PALACKÝ UNIVERSITY IN OLOMOUC

**FACULTY OF SCIENCE**

LABORATORY OF GROWTH REGULATORS

&

DEPARTMENT OF BOTANY

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# A STUDY OF MOLECULAR AND CELLULAR ACTIVITIES OF BRASSINOSTEROIDS AND THEIR DERIVATIVES

Ph.D. Thesis

1501 V BIOLOGY - BOTANY

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**Consultant: Doc. RNDr. Vladimír Kryštof, Ph.D.**

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*Author*

## Bibliographic identification

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| Abstract: | Molecular and cellular effects of two groups of antiproliferative agents, natural brassinosteroids and their synthetic derivatives, were examined in different adherent human cancer cell lines (MCF-7, MDA-MB-468) and in primary endothelial cells (HUVEC, HMEC-1). Their effects were studied by classical biochemical methods (SDS-polyacrylamide gel electrophoresis and immunoblotting, indirect immunofluorescence analysis, flow cytometry, caspase activation assay) and by angiogenesis assays *in vitro* (proliferation, migration, tube formation). Brassinosteroids and their derivatives can inhibit the growth of several human cancer cell lines and inhibit angiogenesis-like behaviour of primary endothelial cells *in vitro*. |
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## Contents

## Abbreviations

| | |
|---|---|
| BAK1 | BRI1-associated receptor kinase 1 |
| BKI1 | BRI1 kinase inhibitor |
| BIN2 | brassinosteroid-insensitive 2 |
| BL | brassinolide |
| BR | brassinosteroid |
| BRI1 | brassinosteroid-insensitive 1 |
| BSA | bovine-serum albumin |
| CDK | cyclin-dependent kinase |
| CS | castasterone |
| 24-epiBL | 24-epibrassinolide |
| 24-epiCS | 24-epicastasterone |
| HMEC-1 | human microvascular endothelial cells |
| 28-homoBL | 28-homobrassinolide |
| 28-homoCS | 28-homocastasterone |
| HRP | horse-radish peroxidase |
| HUVEC | human umbilical vein endothelial cells |
| LRR-RK | leucin-rich repeat receptor kinase |
| PBS | phosphate buffer saline |
| RLK | receptor-like kinase |
| HeLa | human cervical carcinoma cell line |
| MCF-7 | human breast carcinoma cell line |
| Mcl-1 | myeloid cell leukemia sequence 1 |
| PARP-1 | poly(ADPribose)polymerase-1 |
| pRb | retinoblastoma protein |
| p53 | protein p53 |

# 1. Introduction

## 1.1. Brassinosteroids

### *1.1.1. Plant hormones*

The phytohormones known as brassinosteroids (BRs) are low-molecular weight steroid compounds occurring in plants (Khripach et al., 1999). The first brassinosteroid (22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one (brassinolide) was isolated in 1979 from pollen of *Brassica napus* L.(Grove, 1979). Up to date, more than 70 of these phytohormones have been discovered (Sakurai and Fujioka, 1997). BRs have been detected and identified in many different plant species. They are ubiquitously distributed through the plant kingdom from lower to higher plants. BRs occur in most organs of the higher plant, including pollen, anthers, leaves, stems, roots, flowers, seeds and grain (Bajguz and Tretyn, 2003). The young growing tissue has higher levels of BRs than mature tissues. BRs occur endogenously at very low levels. The richest sources of BRs are pollen and immature seeds with ranges of 1–100 $ng.g^{-1}$ fresh weight, while shoots and leaves usually have lower amounts of 0.01–0.1 $ng.g^{-1}$ fresh weight. (Bajguz and Tretyn, 2003). The presence of BRs in roots (0.3 $ng.g^{-1}$ of castasterone in fresh weight) has been confirmed (Kim et al., 2000). Castasterone has been found for the first time in crown galls, for example the galls of *Castanea crenata* Siebold&Zucc. The most widely distributed BRs are castasterone, brassinolide and typhasterol. In plants there is a mixture of brassinosteroids, the composition of which depends on the species (Bajguz and Tretyn, 2003).

BRs are derived from the cholestane skeleton with various hydroxyl substitutions and attached functional groups (Haubrick and Assmann, 2006). Their structural variations come from the type and position of functionality in the A/B rings and the side chain (Fig. 1). Only the 3-oxygenated (22R,23R)-5α-cholestane-22,23-diols of plant origin, bearing alkyl or oxy substituents, conjugated or not to sugars or fatty acids, and exhibiting the characteristic brassinolide activity, are considered as natural brassinosteroids. The 2α,3α-dihydroxylation is necessary for biological activity (Zullo et al., 2003).

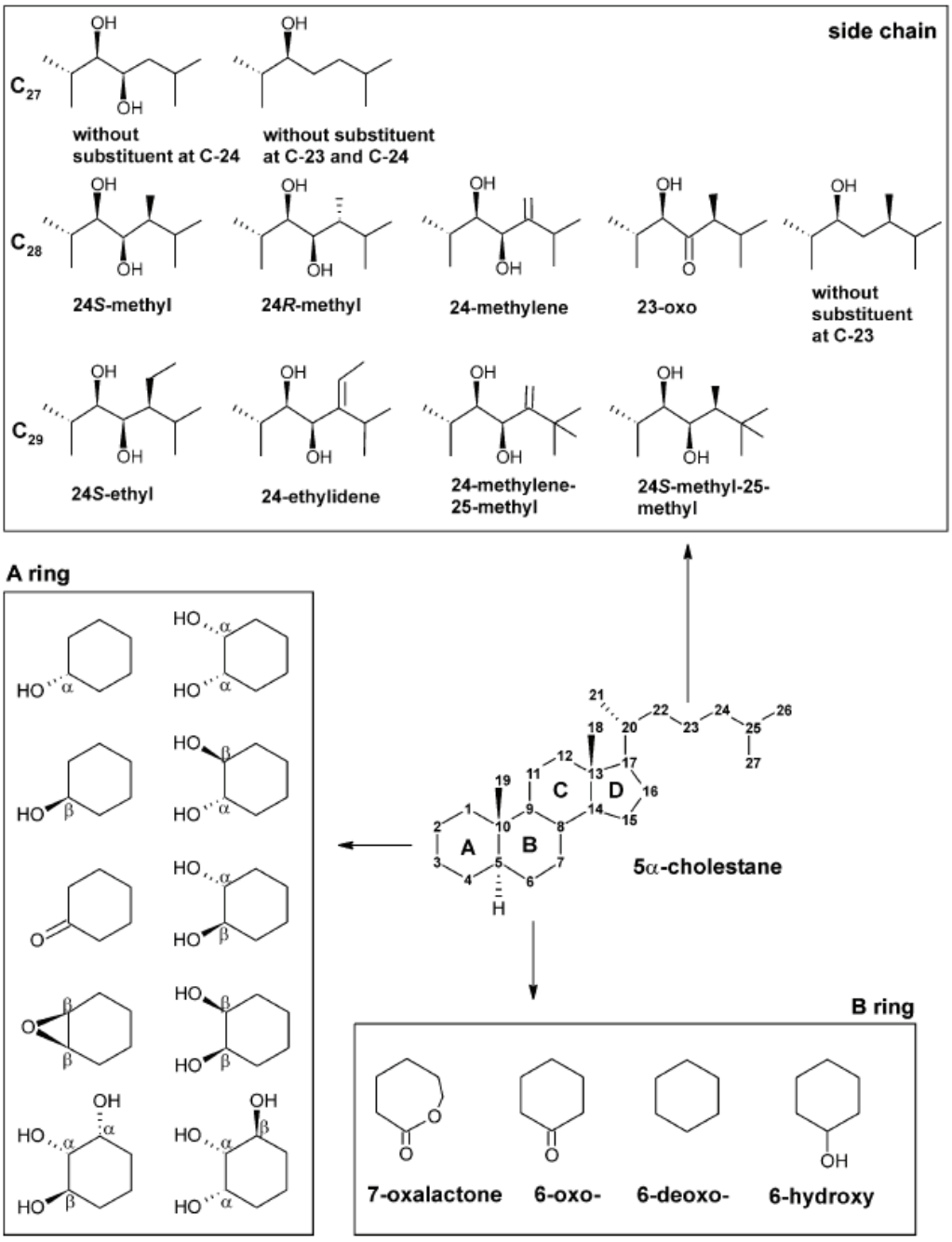
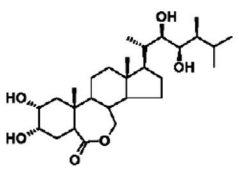



**Fig.1** Different substituents in the A and B rings and on the side chain of naturally occurring brassinosteroids (adopted from Bajguz and Tretyn, 2003*)*.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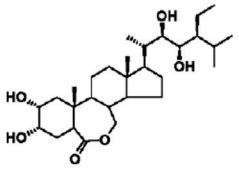
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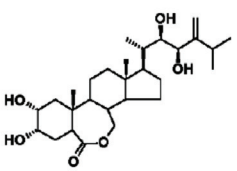
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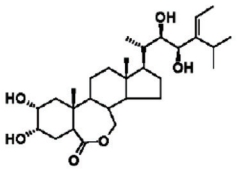
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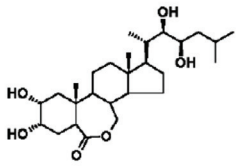
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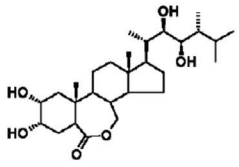
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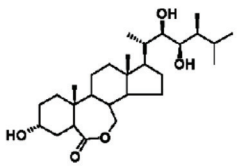
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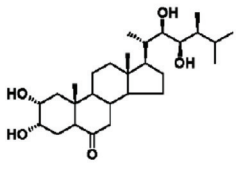
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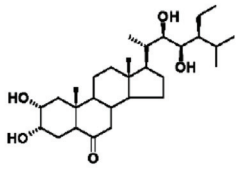
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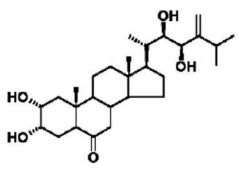
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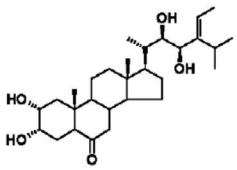
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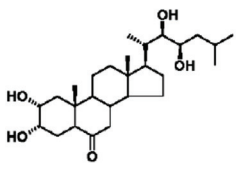
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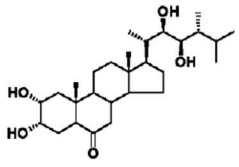
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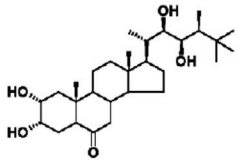
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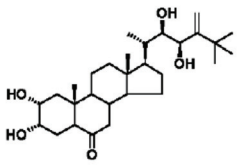
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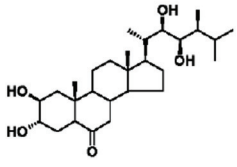
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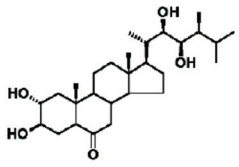
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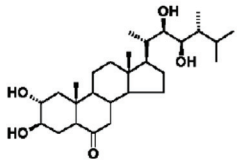
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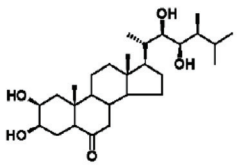
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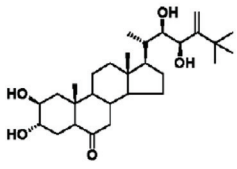
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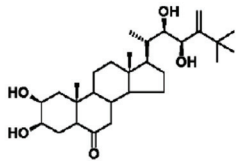
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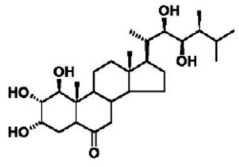
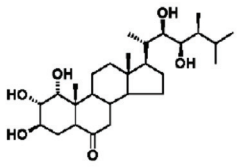

