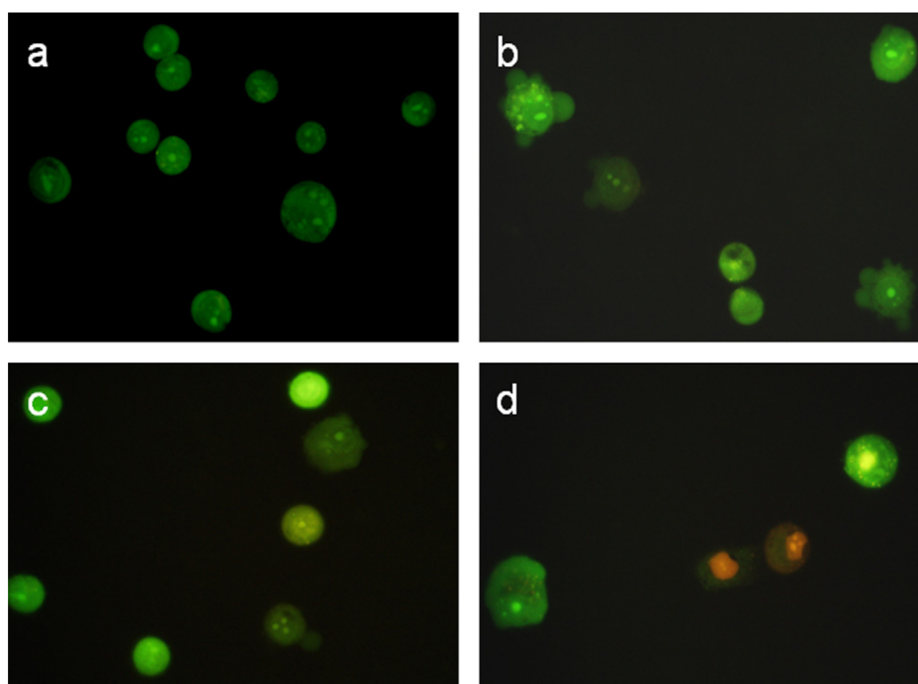

Fig. 4. Fluorescent micrograph of acridine orange and propidium iodide double-stained human breast cancer cells lines (MDA-MB-468). Cells were treated at IC_{50} of BRs for 24 and 48 h. (a) Untreated cells showed normal structure without prominent apoptosis and necrosis. (b) Blebbing and nuclear margination were noticed in 28-homoCS-treated cells after 24 h. (c) Early apoptosis features were seen after 24 h treatment by 24-epiBL representing intercalated acridine orange (bright green) amongst the fragmented DNA. (d) Late apoptosis was seen in 28-homoCS incubated cells for 48 h, whereby positive staining with orange color represents the hallmark of late apoptosis (magnification 40×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

red did not increase. This indicates that most of the cells were not undergoing necrosis and cell death occurred primarily through apoptosis.

Following the TUNEL assays, we examined the molecular mechanism of breast cancer cell apoptosis-induced by the studied BRs using Western blotting to analyse the expression of apoptosis-related proteins in cells collected after 6, 12, and 24 h of treatment with IC_{50} concentrations of either 28-homoCS or 24-epiBL. The BR-mediated changes in the expression of apoptosis-related protein are presented in Fig. 5. Both BR treatments caused expression of the anti-apoptotic Bcl-2 and Bcl-X_L proteins in MCF-7 cells to decrease after 12 h (Fig. 5). Surprisingly, however, increased levels of Bcl-2 were observed in this cell line after 6 and 24 h treatment with 28-homoCS. Furthermore, both BR treatments resulted in slight reductions in expression of the pro-apoptotic uncleaved Bid protein in the MCF-7 cells after 6 and 12 h treatment with both type of BRs and after 24 h treatment with 24-epiBL only (Fig. 5). Such reductions in the level of Bid may indicate cleavage of the protein, although no evidence for this was found. In addition, no significant changes in expression of the pro-apoptotic protein Bax were seen in the BR-treated MCF-7 cells. In the MDA-MB-468 cells, Bcl-2 expression was slightly decreased after the 28-homoCS and 24-epiBL treatments (6, 12 and 24 h), while Bcl-X_L expression was not affected by exposure to either BR. However, MDA-MB-468 cells showed increased levels of Bax expression and reductions in uncleaved Bid expression only when subjected to 6 h of treatment (Fig. 5). To test the possibility that BRs could induce the recruitment of the adaptor protein FADD to the Fas receptor, additional experiments were performed. But Western blot analysis revealed no changes in the Fas and Fas-L expression after the treatment in either type of cell lines (data not shown).