1$\beta$-Hydroxycastasterone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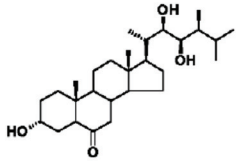
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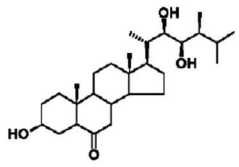
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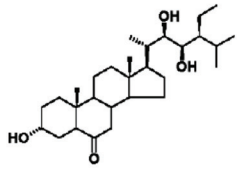
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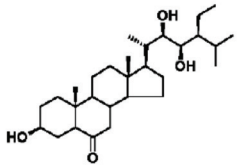
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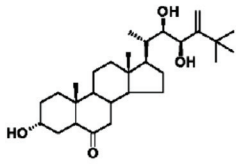
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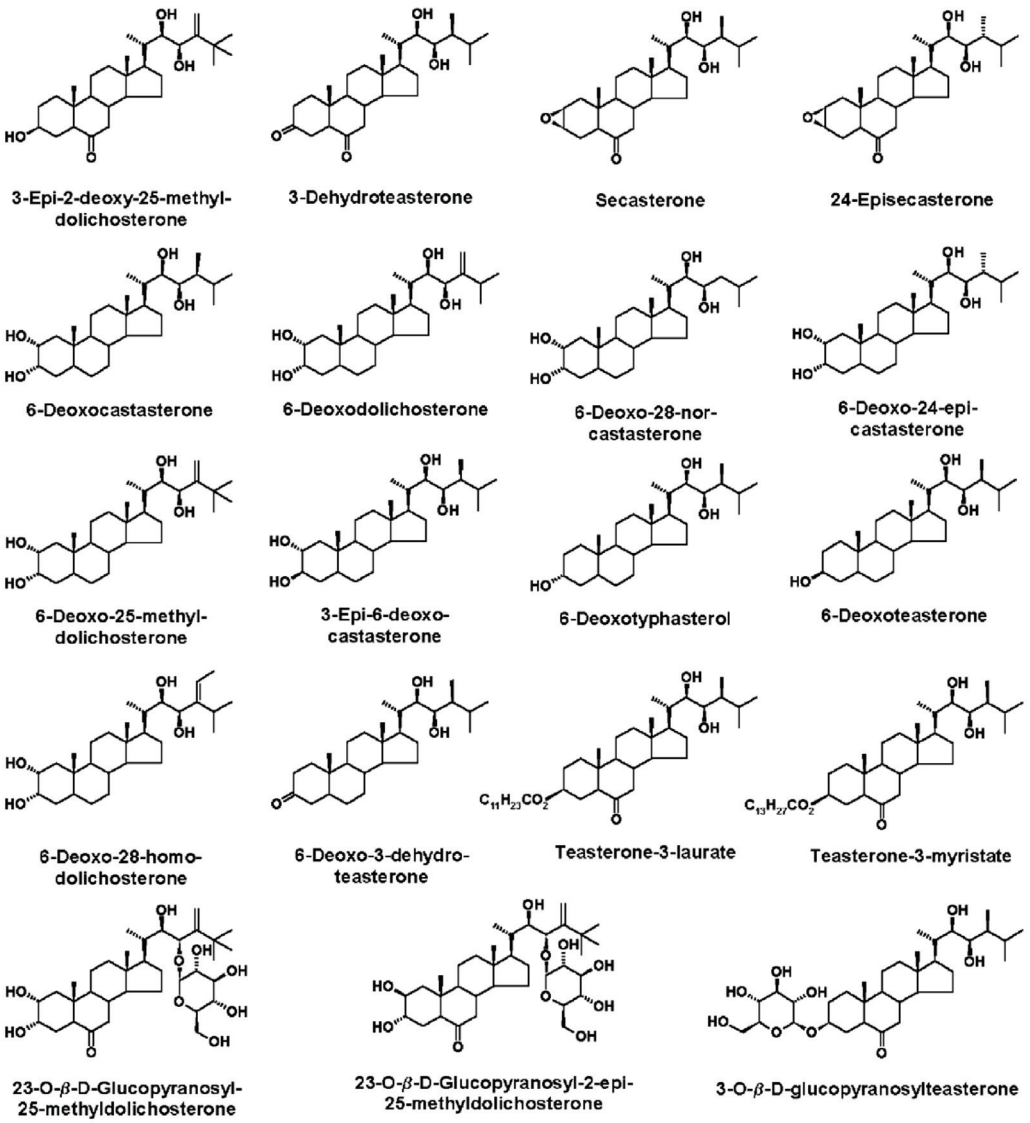


**Fig. 2** Structures of natural brassinosteroids (from Zullo, 2003).

Brassinosteroids (Fig. 2) can be divided with respect to their A/B ring functionalities into following groups (Schmidt et al., 2000):

- 7-oxalactone-B-ring, 7-membered and vicinal 2,3-hydroxyl groups;
- 6-oxo 6-membered B-ring with two hydroxyl groups at position C-2 and C-3;
- 6-oxo ring with 2,3-oriented epoxide group;
- 6-oxo ring with an additional hydroxyl group at position C-1;
- without oxygen functions in the B-ring;
- with hydroxyl group at position C-6.

BRs are required for normal plant growth, reproduction and development. Plants that are deficient either in the biosynthesis or perception of these hormones are typically dark green dwarfs with epinastic leaves, have reduced fertility or are sterile, and exhibit delayed development. The wild-type is partially or fully restored with addition of BRs (Bishop and Koncz, 2002).

BRs play an important role in hormone signalling in plants and in their physiological responses to environmental stimuli, including seed germination, growth, cell division and differentiation, root and stem elongation, bending, reproductive and vascular development, membrane polarization and proton pumping, source/sink relationships, disease resistance, modulation of stress and senescence (Clouse and Sasse, 1998; Haubrick and Assmann, 2006; Nemhauser and Chory, 2004). They stimulate the division and elongation of cells (Bajguz and Tretyn, 2003; Schmidt et al., 1997) and are also involved in regulating plant-specific processes including photomorphogenesis and cell expansion in the presence of a potentially growth-limiting cell wall (Clouse, 2002). A pronounced elongation of hypocotyls, epicotyls, and peduncles of dicotyledons, as well as coleoptiles and mesocotyls of monocotyledons, is caused by the application of brassinosteroids at nanomolar to micromolar levels (Clouse, 1996). The elongation of the cell wall is influenced by brassinosteroids. Plasticity of the cell wall is increased in the presence of brassinosteroid when proton extrusion by H+-ATPases acidifies the apoplast and activates the cell wall-loosening enzymes. Turgor pressure then drives cell expansion as new cell wall and membrane materials are synthesized and secreted (Haubrick and Assmann, 2006). Organization of microtubules and cell elongation rescue is restored by the application of homobrassinolide in dwarf mutants of *Arabidopsis thaliana* L. without the activation of tubulin gene expression (Catterou et al., 2001).

Brassinosteroids enhance resistance to abiotic and biotic stresses in plants (Müssig et al., 2006). BRs can stimulate the immune response in plants when applied at the appropriate concentration and the correct stage of plant development. Stress response regulated by BRs is a result of a complex sequence of biochemical reactions such as induction of protein synthesis, activation or suppression of key enzymatic reactions, and the production of various chemical defence compounds (Bajguz and Hayat, 2009). BRs treatment stimulated levels of proteins, abscisic acid, free proline and agglutinin when applied during exposure to osmotic stress (Vardhini and Rao, 2003). Enhanced levels of heat-shock proteins occurred in thermally-stressed plants.

BRs increased the level of glutathione or and rate of photosynthesis in response to oxidative and/or heavy metal stress (Bajguz and Hayat, 2009). Epibrassinolide treatment caused thermotolerance with increased heat shock protein synthesis and accumulation, as well as increased expression of translational machinery in *B. napus* L. seedlings (Dhaubhadel et al., 2002). These phytohormones can also affect the insect and fungal development in plants (Clouse and Sasse, 1998). BRs treatment had a fungicidal effect which is dependent on concentrations as well as on the method and timing of delivery (Khripach, 1999). In tobacco and rice BR application induced disease resistance against bacterial and fungal pathogens (Haubrick and Assmann, 2006). BRs influence the plant architecture, flowering time, stress tolerance and seed yield (increase by 20-60%) (Divi and Krishna, 2009).

Brassinosteroids interact with other plant hormones (Haubrick and Assmann, 2006). Additive effects of BRs on elongation were seen with gibberellins, and enhancement of lateral enlargement induced by cytokinins, and inhibitory effects of cytokinins, abscisic acid, and ethylene on brassinosteroid-induced elongation have been described for stem tissue (Clouse and Sasse, 1998). An interdependent and possibly synergistic effect between BRs and auxins on stem segment elongation has been reported (Yopp et al., 1981; Katsumi, 1985; Hardtke, 2007). In mung bean epicotyl segments the increased ethylene production was detected in response to brassinolide (Arteca et al., 1983). BRs have an additive effect with gibberellins (Mayumi and Shiboaka, 1995). Abscisic acid and brassinolide both enhance drought tolerance in sorghum (Xu et al., 1994). Co-application of brassinolide and gibberellins, or brassinolide and auxin, resulted in a synergistic increase in hypocotyl elongation in intact *Arabidopsis* seedlings (Tanaka et al., 2003).

### *1.1.2. Biosynthesis of brassinosteroids in plants*

Biosynthesis of BRs occurs as a branch of the terpenoid pathway. Biosynthesis starts with the polymerization of two farnesyl diphosphates to form the $C_{30}$ triterpene squalene. Squalene is then modified by series of ring closures to form the pentacyclic triterpenoid (sterol) precursor cycloartenol. All steroids in plants are derived from cycloartenol by a series of oxidation reactions and other modifications (Fujioka and Yokota, 2003). The basic 5-carbon units of terpenes (isopentenyl pyrophosphate + dimethylallyl pyrophosphate) can be synthesised by two different pathways, namely the

mevalonate and methylerythritol phosphate (MEP) pathways (Hunter, 2007). Biosynthesis of the brassinosteroid precursor campesterol via mevalonate pathway: Mevalonate → Isopentenyl diphosphate → Dimethylallyl diphosphate → Geranyl diphosphate → Farnesyl diphosphate → Squalene → Cycloartenol → 24-Methylenecholesterol → Campesterol

Brassinolide, the most active BR, may be synthesized from campesterol by several pathways (Fujioka and Yokota, 2003). The presence of two parallel pathways from campesterol to castasterone, termed as early and late C-6 oxidation pathways (Fig. 3), was revealed in *Arabidopsis thaliana* L. (Noguchi et al., 2000). Other pathways, termed as the early C-22 oxidation pathway and C-23 oxidation pathway, with shortcut from campesterol to 6-deoxytyphasterol, have also been described (Fujioka et al., 2002; Onishi et al., 2006). In the final step castasterone is converted to brassinolide by the lactonization of the B ring. The most active synthesis of BRs occurs in young and developing organs, consistent with their growth-promoting function in plants (Divi and Krishna, 2009).

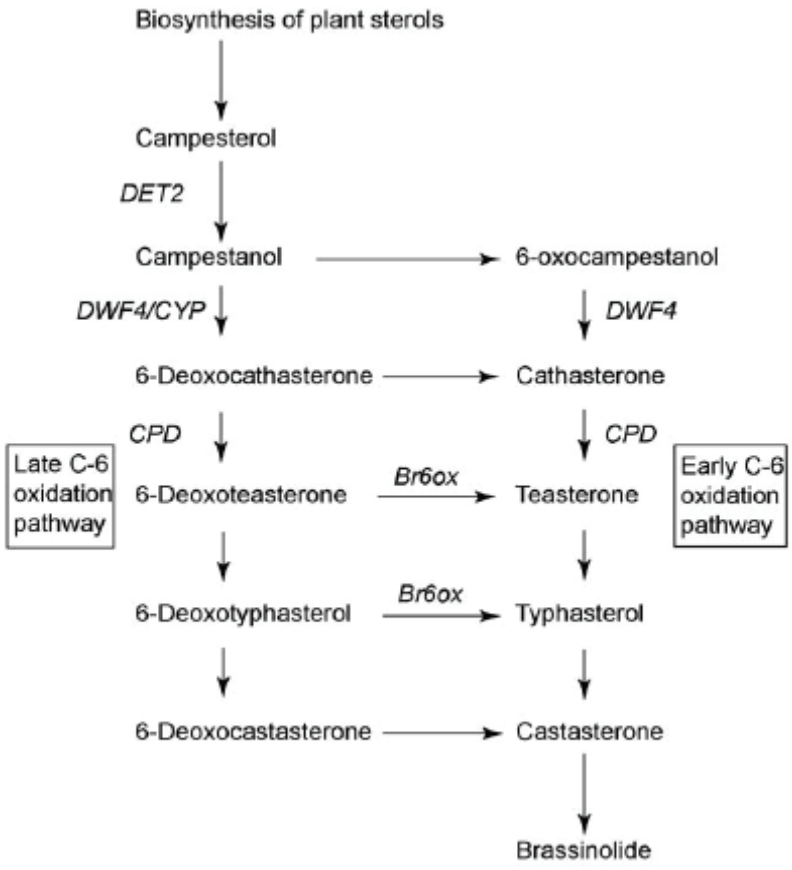


**Fig. 3** Brassinosteroid biosynthetic pathway (from Divi and Krishna, 2009).

### *1.1.3. Metabolism of brassinosteroids*

Up to date 42 brassinosteroid metabolites and their conjugates have been found. The metabolic pathways of BRs vary according to plant species, developmental stages and BR structure. Metabolism of BRs includes structural changes: to the steroidal skeleton (4 metabolic processes; 21 compounds) and to the side-chain (6 metabolic processes; 21 compounds). The most common modifications to the steroidal skeleton are esterification at C-3 (8 compounds) and glycosylation at C-3 (5 compounds). The most common modifications to the side-chain include hydroxylation at C-25 (4 compounds) and side-chain cleavage at C-20/C-22 (3 compounds) (Bajguz, 2007). The endogenous BR levels are influenced by further BR metabolism (Fujioka and Yokota, 2003). In plants there are several types of metabolic process involving BRs: dehydrogenation, demethylation, epimerization, esterification, hydroxylation, side-chain cleavage, sulfonation and glycosylation. The final step is the oxidation of the 6-oxo group of castasterone to the lactone group of brassinolide. Conjugated compounds seem to serve as a pool of inactive BRs that can be converted to active forms by de-conjugation reactions (Bajguz, 2007).

### *1.1.4. Signalling by brassinosteroids in plants*

Relatively little information is available concerning the mechanism of action of BRs in plants at the molecular level. Signalling by BRs, and the resulting genomic responses, are initiated by the binding of a BR molecule to a receptor kinase, brassinosteroid-insensitive 1 (BRI1), localized in the plasma-membrane. BRs bind to the extracellular domain of the BRI1 which is leucine-rich repeat receptor kinase (LRR-RK). This leads to phosphorylation of the BRI1 intracellular serine-threonine kinase domain, causing disassociation from the membrane-bound BRI1 kinase inhibitor 1 (BKI1) and oligomerization with a second receptor kinase, BRI1-associated receptor kinase 1 (BAK1). The active BRI1/BAK1 receptor kinase pair then propagates the signal downstream by inactivating a soluble kinase, brassinosteroid-insensitive 2 (BIN2), which is a negative regulator of BR signaling (Belkhadir and Chory, 2006; Chinchilla et al., 2009; Clouse, 2008; Müssig and Altmann, 2001).

24-Epibrassinolide (24-epiBL) has also been shown to up-regulate transcription of an important positive cell cycle regulator, cyclin D3, gene through which cytokinin

activates cell division in *Arabidopsis thaliana* L. (Hu et al., 2000). In plants, no gene has been identified that encodes a protein for an intracellular receptor of BRs. Only a chaperone heterocomplex, similar to an intracellular steroid receptor in animals, has been identified in wheat germ lysate (Stancato et al., 1996). However, it is likely that in plants an intracellular signalling pathway may exist that regulates gene transcription in a similar fashion to the steroid hormones in animals (Hu et al., 2000).

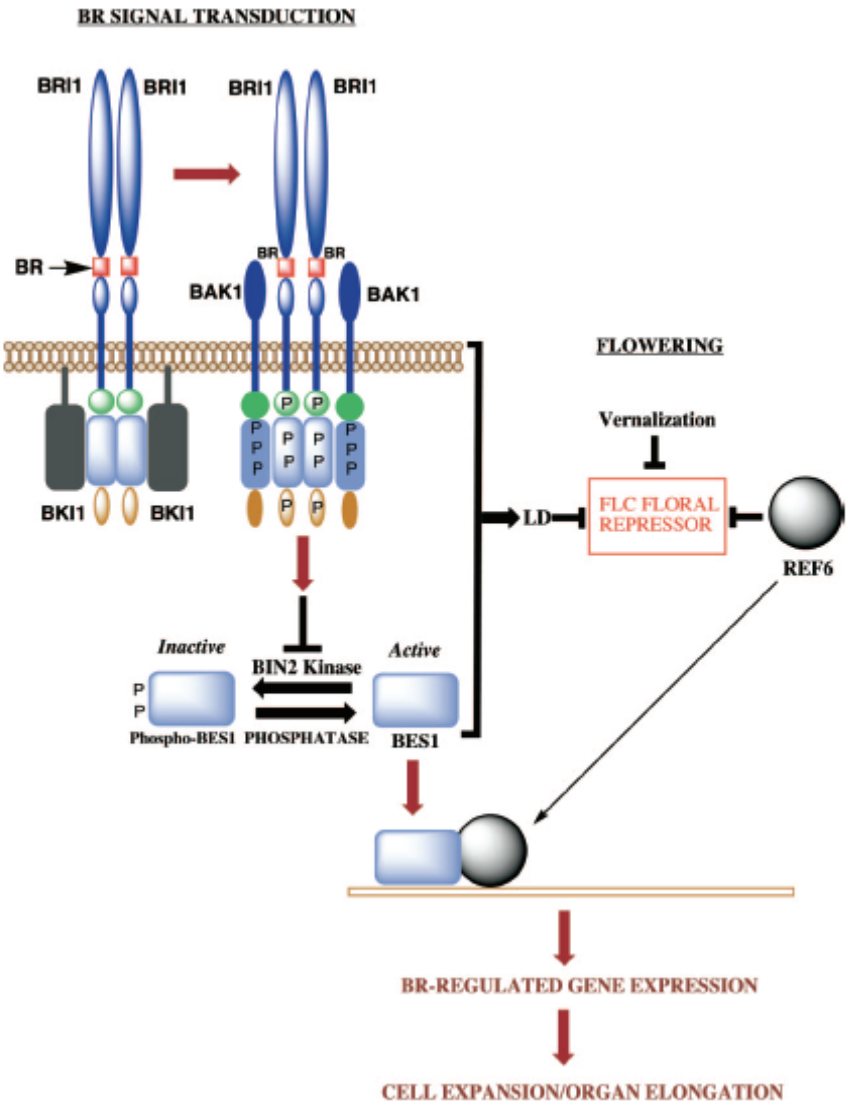


**Fig.4** BR signal transduction in *Arabidopsis* (adopted from Clouse, 2008).

## 1.2. Steroid receptors

Steroid receptors (estrogen, androgen, progesterone, mineralcorticoid and glucocorticoid) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. Natural ligands for steroid receptors are presented in Table 1.

**Table 1.** Nuclear receptor ligands

| Receptor | Ligand |
|---|---|
| Estrogen | 17β-Estradiol |
| Progesterone | Progesterone |
| Androgen | Testosterone |
| Glucocorticoid | Cortisol |
| Mineralocorticoid | Aldosterone |

Estrogen receptors (ER-α and ER-β) show a high degree of similarity when compared at the amino acid level. The amino acid sequence identity between ER-α and ER-β is approximately 97% in the DNA-binding domain and approximately 56% in the ligand-binding domain (LBD), whereas the N terminus is poorly homologous at 24%. Transcriptional activation by ER-α is mediated by two distinct activation functions: the constitutively active AF-1, located in the N-terminal domain of the receptor protein, and the ligand-dependent AF-2, located in the C-terminal domain of the receptor protein. ER-β seems to have a weaker corresponding AF-1 function and thus depends more on the ligand-dependent AF-2 for its transcriptional activation function (Delaunay et al., 2000). ER-α and ER-β can be detected in a broad spectrum of tissues. In some organs, both receptor subtypes are expressed at similar levels, but one or the other subtype usually dominates. ER-α is mainly expressed in, for example, uterus, prostate (stroma), ovary (theca cells), testes (Leydig cells), epididymis, bone, breast, various regions of the brain, liver, and white adipose tissue. ERβ is expressed in, for example, colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelium, and certain regions of the brain (Dahlman-Wright et al., 2006). Like other steroid hormone receptors, ERs act as dimers to regulate transcriptional activation. Transcriptional control by ERs requires interaction with coregulator complexes, either

coactivators for stimulation or corepressors for inhibition of target gene expression (Klinge, 2000). Estrogens are also implicated in the development or progression of numerous diseases, for example various types of cancer (breast, ovarian, colorectal, prostate, and endometrial), osteoporosis, neurodegenerative and cardiovascular diseases, insulin resistance, lupus erythematosus, endometriosis, and obesity (Deroo and Korach, 2006).