The blots showed degradation of caspase-3 into cleaved fragments (part of apoptotic cascade) after 24 h of each BR treatment in MDA-MB-468 cells, but no caspase-3 expression occurred in MCF-7 cells, due to its mutation (Fig. 5). Furthermore, a slight decrease in poly-(ADP-ribose) polymerase (PARP) expression was observed in the MDA-MB-468 cells after the BR treatments. Such reduction in the PARP level may indicate cleavage of the protein, although no evidence for this alteration was found over a 24 h period (Fig. 5). In MCF-7 cells, the expression of PARP was at the limit of detection.

4. Discussion

Several effective, relatively safe drugs for treating breast cancer have been developed that target regulatory points in the endocrine system called selective ER modulators (SERMs), e.g. tamoxifen and raloxifen [25]. In addition, we previously demonstrated that some BRs can effectively inhibit human estrogen sensitive/insensitive breast cancer cell growth without affecting the normal non-tumor cell growth of BJ fibroblasts [19]. In a previous study, cells were exposed to six serial 4-fold dilutions of the tested drug for 72 h. The proportions of surviving cells were then estimated and IC_{50} values were calculated. No BR-mediated loss of viability was observed in the BJ fibroblasts ($IC_{50} > 50\,\mu M$), suggesting that BRs induce different responses in cancer and normal cells [19]. We found that low micromolar concentrations of 28-homoCS significantly inhibited proliferation of MCF-7 cells to a lesser extent that of MDA-MB-468 cells. The natural BRs 28-homoCS and 24-epiBL were also found to induce significant arrest of the cell cycle at the G_1 phase in both cell lines. Accordingly, the growth-inhibiting effects of antiestrogens in ER-positive breast cancer cells and normal epithelial cells result from the arrest of the cell cycle in the G_0/G_1 phase [26,27]. Hence, in the presented study we used estrogen-sensitive (MCF-7) and estrogen-insensitive (MDA-MB-468) breast cancer cell lines to obtain further information regarding the mechanisms behind the antiproliferative and pro-apoptotic activity of the natural BRs 28-homoCS and 24-epiBL. These cell lines were chosen as they are representative of a wide range of breast cancer phenotypes. The MCF-7 cell line is derived from pleural metastasis of breast car-

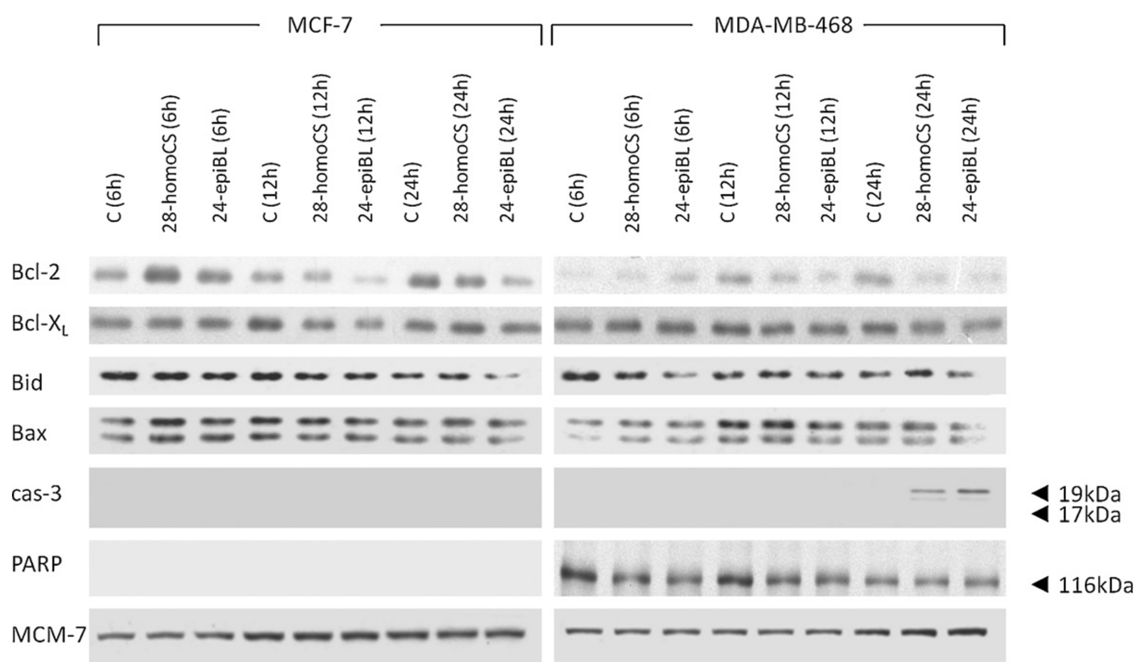

Fig. 5. Western blot comparison of apoptosis-related proteins Bcl-2, Bcl-X_L, Bax, Bid, caspase-3 (cleaved form) and PARP in MCF-7 and MDA-MB-468 breast cancer cells treated with 28-homoCS or 24-epiBL for 6, 12, and 24 h, and untreated controls. MCM-7 was used as a protein loading marker, the experiment was repeated three times with similar results, and the presented data are from a single representative run.

cinoma [53]. This cell line is generally a well-established model for investigating steroid hormone receptors and for testing antiestrogens. It is known that antiestrogen treatment is accompanied by elevated numbers of cells in the G_0/G_1 phase of the cell cycle together with a decreased number in the S phase [54]. MCF-7 cells contain the wild-type tumor suppressor gene p53 and are pRb positive [55]. This cell line has been used as a model for well-differentiated epithelial cells that react to the changes in the nearest neighbour by the expression of specific morphological and functional features. In contrast, the MDA-MB-468 breast carcinoma cell line is hormone-insensitive, Rb-negative and has a mutant p53 [56]. ERs are among the most important targets in breast cancer therapy, and both ER-α and ER-β are known to be expressed in MCF-7 cells, whereas MDA-MB-468 cells lack ER-α expression due to epigenetic silencing [28]. Immunofluorescence analysis detected ER-β expression in the cytoplasm of control MCF-7 cells, whereas striking relocalization of ER-β from the cytoplasm to the nucleus was observed after 28-homoCS treatment in these cells. Furthermore, ER-β was found predominantly at the periphery of the nuclei in the 24-epiBL-treated cells, while ER-α labeling was entirely restricted to the nuclei in MCF-7 control cells, and cytoplasmic speckles of ER-α immunofluorescence appeared in corresponding cells treated with BRs. These changes were also accompanied by down-regulation of the ERs after BR treatment. In contrast, MDA-MB-468 cells showed no changes in ER-β expression. Hence, these results suggest that BRs have similar effects to those of ER antagonists. Tamoxifen, a partial ER antagonist, has also been shown to cause ER nuclear accumulation, while fulvestrant is a pure ER antagonist that down-regulates and prevents the nuclear transport of ERs [29]. The molecular mechanism whereby BRs exert their antiproliferative effects is still not fully understood, but they presumably interact with steroid hormone receptors (ms in preparation).