There are differences between action of steroids in animal cells and in plant cells. In the classic animal model there are steroid receptors located in the cytosol or nucleus, which bind the lipophilic steroids that diffuse through the plasma membrane. Ligand binding induces a conformational change and dimerization with another receptor that allows the ligand/receptor complex to bind DNA and directly modify gene expression over a time period of hours or even days (Lösel and Wehling, 2003). Steroid hormones might also have very nongenomic activity with rapid action on cells, too rapid to invoke transcriptional mechanisms. Binding sites for estrogen were identified in the membrane of endometrial cells that triggered the induction of cyclic AMP (Pietras and Szego, 1977).

Steroid hormone receptors exert their influence in embryonic development and adult homeostasis as hormone-activated transcriptional regulators. Their modular structure, consisting of a DBD, nuclear localization signals, a ligand-binding domain (LBD), and several transcriptional activation functions, is conserved with other members of the nuclear receptor family. All unliganded steroid hormone receptors are associated with a large multiprotein complex of chaperones, including Hsp90 and the immunophilin Hsp56, which maintains the receptors in an inactive but ligand-friendly conformation in contrast to other nuclear receptors (Pratt, 1993). Steroid receptors interact in vitro directly with components of the transcription initiation complex. The binding of ligands should be influenced by coactivators that would act as bridging factors between steroid receptors and the transcription initiation complex. Steroid receptors do not only stimulate gene activity, but they are also able to repress the transcription by competition for the DNA-binding site, by competition for common mediators to the transcription initiation complex, or by sequestration of the transcription factors into inactive forms (Beato et al., 1995).

The mechanisms of action of BRs in animal cells are still largely unknown, but it seems possible that they may interact with one or more of the numerous steroid-binding proteins. The ability of BRs to modulate steroid responses in reporter cell lines is

strong. On the other hand, direct binding of the studied steroids at least to steroid receptors ER-α and ER-β is weak. It seems possible that there are multiple effects, both steroid receptor-dependent and independent.

### 1.3. Anticancer and antiproliferative activities of brassinosteroids

The brassinosteroids are essential for many growth and development processes in plants. On the other hand, information about the effects of BRs and their synthetic analogues on animal cells is still very limited. Contradictory effects of BRs on cell division in different plant species and cultured cell lines have been reported (Jiang et al., 2003; Jun et al., 2008; Kesisis et al., 2007; Khripach et al., 1999). The effect of BRs on cell division has been shown to be mainly promotive. They substitute for the effects of cytokinins: both BRs and cytokinins induce *cycD3* gene expression and promote cell division during the early cell culture phases, suggesting that BRs are rate-limiting factors in the induction of the cell cycle (Hu et al., 2000). There is also one report describing possible effects of 24-epiBL on cultured hybridoma mouse cells. Typical effects of 24-epiBL were: (I) an increase in the value of mitochondrial membrane potential; (II) a drop of intracellular antibody level; (III) an increase in the fraction of the cells in $G_0/G_1$ phase; and *vice versa* (IV) a decrease in proportion of cells in S phase. Furthermore, the density of viable cells was significantly higher at 24-epiBL concentrations $10^{-13}$ and $10^{-12}$ mol/L (Franěk et al., 2003).

An inhibitory effect of the natural BRs, 24-epiBL and 28-homoCS, on the growth and viability of different normal and cancer cell lines has been reported recently (Malíková et al., 2008). 28-HomoCS and 24-epiBL were shown to have effects on the viability (in Calcein AM assays) of BJ fibroblasts and human cancer cell lines of various histopathological origins. Cell lines tested included: the T-lymphoblastic leukaemia CEM, breast carcinoma MCF-7, lung carcinoma A-549, chronic myeloid leukaemia K562, multiple myeloma RPMI 8226, cervical carcinoma HeLa, malignant melanoma G361 and osteosarcoma HOS cell lines. Treatments with 28-homoCS and 24-epiBL resulted in potent, dose-dependent reductions in the viability of CEM and RPMI 8226 cells, albeit at different levels. Estrogen- and androgen-sensitive and insensitive breast and prostate cancer cell lines were shown to respond differently to treatment with natural BRs. Most breast cancers consist of a mixture of estrogen-

sensitive and estrogen-insensitive cells, and the key to the control of breast cancer seems to lie in the elimination of both cell types. Hormone-sensitive cell lines were more susceptible to BRs treatment. This finding could point to possibly modulation of steroid receptor response by natural BRs. A cytotoxic effect of natural BRs was observed in cancer cells, but not in untransformed human fibroblasts, suggesting that BRs induce different responses in cancer and normal cells. Therefore, these plant hormones provide promising leads for potential anticancer drugs (Malíková et al., 2008). Additionally, brassinosteroids may perturb cell cycling in breast and prostate cancer cell lines. Using flow cytometry, it was shown that treatment with 28-homoCS and 24-epiBL induced blocks in the $G_1$ phase of the cell cycle in the breast and prostate cell lines, with concomitant reductions in the percentages of cells in the S phase of the cell cycle (Malíková et al., 2008). In the MCF-7 breast cancer cell model, the most widely studied experimental system in this context, the typical growth inhibitory response to antiestrogens is manifested by similar reductions in the proportions of cells synthesizing DNA (S phase) after antiestrogen treatment that coincide with increases in the proportions of cells in $G_0/G_1$ phase (Parl, 2000).

Brassinosteroids seem to impart their growth inhibitory and cell cycle effects via the cell cycle machinery and may be developed as an agent for the management of breast cancer. All carcinomas should be affected by antiangiogenic effects of BRs and their synthetic derivatives. These properties may lead to development of novel natural product-derived anticancer drugs.

### 1.4. Angiogenesis

Angiogenesis, the growth of new blood vessels in animals, is essential for organ growth (Carmeliet, 2005; Folkman, 1974; Folkman and Shing, 1992) as well as for growth of solid tumors and metastasis (Bhat and Singh, 2008; Kesisis et al., 2007; Risau, 1997). Endothelial cells are the main players in angiogenesis (Bagley et al., 2003) and could be a target for antiangiogenic therapy because they are non-transformed and easily accessible to achievable concentrations of antiangiogenic agents. They are also unlikely to acquire drug resistance, because endothelial cells are genetically stable, homogenous and have a low mutational rate (Bhat and Singh, 2008). The vascularization of tumors plays a crucial role in cell nutrition and oxygen distribution. Tumor angiogenesis

targeting in the sense of the discovery of novel drugs includes inhibition of proteolytic enzymes that break down the extracellular matrix surrounding existing capillaries, inhibition of endothelial cell proliferation, migration and enhancement of tumor endothelial cell apoptosis. Potent angiogenic inhibitors capable of blocking tumor growth appear to have the potential for the development of novel generations of anticancer drugs (Gourley and Williamson, 2000; Ranieri and Gasparini, 2001; Singh and Agarwal, 2003).

Many natural products that inhibit angiogenesis have been reported including compounds with steroid structure. These discoveries include among others an active component of chilli peppers capsaicin (Min et al., 2004); a low molecular-weight natural product isolated from *Dendrobium chrysotoxum* Lindl, erianin (Gong et al., 2004); small molecule compound from extracts of the seed cone of *Magnolia grandiflora* L., honokiol (Bai, 2003); a natural product from marine sponges, laulimalide (Lu et al., 2006); the plant alkaloid, sanguinarine (Eun and Koh, 2004); and a sesquiterpene purified from fruits of *Torilis japonica* (Houtt.) DC., torilin (Kim et al., 2000).

Recently, several steroids (i.e. 2-methoxyestradiol, progestin, medroxyprogesterone acetate, glucocorticoids such as dexamethasone and cortisone) have been shown to have antiangiogenic activity (Mabjeesh et al., 2003; Pietras and Weinberg, 2005). To date, there is no information on the influence of natural BRs on endothelial cells including their effects on antiangiogenic behaviour of these cells. Therefore the effects of naturally occurring BRs and their synthetic analogues on cell proliferation and cycling in human microvascular endothelial or umbilical vein endothelial cells, and specifically on the migration and formation of tubes by these cells were studied.

## 2. Aims and Scopes

This thesis is concerned with the chemistry and biology of brassinosteroids. It is focused on the characterization of the cellular and molecular actions of natural brassinosteroids and their synthetic derivatives, particularly those exhibiting antiproliferative properties. A library of natural BRs and synthetic analogues, available in the Laboratory of Growth Regulators, Palacký University in Olomouc, offers a unique opportunity to demonstrate how plant compounds structurally related to humans steroids act in animal cells. Several compounds with the best antiproliferative activities were chosen from the library and their biological and biochemical effects were investigated in different model systems. The partial objectives of the work underlying this doctoral thesis were:

1. Characterization of antiangiogenic properties of natural brassinosteroids and their synthetic derivatives on human endothelial cells (see chapter 9.1. - Hoffmannová, L., Zahler, S., Liebl, J., Kryštof, V., Sedlák, D., Bartůněk, P., Kohout, L., Brosa, C., and Strnad, M. Antiangiogenic activities of natural brassinosteroids and their synthetic analogues in human endothelial cells. Phytochemistry, submitted).

2. Effects of natural brassinosteroids on cell cycle and apoptosis in cells derived from breast cancers (see chapter 9.2. - Steigerová, J., Oklešťková, J., Levková, M., Hoffmannová, L., Kolář, Z., Strnad, M. Brassinosteroids cause cell cycle arrest and apoptosis of human breast cancer cells via cell cycle machinery. Chem Biol Interact., submitted).

3. Characterization of molecular and cellular activities of natural brassinosteroids and their synthetic analogues in different cancer cell lines (see chapter 9.3. - Hoffmannová, L., Steigerová, J., Oklešťková, J., Kohout, L., Kolář, Z., Strnad, M. Anticancer structure-activity relationships of cholestane brassinosteroid derivatives. Manuscript in prep.).

## 3. Materials and Methods

For characterization of the actions of BRs various biological and biochemical methods were used:

**General Experimental Procedures**

The natural BRs 28-homocastasterone (28-homoCS) and 24-epibrassinolide (24-epiBL) were obtained from Olchemim Ltd., Czech Republic. The synthetic derivatives of brassinosteroids were obtained from the Steroid Museum of the Steroid Department of the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic. Stock solutions (10 mM) were prepared in DMSO. The following specific antibodies were used to detect the relevant proteins: anti-ER-α (clone D-12), anti-ER-β (clone H-150), anti-PR (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-PCNA (clone PC-10, gift from B. Vojtěšek, Masaryk Memorial Cancer Institute, Brno, Czech Republic). Phenol red-free Dulbecco´s Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were from Invitrogen (Carlsbad, CA, USA). L-glutamine, penicillin, streptomycin were purchased from Sigma (MO, USA). Calcein AM was obtained from Molecular Probes (Invitrogen Corporation, CA, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) except for chemicals with specifications below, and were of reagent grade.