Steroid hormones, such as estrogen, play important roles in the growth and development of their target tissues, including the mammary gland, where they interact with other hormones, growth factors, and cytokines in the regulation of cell proliferation and differentiation [30]. Therefore, we studied the BR-mediated modulation of the G_1 phase cell cycle regulatory events in human breast cancer cells, focusing on changes in the expression patterns of proteins involved in the cyclin/CDK/CKI regulatory system (Fig. 3B and C). Initially, we evaluated effects of the BR treatments on levels of the major cyclins involved in the G_1 cell cycle phase (cyclin D_1 and E), and found that they resulted in reductions in the expression of these proteins in both of the studied cell lines. Similarly, we found reductions following BR treatment in CDK2 and CDK4 in MCF-7 and MDA-MB-468 cells, respectively. The pronounced decreases of CDK levels were found after the 24 h-treatment with 24-epiBL. Western blot analysis also showed up-regulation of the CKIs $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ following BR treatment at the specific time points. There were increases in $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ expression in MCF-7 cells after 6 h of 28-homoCS treatment. In the case of 24-epiBL treatment of MCF-7, reductions in $p27^{Kip1}$ expression after 6 and 12 h treatment were found, while $p21^{Waf1/Cip1}$ expression was unaffected. In the MDA-MB-468 cells, significant increases in $p21^{Waf1/Cip1}$ expression were observed after 12 and 24 h treatment with 24-epiBL, while $p27^{Kip1}$ expression was decreased at all time points. Indeed, the BRs appeared to target cyclin D_1, cyclin E, $p21^{Waf1/Cip1}$, and $p27^{Kip1}$ in a similar manner to the antiestrogens, such as tamoxifen [31], and these results are entirely consistent with a BR-mediated inhibition of tumor growth through cell cycle arrest at the G_1 phase and also the induction of apoptosis. Many studies have shown that these CKIs regulate the progression of cells in the G_0/G_1 cell cycle phase, and their induction has been observed to block the G_1/S transition, resulting in G_1 phase arrest [32]. Moreover, estrogen/ER complexes bind directly to a cAMP-response element and a more distal Sp1 site on the cyclin D_1 promoter, which leads to increases in cyclin D_1 mRNA levels [51,52]. In addition, ER-β competes with ER-α in the induction of cyclin D_1 transcription [7]. Estrogen also rapidly activates cyclin E-CDK2 complexes and, by relieving the inhibition mediated by the CDK inhibitor $p21^{Waf1/Cip1}$, accelerates the transition from the G_1 to S phase [33]. Another key element of the G_1 phase regulatory apparatus is the CKI $p27^{Kip1}$, which inhibits the activity of CDK2 in the G_0 and early G_1 phases, and acts as an assembly factor for cyclin D/CDK4/CDK6

complexes in the early G_1 phase [34]. Hence, the functional expression of $p27^{Kip1}$ is essential for normal proliferative responses in the mammary epithelium [35].

The CKI $p21^{Waf1/Cip1}$ also binds to CDK/cyclin complexes, inhibiting the phosphorylation of pRb and thereby inhibiting the G_1 to S phase transition. Therefore, the reductions in levels of phosphorylated pRb observed in this study were presumably due to BR-triggered expression of the CKIs, which in turn decreased the activities of CDK/cyclin complexes. A precedent for this mechanism is provided by the natural monoterpenes that cause G_1 arrest and increase in $p21^{Waf1/Cip1}$ expression [36]. Another example of an agent with similar properties is aragusterol A, a potent steroid that has been isolated from marine sponges and observed to cause G_1 arrest through down-regulation of Rb phosphorylation [37]. Furthermore, the cell division inhibitory properties of antiestrogens are also usually associated with CDK inhibition and reductions in pRb phospohorylation [38]. However, these compounds represent several classes of substances, and have markedly differing effects on ER-α-positive breast cancer cells, for instance tamoxifen and raloxifen block cells in the G_1 phase, whereas fulvestrant (ICI 182 780) renders them inactive [39]. These results suggest that cytotoxic BRs are capable of restoring proper checkpoint control in both of the studied human breast carcinoma cell lines.

The tumor suppressor p53 protein is a negative regulator of the cell division cycle and is inactive in 30% of all breast cancer cells. Normally, p53 levels rise in response to DNA damage, stresses or activation by oncogenes, and subsequently induce apoptosis or prevent cells from entering the S phase of the cell cycle [34]. MCF-7 cells contain wild-type p53, whereas MDA-MB-468 cells produce a mutated form that is unable to bind to DNA. Since ER-α binds directly to p53 [40], it has been suggested that p53 is involved in the antiestrogen response in breast cancer cells. Furthermore, the product of the MDM-2 oncogene, which is overexpressed in various cancers and encodes an E3 ubiquitin ligase, regulates the stability of ER-α by forming a ternary complex with p53 and ER-α [41]. In turn, p53 and MDM-2 regulate the estrogen-dependent down-regulation of ER-α and, possibly, susceptibility to antiestrogens [41]. Previous reports have suggested that one of the mechanisms underlying the pro-apoptotic effect of antiestrogens could be the relief from ER-α mediated p53 inhibition [40]. It is also possible that this mode of action may contribute to the response of breast cancer to BR treatments.