**Cell cultures**

The screening cell lines (T-lymphoblastic leukaemia cell line CEM, breast carcinoma cell line MCF-7 (estrogen-sensitive), breast adenocarcinoma cell line MDA-MB-468 (estrogen-insensitive), cervical carcinoma cell line HeLa, human glioblastoma cell line T98, multiple myeloma cell line RPMI 8226, and human fibroblasts BJ) were obtained from the American Type Culture Collection (Manassas, VA, USA).

**Endothelial cell cultures**

Human microvascular endothelial cells (HMEC-1) were a kind gift of Dr. F.J. Candal from the CDC, Atlanta, GA, USA. These cells were cultured in endothelial cell growth medium (Provitro, Berlin, FRG) supplemented with 10% serum. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by collagenase

digestion and used at passage two or three as described previously (McGregor et al., 1994).

**Proliferation assay**

The cell suspension of approximate density of $1.25 \times 10^5$ cells/ml was redistributed into 96-well microtiter plates and after 12 h of stabilization the synthetic derivatives to be tested were added at different concentrations. Compounds were dissolved in DMSO before addition to cultures. Control cultures were treated with DMSO alone. The final concentration of DMSO in the reaction mixture never exceeded 1%. Four-fold dilutions of the intended test concentration were added at time zero in 20 µl aliquots to the microtiter plate wells. Usually, each test compound was evaluated at six 4-fold dilutions. In routine testing, the highest well concentration was 50 µmol/l, but was varied depending on the agent. After 72 h of culture, the cells were incubated with Calcein AM solution (Molecular Probes) for 1 h. Fluorescence of viable cells was quantified with Fluoroskan Ascent (Microsystems). Each compound was tested in triplicate and the entire test was repeated at least 3 times. The $IC_{50}$ value, the drug concentration reducing the number of cells by 50%, was calculated from the obtained dose response curves.

**Flow cytometry**

Flow cytometry was used to evaluate the number of cells in the particular phases of the cell cycle, including sub$G_1$ peak detection. Control and treated cells after 24 and 48 h of incubation were washed twice with PBS and centrifuged at 360 g for 10 min at 4°C, washed twice in cold PBS, and fixed with chilled ethanol (70%; v/v) by low-speeded vortexing. To analyse DNA content, propidium iodide staining was used. The cells were analyzed using a Cell Lab Quanta flow cytometer (Beckman Coulter, CA, USA). In a histogram analysis, distribution of cells into the sub$G_1$ (apoptotic cells), the $G_0/G_1$, S and the $G_2$/M peak was quantified using Multicycle AV software (CA, USA).

**Migration assay**

Confluent HUVEC monolayers were scratched and immediately treated either with starvation medium M199 (serum free, negative control; PAN Biotech, Aidenbach, Germany) or full endothelial cell growth medium containing different substances. After 16 h, images were taken with a TILLvisiON-system (TILL Photonics, Lochham,

Germany) connected to an Axiovert 200 microscope (Zeiss, Germany). Migration was expressed as the ratio of pixels covered by cells to the total number of pixels in the wound area (S.CO LifeScience, Garching, Germany).

**Tube Formation**

Ibidi µ-slides (18-well, Ibidi GmbH, Munich, Germany) were coated with Matrigel® (Schubert & Weiss-OMNILAB, Munich, Germany). Medium containing $1\times10^4$ HMECs and the compound to be tested were added to the wells. After 16 h images were taken using a TILLvisiON-system connected to an Axiovert 200 microscope. Formation of tubes was expressed as the number of tubes and number of nodes on treated cells compared with untreated control cells, using specific software (S.CO LifeScience, Garching, Germany).

**Activity of caspases-3/7 in breast cancer cells**

Treated cells were harvested by centrifugations and homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, inhibitors of proteases, pH 7.4) on ice for 20 min. The homogenates were clarified by centrifugation at 10,000g for 20 min at 4ºC; the proteins were quantified by the Bradford method and diluted to the same concentration. Lysates were then incubated for 1 h with 100 mM Ac-DEVD-AMC as a substrate (Sigma–Aldrich) in an assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 mM Ac-DEVD-CHO as a caspases-3/7 inhibitor (Sigma–Aldrich). The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems) at 346/442 nm (excitation/emission wavelengths).

**SDS–polyacrylamide gel electrophoresis and immunoblotting**

For immunoblotting, harvested cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 mM PMSF, 1 mM DTT, 10 mg/ml of aprotinin and leupeptin). Proteins in lysates were quantified by the Bradford method and then diluted with Laemmli electrophoresis buffer. Proteins were then separated electrophoresis on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, CA, USA) and stained with Ponceau S to check equal protein loading. The membranes were blocked with 5% (w/v) BSA and 0.1% Tween-20 in TBS for 2 h and probed with the specific

primary antibodies overnight. After washing in TBS and TBS containing 0.1% Tween-20, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies and visualized with chemiluminiscence detection reagent (West Pico Supersignal; Thermo Fisher Scientific, Rockford, USA). To confirm equal protein loading, immunodetection was performed with anti-PCNA monoclonal antibody. The experiments were repeated three times. The protein expressions in treated cells were compared to untreated controls.

**Statistical Analysis**

All experiments were performed in triplicates in at least three independent experiments. All quantitative data are presented as mean ± standard error of the mean (SEM) or as mean ± standard deviation of the mean (SDM) using SigmaPlot 10 or GraphPad Prism 5.0 software. For comparisons with control, a one way ANOVA was used.

# 4. Results

## 4.1. Antiangiogenic activities of natural brassinosteroids and their synthetic analogues

(See chapter 9.1.)

Antiangiogenic activity of the brassinosteroid plant hormones was investigated in human umbilical vein endothelial cells (HUVEC) and in human microvascular endothelial cells (HMEC-1). All the tested compounds, two natural brassinosteroids (24-epiBL, 28-homoCS) and two synthetic analogues of brassinosteroids (BR4848, KAR 6299), inhibited the growth of HMEC-1 cells in a dose-dependent manner. At 30 μM, 24-epiBL and 28-homoCS reduced the number of viable cells to approx. 50%, while both BR4848 and KAR 6299 were similarly effective but at a threefold lower concentration. After 30 min at 30 μM, BR4848 inhibited cell adhesion to a plastic surface more strongly than to a plastic surface coated with collagen (from 100% for untreated cells to 47% on a plastic surface and from 100% to 81% on a collagen-coated surface). Furthermore, KAR 6299 also decreased the number of cells adhering to a plastic surface more strongly (73%) than to a plastic surface coated with collagen (90%). This effect may contribute to the antiproliferative activity of the tested compounds as many cells require adhesion to a solid surface in order to proliferate.

Flow cytometric analysis shown that treatment with 24-epiBL or 28-homoCS only slightly increased the proportion of cells in the $subG_1$ fraction in HMEC-1 cells, when compared to the untreated controls. In contrast, after 48 h BR4848 and KAR 6299 treatment enhanced the number of $subG_1$ (apoptotic) cells 7-fold and 13-fold respectively compared to untreated controls. Moreover, BR4848 significantly induced block in the $G_2$/M phase. The tested steroids were more effective in causing cell cycle arrest and in inducing apoptosis than were the natural brassinosteroids, which only had cytostatic effects.

Natural BRs and their derivatives inhibited migration of HUVEC cells. At a concentration of 30 μM, 24-epiBL and 28-homoCS reduced migration to 59% and 40%, respectively, of that in the corresponding controls. The BR analogues were more potent: KAR 6299 inhibited migration at the same concentration to 38% and BR4848 application led to almost complete inhibition of migration (8%) compared with untreated cells.

Natural BRs caused only a slight inhibition of tube formation. Treatment with 24-epiBL or 28-homoCS reduced the number of tubes as well as the number of nodes; node number is one of the measured antiangiogenic parameters. BR4848 and KAR 6299 decreased the number of tubes by 2% and 34%, respectively, of the number in control treatments. In contrast, the number of nodes was reduced by BR4848 treatment, but not with KAR 6299. Surprisingly, KAR 6299 treatment enhanced formation of nodes, while BR4848 blocked the creation of longer tubes.

Because of the similarity of BRs to human steroids, we have also studied interactions of BRs with human steroid receptors using reporter assays and a competition binding assay. Reporter assays showed that 24-epibrassinolide was a weak antagonist of estrogen-receptor-α (ER-α); the synthetic BRs, KAR 6299 and BR4848, showed agonistic effects on ER-α, estrogen-receptor-β (ER-β) and androgen receptor (AR). Despite the results of the reporter assays, we were unable to demonstrate direct binding of the tested steroids to ER-α and ER-β in competition binding assays, with the exception of KAR 6299 which showed weak binding to ER-α and to ER-β. 24-EpiBL, 28-homoCS and BR4848 did not bind to estrogen receptors. While all tested compounds showed some effects in our angiogenesis assays *in vitro* at micromolar concentrations, their ability to modulate steroid responses in reporter cell lines was significantly stronger. On the other hand, direct binding of the studied steroids at least to steroid receptors ER-α and ER-β is weak. Therefore, we can only speculate about the action of natural BRs and synthetic derivatives on angiogenesis. It seems possible that there are multiple effects, both steroid receptor-dependent and independent. A similar possibility is also true for 2-methoxyestradiol which binds to estrogen receptors, but the mechanism of action of which in the antiangiogenic response is probably unrelated to the receptor pathway.

### 4.2. Effects of natural brassinosteroids on the cell cycle and apoptosis in breast cancer cells

(See chapter 9.2.)

We showed that the BRs 28-homoCS and 24-epiBL have dose-dependent effects on the viability of estrogen-sensitive MCF-7 cells and estrogen-insensitive MDA-MB-468 cells. BRs inhibited cell growth and induced blocks in the $G_1$ cell cycle phase with

concomitant reductions in the percentages of cells in the S phase in breast cancer cells. Cell cycle blockade was accompanied by reductions in cyclin-dependent kinases (CDKs) 2/4/6, cyclin $D_1$ and E expression and pRb phosphorylation, together with up-regulation of the cyclin-dependent kinase inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, which inhibit cyclin/CDK complexes. Both BR treatments caused expression of the anti-apoptotic Bcl-2 and Bcl-$X_L$ proteins in MCF-7 cells. The protein levels of p53 and MDM-2 were slightly affected by BR treatments. It was shown up-regulation of caspases 6 and 9 and degradation of caspase-3 into cleaved fragments (part of apoptotic cascade) after 24 h of each BR treatment in MDA-MB-468 cells. In addition, BR application to MDA-MB-468 cells resulted in $G_1$ phase arrest and increases in the sub$G_1$ fraction, which represents apoptotic bodies. It was confirmed that the BR-mediated apoptosis ocuured in both cell lines, although changes in the expression of apoptosis-related proteins were modulated differently by the BRs in each cell line.

Changes in ER-α and ER-β localization patterns were observed in MCF-7 cells after 24 h of BR treatment using immunofluorescence detection. Strong, uniform ER-α immuno-nuclear labelling 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. 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. These changes were also accompanied by down-regulation of the ERs following BR treatment (submitted manuscript). The studied BRs seem to exert potent growth inhibitory effects via interactions with the cell cycle machinery, and they could be valuable leads for agents for managing breast cancer.

### 4.3. Anticancer structure-activity relationships of cholestane brassinosteroid derivatives

(See chapter 9.3.)

The next goal was to determine the biological effects of synthetic cholestane derivatives of brassinosteroids in cancer cells. Firstly, the effects of cholestane derivatives on viability of normal and cancer cell lines of different histopathological origin were investigated. To evaluate the cytotoxic properties of several derivatives and some related steroids (cholesterol), we used cells of a T-lymphoblastic leukemia cell line,

CEM, a myeloma cell line, RPMI 8226, a breast carcinoma cell line, MCF-7, a cervical carcinoma cell line, HeLa, a human glioblastoma cell line, T98, and, as controls, a normal human skin fibroblast cells, BJ. Treatment with cholestane derivatives resulted in a potent and dose- dependent decrease in the viability of CEM, RPMI 8226 and HeLa cells, albeit at different concentrations. A high cytotoxicity was observed after application of 1933, KAR 6299, 1966 and SK468. Cholesterol, which is a non-cholestane derived plant and animal sterol, was however inactive or exhibited almost zero cytotoxic activity. In the BJ human fibroblasts a cholestane derivative-mediated loss of viability was not observed. These results suggest that cancer cells and normal cells respond differentially to cholestane derivatives. At present, only a few natural agents are known to posses the potential ability for selective/preferential elimination of cancer cells without affecting growth of normal cells. This study also provides the first evidence of cholestane derivatives as anticancer compounds with antiproliferative properties. To conclude the most active compound from the SAR study of cholestane derivative cytotoxicity on cancer cells was KAR 6299 and 1966. Changing the 6-oxo-7-oxalactone to a 6-oxo functionality dramatically increased the growth inhibitory activity of the cholestane derivatives. Thus, KAR 6299 produced about 3-times stronger response than M22. The 24R side chain is also a decisive group which increases the cytotoxicity of cholestane derivatives. Results point to the use cholestane derivatives as pharmaceuticals for inhibition of hyperproliferation in tumors.

Flow cytometry analysis showed an increase in the proportion of cells in the sub$G_1$ phase of the cell cycle (apoptotic cells) in MCF-7 and MDA-MB-468 cell lines after treatment with cholestane derivatives KAR 6299 or 1966. KAR 6299 treatment increased the proportion of cells in sub$G_1$ and $G_0/G_1$ phase and decreased the proportion in S phase. Treatment with 1966 caused a strong increase in the proportion of sub$G_1$ phase cells (apoptotic cells) and a concomitant decrease in other cell phases. Cholestane derivatives resulted in a decrease in the percentage of cells of both cell lines found in the S phase of the cell cycle, but a stronger effect was detected in MCF-7 estrogen-sensitive breast cancer cells.

Using a fluorogenic substrate Ac-DEVD-AMC and/or caspase-3/7 inhibitor Ac-DEVD-DHO, the activity of caspase-3/7 in MDA-MB-468 cells exposed to KAR 6299 or 1966 was determined. Caspases-3/7 start the apoptotic cascade in cells. KAR 6299 strongly induced the activity of caspase-3/7; after treatment for 24 h at concentration 30

μM a fivefold increase in the effector caspases was observed compared to the untreated control. Derivative 1966 only weakly affected the activity of caspases-3/7.

To detect changes in apoptosis related protein expression in breast cancer cell lines, cells treated for 24 and 48 h with cholestane derivatives (at 5, 15, 30 and 40 μM) were used for Western blot immunodetection. Expression of a tumor suppressor protein p53 was observed in controls of breast cancer cell lines and KAR 6299 and 1966 caused its enhanced expression after 24 and 48 h. Enhanced expression of p53 correlated with a decreased expression of antiapoptotic Mcl-1 protein in cells treated with KAR 6299 and 1966. These results confirm that cholestane derivatives KAR 6299 and 1966 can support apoptosis with caspase-3/7 activation.

The cholestane derivatives treatment causes the dose-dependent activation of apoptosis. This suggests, together with antiangiogenic activities of BRs, that these plant hormones might become important compounds in development of new anticancer drugs.

## 5. Conclusions

The presented doctoral thesis deals with the antiangiogenic properties of naturally occurring brassinosteroids and their synthetic derivatives in human endothelial cells and with the biological and anticancer activity of new BR-analogues in cancer cells *in vitro*. To analyze the mechanisms of actions of the studied compounds biochemical methods were used, including cytotoxicity studies, flow cytometry, protein SDS-PAGE electrophoresis and immunoblotting, immunofluorescence labelling, and antiangiogenic assays.
The most important results from the work are as follows:

The first evidence that BRs can inhibit *in vitro* angiogenesis of primary endothelial cells at micromolar concentrations was obtained. The inhibition of proliferation and migration of human endothelial cells by BRs was demonstrated and obtained evidence that BRs initiate cell death, probably by apoptosis. Analogues of BRs were found to be more effective than natural BRs. The observed inhibition of migration and tube formation points to antiangiogenic activity of BRs. This finding may be significant in terms of the potential use of BRs in preventing development of metastasis. If migration of endothelial cells is inhibited then tumor(s) cannot be efficiently supported with oxygen and nutrients and their growth is blocked.

This work demonstrates that natural and synthetic brassinosteroids cause growth inhibition, cell cycle blockade and initiation of apoptosis of many different cancer cell lines. The most effective compounds in various cancer cell lines derived from different tumors were the synthetic derivatives KAR 6299 and 1966, which were active in the micromolar range. The antiproliferative properties may be of value in the development of a new brassinosteroid-derived generation of anticancer drugs.

Mechanisms of action of BRs in human cancer and endothelial cells were studied using cellular and molecular properties in the description of BR effects. The possible involvement of steroid receptors in BR action was investigated. However, these compounds did not bind directly to steroid receptors and it seems that brassinosteroids function via steroid receptor-independent pathway(s).

# 6. Summary in Czech (Souhrn)

**Studium buněčných a molekulárních účinků brassinosteroidů a jejich derivátů**

Předkládaná disertační práce se zabývá charakterizací buněčných a molekulárních procesů ovlivněných brassinosteroidy a jejich syntetickými deriváty. Tyto látky jsou v současnosti zkoumány jako regulátory růstu a vývoje rostlin, ale i jako látky inhibující proliferaci lidských nádorových a endoteliálních buněk. Získané výsledky byly odeslány do tisku nebo publikovány formou článků v mezinárodních časopisech nebo zveřejněny jako patentové přihlášky.

První část této práce je zaměřena na antiangiogenními účinky brassinosteroidů na lidské endoteliální buňky HUVEC (lidské endoteliální buňky z pupečníku) a HMEC-1 (lidské kapilární endoteliální buňky). Tyto buňky jsou prvními prekurzory nově vznikajících kapilár. Angiogeneze je jedním z klíčových procesů při růstu nádorů. Umožňuje jejich zásobení krví a tudíž i dostatek živin a kyslíku pro růst dalších nádorových buněk. Pro testování byly použity tyto *in vitro* modely angiogeneze: proliferace, migrace a tvorba tubulů, tzv. „spojek“, mezi endoteliálními buňkami. Výše uvedené metody studia angiogeneze byly zavedeny do rutinní praxe testování protinádorových účinků přírodních látek v Laboratoři růstových regulátorů. Po aplikaci mikromolární dávky přirozených brassinosteroidů nebo jejich derivátů došlo k inhibici proliferace, migrace a tvorby tubulů (tube formation) lidských endoteliálních buněk. Přirozené brassinosteroidy i jejich syntetické deriváty tedy blokují angiogenezi *in vitro.*

Následně byl sledován vliv brassinosteroidů a jejich syntetických analog na lidské nádorové buňky odvozené od různých typů nádorů. Jako studovaný model byly použity především hormonálně závislé lidské prsní nádorové buňky, u kterých lze předpokládat vyšší senzitivitu k aplikaci steroidních látek. U těchto buněk byl studován vliv na proliferaci buněk, buněčný cyklus a apoptózu. Testování cytotoxicity potvrdilo inhibiční účinky těchto rostlinných hormonů v řádu μM na nádorové buňky. Přirozené brassinosteroidy neměly téměř žádný vliv na buněčný cyklus nádorových buněk. Jejich deriváty ale způsobovaly zablokování buněčného cyklu v jeho jednotlivých fázích nebo iniciovaly programovanou buněčnou smrt. Normální fibroblasty přitom studovanými látkami zůstaly téměř neovlivněny, což je výhodné pro jejich potencionální využití v praxi.

Dále bylo zjištěno, že testované brassinosteroidy a jejich analoga modulují odpověď lidských steroidních receptorů, konkrétně estrogenového, androgenového a progesteronového. Přímo na receptor se však váží pouze slabě. Jde zřejmě o působení brassinosteroidů drahou nezávislou na steroidních receptorech. Tyto rostlinné hormony by tedy mohly být s úspěchem použity jako protinádorové léčivo, které nejen blokuje buněčný cyklus a/nebo způsobuje indukci programované buněčné smrti selektivně v nádorových buňkách, ale navíc také inhibuje angiogenezi.

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## 8. List of papers

1. **Hoffmannová, L.**, Zahler, S., Liebl, J., Kryštof, V., Sedlák, D., Bartůněk, P., Kohout, L., Brosa, C., and Strnad, M. Antiangiogenic activities of natural brassinosteroids and their synthetic analogues in human endothelial cells. Phytochemistry, submitted.
2. Steigerová, J., Oklešťková, J., Levková, M., **Hoffmannová, L.**, Kolář, Z., Strnad, M. Brassinosteroids cause cell cycle arrest and apoptosis of human breast cancer cells via cell cycle machinery. Chem Biol Interact., submitted.
3. **Hoffmannová, L.**, Steigerová, J., Oklešťková, J., Kohout, L., Kolář, Z., Strnad, M. Anticancer structure-activity relationships of cholestane brassinosteroid derivatives. Manuscript in prep.
4. Oklešťková, J., **Hoffmannová, L.**, Steigerová, J., Kohout, L., Kolář, Z., Strnad, M., Natural brassinosteroids for use for treating hyperproliferation, treating proliferative diseases and reducing adverse effects of steroid dysfunction in mammals, pharmaceutical composition and its use, International Application Published Under the Patent Cooperation Treaty (PCT), World Intellectual Property Organization WO2008CZ00097 20080820, 2008.
5. **Hoffmannová, L.**, Steigerová, J., Oklešťková, J., Kohout, L., Chodounská, H., Hniličková, J., Kasal, A., Černý, I., Kolář, Z., Strnad, M., Cholestane derivatives as antiproliferative and antiangiogenic pharmaceuticals and pharmaceutical preparations containing these compounds, Patent application, Czech Industrial Property Office CZ PV 2009-725, 2009.