Our data provide evidence for BR-mediated induction of $p21^{Waf1/Cip1}$, which appears to be p53-dependent in the MCF-7 cells. In contrast, the protein levels of p53 and MDM-2 were not affected by BR treatments in the MDA-MB-468 cells. It has been suggested that BRs induce breast cancer cell apoptosis, at least in part, by relieving the inhibitory activity of ER-α on p53 activity. However, our results indicate that the BRs exert growth inhibitory and cell cycle dysregulatory effects regardless of cell p53 status. Hence, confirmation of a definite association between cancer cell p53 status and the biological effects of BRs will require further study.

Cell cycle analysis also demonstrated that BR treatment of MDA-MB-468 cells resulted in an increase of the subG_1 fraction, which represents apoptotic bodies [19]. However, no such increase in the subG_1 fraction was observed in MCF-7 cells, prompting additional tests to examine if the BRs really do induce apoptosis in the cancer cell lines. TUNEL and the acridine orange–propidium iodide double staining demonstrated that apoptosis occurred in both cell lines following BR treatment.

Tamoxifen has been shown to increase both caspase-3 activity in ER-insensitive breast cancer cells [42] and the expression of caspase-6, -7, and -9 in MCF-7 cells [43]. In a previous study, we have also shown that both of the studied BRs can induce moderate apoptosis in MCF-7 cells, similar to the effects of the antiestrogens described by Gompel et al. [44]. Hence, these results confirm that 28-homoCS and 24-epiBL can promote apoptosis by modulating the expression and/or activity of Bcl-2 proteins and caspase-3 in breast carcinoma cell lines.

The possible involvement of apoptosis-related proteins in the molecular mechanisms of BR-induced apoptosis in breast cancer cells was also examined. BR treatment resulted in reductions in levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic uncleaved protein Bid. Such reductions in the level of Bid may indicate cleavage of the protein, although no evidence for this was found. The expression of the pro-apoptotic protein Bax was generally unchanged, although slight increases in the expression of this protein were detected in MDA-MB-468 cells treated with either BR for 6 h. Other studies have revealed that Bcl-2 forms a heterodimer with Bax, thereby potentially neutralizing its pro-apoptotic effects [45], and Bcl-2 is known to prevent the release of caspases [46]. However, MDA-MB-468 cells treated with the studied BRs showed degradation of caspase-3 into cleaved fragments. MCF-7 cells are caspase-3 deficient [47].

Results of previous studies of the effects of antiestrogens on members of the Bcl-2 family have been conflicting. For instance, estradiol has been shown to up-regulate Bcl-2 in ER-sensitive cells [44], while antiestrogens counteract this effect [48]. In addition, androgens have been shown to decrease pro-apoptotic Bak protein levels in MCF-7 cells, whereas aromatase inhibitors or antiestrogens increase Bak levels [43]. However, other studies have reported no (anti)estrogenic effects on either Bax or Bak protein levels [44]. Therefore, there is no clear consensus regarding the roles of these molecules in the induction of apoptosis. However, the anti-apoptotic Bcl-X_L protein is produced in small amounts in breast cancer cells and is unaffected by antiestrogens, whereas tamoxifen and raloxifen have been shown to up-regulate pro-apoptotic proteins [49,50]. Thus, the activity of pro-apoptotic compounds in breast cancer cells is probably triggered through multiple pathways, which may be either dependent or independent of ER signaling.

The presented study demonstrates several effects of natural cytotoxic BRs, and provides indications of the molecular mechanisms that may be involved. The studied BRs were capable of causing cell cycle arrest and some apoptotic changes in the investigated breast cancer cell lines. A major finding was that BRs can cause cell cycle blockade and apoptosis of hormone-sensitive and -insensitive human breast cancer cells. This finding is important, since breast cancer progresses from an estrogen-responsive to a late estrogen-insensitive (metastatic) form, and at the time of clinical diagnosis, most breast cancers include a mixture of estrogen-sensitive and -insensitive cells. Therefore, eliminating both carcinoma cell types may be crucial for effective control of breast cancer, and these results suggest that BRs and their analogues could play valuable therapeutic roles. However, additional studies are required to explain the differences between responses of cancer cells and normal cells to 28-homoCS and 24-epiBL applications. Hence, future studies will need to improve our understanding of the genetic and proteomic changes, and identify the regulatory pathways involved in BR-induced apoptosis in disease states.

Conflict of interest

The authors of this manuscript do not have any conflict of interest related to publishing of this study.

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