## 9. Supplement

## 9.1. Supplement I

Hoffmannová, L., Zahler, S., Liebl, J., Kryštof, V., Sedlák, D., Bartůněk, P., Kohout, L., Brosa, C., and Strnad, M. Antiangiogenic activities of natural brassinosteroids and their synthetic analogues in human endothelial cells. Phytochemistry, submitted.

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Abstract: Antiangiogenic activity of the brassinosteroid plant hormones (BRs) was investigated in human umbilical vein endothelial cells (HUVEC) and in human microvascular endothelial cells (HMEC-1). All the tested compounds inhibited the growth of HMEC-1 cells and inhibited migration of HUVEC cells. Because of the similarity of BRs to human steroids, we have also studied interactions of BRs with human steroid receptors. Of the natural BRs, 24-epibrassinolide was found to be a weak antagonist of estrogen-receptor-α (ERα). Synthetic BRs cholestanon and BR4848 showed agonistic effects on ERα, estrogen-receptor-β (ERβ) and androgen receptor (AR). Our results provide the first evidence that BRs can inhibit in vitro angiogenesis of primary endothelial cells at micromolar concentrations. BRs constitute a novel group of human steroid receptor activators with capacity to inhibit angiogenesis, which provides a basis for development of brassinosteroid-derived anticancer drugs.

**Title**

# Antiangiogenic activities of natural brassinosteroids and their synthetic analogues in human endothelial cells


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**Abstract**

Antiangiogenic activity of the brassinosteroid plant hormones (BRs) was investigated in human umbilical vein endothelial cells (HUVEC) and in human microvascular endothelial cells (HMEC-1). All the tested compounds inhibited the growth of HMEC-1 cells and inhibited migration of HUVEC cells. Because of the similarity of BRs to human steroids, we have also studied interactions of BRs with human steroid receptors. Of the natural BRs, 24-epibrassinolide was found to be a weak antagonist of estrogen-receptor-α (ERα). Synthetic BRs cholestanon and BR4848 showed agonistic effects on ERα, estrogen-receptor-β (ERβ) and androgen receptor (AR). Our results provide the first evidence that BRs can inhibit *in vitro* angiogenesis of primary endothelial cells at micromolar concentrations. BRs constitute a novel group of human steroid receptor activators with capacity to inhibit angiogenesis, which provides a basis for development of brassinosteroid-derived anticancer drugs.

**Keywords:**

brassinosteroids; human umbilical vein endothelial cells; angiogenesis; migration;

steroid receptors

## 1. Introduction

Brassinosteroids (BRs) comprise one of several groups of phytohormones, which are low-molecular weight organic compounds occurring in plants, particularly in flowers, pollen and seeds. The first brassinosteroid (brassinolide) was isolated in 1979 from pollen of *Brassica napus* L (Grove et al., 1979). Up to date, more than 70 of these plant growth regulators have been discovered (Sakurai and Fujioka, 1997). BRs are ubiquitously distributed through the plant kingdom and play an important role in hormone signalling and physiological response in plants, including growth, cell differentiation, root and stem elongation, disease resistance, stress tolerance and senescence (Clouse and Sasse, 1998; Nemhauser and Chory, 2004) They stimulate the division and elongation of cells (Schmidt et al., 1997; Bajguz and Tretyn, 2003) and are also involved in regulating plant-specific processes including photomorphogenesis and cell expansion in the presence of a potentially growth-limiting cell wall (Clouse, 2002). Brassinosteroids enhance resistance to abiotic and biotic stresses in plants (Müssig et al., 2006).

Insufficient evidence is available to know how BRs work in plants at the molecular level. Signalling by BRs is mediated by the receptor kinase brassinosteroid-insensitive 1 (BRI1). Its extracellular domain binds BRs and the signal is mediated by an intracellular kinase domain that autophosphorylates on Ser and Thr residues and may also phosphorylate other substrates. BRI1 also mediates genomic effects by steroid signal transduction across plasma membrane (Müssig and Altmann, 2001). 24-epibrassinolide (24-epiBL) has also been shown to up-regulate transcription of an important positive cell cycle regulator, cyclin D3, in *Arabidopsis thaliana* (Hu et al., 2000). In plants, no gene has yet been identified that encodes a protein for an intracellular receptor of BRs. Only a chaperone heterocomplex, similar to an intracellular steroid receptor in animals, has been identified in wheat germ lysate (Stancato et

al., 1996). Nevertheless, it is likely that in plants an intracellular signalling pathway may exist that regulates gene transcription in a similar fashion to the steroid hormones in animals (Hu et al., 2000).

BRs are now studied very intensively, especially in relation to their inhibitory effect in animal and human cells. A cytostatic effect of BRs on human cancer cells *in vitro* has been discovered recently (Malíková et al., 2008). The results also provide the first evidence that natural BRs impart a growth-inhibitory response in cancer cell lines without affecting proliferation of normal cells. Modulators of steroid receptors are an important class of anticancer therapeutic drugs that primarily influence gene transcription. Recently, several steroids (for example 2-methoxyestradiol, progestin, medroxyprogesterone acetate, glucocorticoids such as dexamethasone and cortisone) surprisingly have been shown to have antiangiogenic activity (Pietras and Weinberg, 2005; Mabjeesh et al., 2003).

In animals the growth of new blood vessels (known as angiogenesis) is essential for organ growth (Folkman et al., 1974, 1992; Carmeliet, 2005) as well as for growth of solid tumors and metastases (Bhat and Singh, 2008; Kesisis et al., 2007; Risau, 1997). Endothelial cells are the main players in angiogenesis (Bagley, 2003) and could be a target for antiangiogenic therapy because they are non-transformed and easily accessible to achievable concentrations of antiangiogenic agents. They are also unlikely to acquire drug resistance, because endothelial cells are genetically stable, homogenous and have a low mutation rate (Bhat and Singh, 2008). The vascularization of tumors plays a crucial role in cell nutrition and oxygen distribution. The targeting of tumour angiogenesis is an important goal in the development of novel anti-cancer drugs. Possibilities for targeting include inhibition of proteolytic enzymes that break down the extracellular matrix surrounding existing capillaries, inhibition of endothelial cell proliferation and migration and enhancement of tumor

endothelial cell apoptosis (Gourley and Williamson, 2000; Ranieri and Gasparini, 2001; Singh and Agarwal, 2003).

To date, there is no information on the influence of natural BRs on endothelial cells. Therefore we studied cell proliferation and cycling in human microvascular endothelial and human umbilical vein endothelial cells, and specifically migration and formation of tubes following treatment with two naturally occurring BRs and their two synthetic analogues (Figure 1). The mechanisms of action of BRs in animal cells are still largely unknown, but it seems possible that they may interact with one or more of the numerous steroid-binding proteins, as suggested in this work. In animals, steroid hormones regulate gene transcription by binding to a nuclear receptor.

## 2. Results and discussion

Many natural products that inhibit angiogenesis have been reported including compounds with a steroid structure. These discoveries include among others (i) an active component of chilli peppers, capsaicin (Min et al., 2004); (ii) a low molecular-weight natural product isolated from *Dendrobium chrysotoxum* Lindl, erianin (Gong et al., 2004); (iii) small molecule compound from extracts of the seed cone of *Magnolia grandiflora*, honokiol (Bai et al., 2003); (iv) a natural product from marine sponges, laulimalide (Lu et al., 2006); (v) a plant alkaloid, sanguinarine (Eun and Koh, 2004); and (vi) a sesquiterpene purified from fruits of *Torilis japonica*, torilin (Kim et al., 2000).

The plant hormone BRs are essential for many growth and development processes in plants (Clouse, 2002; Clouse and Sasse, 1998; Fujioka and Sakurai, 1997; Khripach et al., 1999). On the other hand, information about the effects of BRs and their synthetic analogues in animal cells is still very limited. An inhibitory effect of the natural BRs, 24-epiBL and 28-

homoCS, on the growth and viability of different normal and cancer cell lines was reported recently (Malikova et al., 2008). A cytotoxic effect of natural BRs was observed in cancer cells, but not in untransformed human fibroblasts. Clearly, BRs can induce different responses in cancer cells and normal fibroblasts. Natural BRs have been shown recently to arrest the cell cycle and cause apoptosis of human breast cancer cells, irrespective of their estrogen status (Malikova et al., 2008).

For the reasons outlined above, we investigated the effects of two natural BRs and selected synthetic analogues in human endothelial cells *in vitro*. All four tested compounds inhibited the growth of HMEC-1 cells in a dose-dependent manner (Figure 2). 24-EpiBL and 28-homoCS reduced the number of viable cells at 30 μM concentration to approx. 50%, while both BR4848 and cholestanon were equally effective but at a threefold lower concentration. Moreover, at 30 μM BR4848 inhibited more strongly cell adhesion after 30 min to a plastic surface (from 100% for untreated cells to 47%) than to a plastic surface coated with collagen (to 81%). Furthermore, cholestanon more strongly decreased the number of cells adhering to a plastic surface (73%) than to a plastic surface coated with collagen (90%) (data not shown). This effect may contribute to the antiproliferative activity of the tested compounds as many cells require adhesion to a solid surface in order to proliferate.

Flow cytometric analysis demonstrated that BR treatment with 24-epiBL or 28-homoCS did not induce noticeable changes in cell cycle distribution (Figure 3). In contrast, after 24 h treatment with BR4848 at 10 and 30 μM strongly increased the proportion of cells in the $G_2$/M fraction with a concomitant reduction in the percentage of cells in the S-phase. In contrast, 30 μM cholestanon treatment increased the number of S-phase cells (Figure 3). Treatment with 24-epiBL or 28-homoCS only slightly increased the proportion of cells in the sub$G_1$ fraction in HMEC-1 cells when compared to the untreated controls. In contrast, after 48 h BR4848 and cholestanon treatment enhanced the number of sub$G_1$ (apoptotic) cells 7-fold

and 13-fold respectively compared to untreated controls (supplementary information Figure S1). The tested steroids were more effective in causing cell cycle arrest and in inducing apoptosis than were the natural brassinosteroids, which only had cytostatic effects (Figure 3). Similar effects have been described for several other steroids when tested on human cells. For example, in human acute leukemia Jurkat T cells, 17α-estradiol induces cell cycle arrest at $G_2$/M and initiates apoptosis (Jun et al., 2008). Another estrogen 17β-estradiol causes $G_2$/M arrest together with induction of apoptosis in MDA-MB-231 breast cancer cells that are ER-α negative and therefore the compound, which normally binds to and activates this receptor, probably acts via an ER-α-independent pathway (Nomoto et al., 2002). Such antiproliferative and proapoptotic effects are typical also for 2-methoxyestradiol, an endogenous metabolite of 17β-estradiol (Lee et al., 2008).

One of aims of this work was to investigate whether BRs can influence the ability of human endothelial cells to migrate and form tubes *in vitro*. We evaluated the effects of the tested steroids on the migration of HUVEC cells using a scratch assay and formation of tubes by HMEC-1 cells. At a concentration of 30 μM, 24-epiBL and 28-homoCS reduced migration to 59% and 40%, respectively, and only a slightly inhibited tube formation as well as the number of nodes (Figure 4). The BR analogues were more potent; cholestanon inhibited migration at the same concentration to 38% and BR4848 application led to almost complete inhibition of migration (8%) compared with untreated cells (Figure 4). BR4848 and cholestanon decreased the number of tubes by 2% and 34%, respectively, of the number in control treatments. In contrast, the number of nodes was reduced by BR4848 treatment, but not with cholestanon (Figure 5). Surprisingly, cholestanon treatment enhanced formation of nodes, while BR4848 blocked the creation of longer tubes.

Since little is known about the action of plant steroids on the vertebrate steroid hormone receptors and given that most effects observed in this study might be mediated by

these receptors, we decided to profile interactions between these compounds and steroid receptors using selective stable reporter cell-lines. Briefly, none of the natural brassinosteroids showed an agonistic effect on any of the steroid receptors over the range of concentrations tested. On the other hand, 24-epiBL weakly antagonized E2 on ERα with $IC_{50}$ = 14.8 μM and 28-homoCS was an even weaker antagonist of ERα. ERβ was not affected by these two compounds (Figure 6). Cholestanon was the most potent agonist of both ERα and ERβ with $EC_{50}$ = 3.6 nM and $EC_{50}$ = 21.9 nM, respectively. Furthermore, it exhibited the full agonistic profile on ERα and acted as a supragonist on ERβ in contrast to BR4848, which achieved only 50 % of the full activity of 17β-estradiol on ERα over the range of concentrations tested and showed only barely detectable activity on ERβ. AR was activated to the strongest level by cholestanon, which acted as a partial agonist of this receptor reaching half efficacy of the fully activated receptor. Despite the results of the reporter assays, direct binding of the tested steroids to ERα and ERβ in competition binding assays with was not demonstrated, with the exception of cholestanon which showed weak binding to ERα and to ERβ (supplementary table S2).

We also analyzed effect of BRs and analogues on steroid receptor levels in HUVEC endothelial cells by immunoblotting (Figure 7). Treatment of HUVEC with all four compounds markedly increased ER-α expression. The impact of BR4848 and cholestanon on ER-α protein correlates with their activation of an ER-α response in the reporter cell line (Figure 6); cholestanon, which is an approximately four-fold stronger agonist of ER-α than is BR4848, responded in a dose dependent manner and the effect on ER-α protein levels was observed even with the lowest concentration tested. Interestingly, treatment with 24-epiBL increased protein levels of PR, while 28-homoCS and cholestanon led to downregulation of PR protein expression, although we did not observe any interaction in the cellular reporter assay for PR response (supplementary table 1).

The anticancer activity of 2-methoxyestradiol is at least partially explained by its ability to limit angiogenesis (Mabjeesh et al., 2003). The molecular mechanism of this effect has not been fully elucidated, but 2-methoxyestradiol downregulates hypoxia-inducible factor-1 (HIF) at the posttranscriptional level and thus inhibits HIF-1-induced transcription of vascular endothelial growth factor (VEGF). Moreover, 2-methoxyestradiol, at concentrations that are effective *in vivo*, depolymerizes microtubules in tumor cells. Compromised microtubular cytoskeleton and suppressed transcriptional activity of HIF-1 are common characteristics of antiangiogenic potential of microtubule-targeting drugs used in the clinics (Mabjeesh et al., 2003). Despite the fact that physiological levels of 2-methoxyestradiol do not exert estrogenic activity, it binds to and activates ER in pharmacological concentrations (Sutherland et al., 2005). It therefore cannot be excluded that the antiangiogenic properties of 2-methoxyestradiol are caused by its ability to agonize ER. A similar discrepancy in the concentrations of tested BRs also emerged in this work. While all tested compounds showed some effects in our angiogenesis assays *in vitro* at micromolar concentrations (Figures 4 and 5), their ability to modulate steroid responses in reporter cell lines was significantly stronger (Figure 6 and Supplementary table 1). On the other hand, direct binding of the studied steroids at least to steroid receptors ERα and ERβ is weak (Supplementary table 2). Therefore, we can only speculate about the action of natural BRs and synthetic derivatives on angiogenesis. It seems possible that there are multiple effects, both steroid receptor-dependent and independent. A similar possibility is also true for 2-methoxyestradiol which binds to estrogen receptors, but the mechanism of action of which in the antiangiogenic response is probably unrelated to the receptor pathway. The mechanism of action of BRs in animal cells thus remains unknown, but it is possible that BRs may involve interactions with steroid receptors and influence steroid signaling.

## 3. Conclusions

We have demonstrated for the first time that natural BRs and their synthetic analogues inhibit angiogenesis-critical behaviour of human endothelial cells. Analogues of BRs have been found to be more effective than natural BRs, they also reduced tube formation by HMEC-1 cells. The inhibition of migration and tube creation points to antiangiogenic activity of BRs. This finding may be significant in terms of the potential use of BRs in preventing development of metastasis. If the migration of endothelial cells is inhibited then the tumor(s) cannot be efficiently supported with oxygen and nutrients and its growth is blocked. This suggests that BRs might become important compounds in development of new anticancer drugs. We are continuing to intensively investigate the molecular and cellular action of BRs.

## 4. Experimental

### *4.1. General Experimental Procedures*

The natural BRs 28-homocastasterone (28-homoCS) and 24-epibrassinolide (24-epiBL) (Figure 1) were obtained from Olchemim Ltd., Czech Republic. The synthetic derivative of brassinosteroids, 2α,3α-dihydroxy-5α-cholest-7-on-17β-yl (Boc)-D,L-valinate, was synthesized as described below. 3β-Cholestan-7-on (cholestanon) was obtained from the Steroid Museum of the Steroid Department of the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic. Stock solutions (10 mM) were prepared in DMSO. The following specific antibodies were used to detect the relevant proteins: anti-ERα (clone D-12), anti-ERβ (clone H-150), anti-PR (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-PCNA (clone PC-10, gift from B. Vojtěšek, Masaryk Memorial Cancer Institute, Brno, Czech Republic). 17β-estradiol, dexamethasone, ICI

182 780, dihydrotestosterone, mifepristone, progesterone, nilutamide, and aldosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenol red-free Dulbecco´s Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were from Invitrogen (Carlsbad, CA, USA); Hyclone Charcoal/Dextran Treated Fetal Bovine Serum (C/D FBS) from Thermo Fisher Scientific Inc. (Waltham, MA, USA). One-Glo™ Luciferase Assay System was obtained from Promega (Madison, WI, USA). Panvera´s Fluorescence polarization-based ER Competitor Assays, RED, for ERα and ERβ were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) except for chemicals with specifications below, and were of reagent grade.

*4.2. 5α-Cholest-2-en-7-on-17β-yl(Boc)-D,L-valinate (BR4848)*

17-Hydroxy-5α -cholest-2-en-7-one (Kohout, 1989) (405 mg) and Boc-D,L-valine ((*tert*.-butoxycarbonyl-amino)-3-methylbutyric acid; Aldrich) (209 mg) were added to benzene (15 ml). 3 ml of benzene were distilled off and after cooling, dicyclohexylcarbodiimide (165 mg) and N,N-dimethylaminopyridine (2 mg) were added. The mixture was stirred for 20 hours at room temperature. Then the mixture was poured into water, extracted with diethylether, the separated organic layer was extracted with water, dried on magnesium sulphate and the solvent was evaporated under reduced pressure. The residue was chromatographed on a silica gel (100 g) column and eluted with petrolether containing 10 % (v/v) diethylether. Working up of appropriate fractions produced 470 mg of an oil of 5α-cholest-2-en-7-on-17β-yl (Boc)-D,L-valinate. $^{1}$H NMR spectrum: 0.72 s (3H, 18-H), 0.81 s (3H, 19 H), 0.94 t (3H, J = 7.2 Hz, $CH_3$ of methyl butyrate, 1.45 s, (9H, Boc), 4.30 m (1H, $W_{1/2}$ = 6 Hz, NH-C<u>H</u>-CO), 4.70 dd (1H, J = 7.5, J´= 9.2 Hz, H-17α), 4.99 d (1H, J = 7.9, HN), 5.55 and 5.69 (2 m ($W_{1/2}$ = 8 + 8 Hz, H-2 and H-3). IR spectrum ($CCl_4$): 3444 (NH), 3068,

3027, 1657 (C=C), 1737 (ester CO), 1714 (carbamate CO), 1497 (amide), 1392, 1367 (*tert*-butyl), 1249, 1199, 1164, 1063, 1024 (C-O) $cm^{-1}$. MS: m/e 488 $(M+H)^+$. For $C_{29}H_{45}O_5N$ (487.69) calculated: 71.42 % C, 9.30 % H, 2.87 % N, found: 71.07 % C, 9.40 % H, 3.08 % N.

*4.3. 2α,3α-Dihydroxy-5α-cholest-7-on-17β-yl(Boc)-D,L-valinate*

N-Methyl-morpholine-N-oxide (350 mg) and a solution of osmium tetroxide in *tert* butanol (0.26 ml containing 26 mg of osmium tetroxide) were added to a solution of 5α-cholest-2-en-7-on-17β-yl (Boc)-D,L-valinate from preceding experiment (262 mg) in a mixture of tehrahydrofurane (22 ml) and acetone (22 ml). The water (0.7 ml) was then added and the reaction mixture was stirred for 22 h at room temperature. A 5% water solution of sodium thiosulphate was then added and after 30 minutes stirring the mixture was poured into water. The aqueous solution was extracted with diethylether, the separated organic layer was extracted with water, dried over natrium sulphate and the solvent was evaporated under reduced pressure. The residue was chromatographed on a silica gel (40 g) column and eluted with chloroform containing 10 % (v/v) diethylether. Working up of appropriate fractions resulted in 270 mg of 2α,3α-dihydroxy-5α-cholest-7-on-17β-yl (Boc)-D,L-valinate as a solid material. $^1$H NMR spectrum: 0.77 s (3H, 18-H), 0.81 s (3H, 19 H), 0.93 t (3H, J = 7.2 Hz, $CH_3$ of methyl butyrate), 1.45 s, (9H, Boc), 3.73 m (1H, $W_{1/2}$ = 22, H-2β), 4.05 m (1H, $W_{1/2}$ = 6, H-3β), 4.38 m (1H, $W_{1/2}$ = 16 Hz, NH-C<u>H</u>-CO), 4.68 dd (1H, J = 7, J´= 9.5 Hz, H-17α), 5.01 dm (1H, J = 8.0, HN). IR spectrum ($CCl_4$): 3629 (OH), 3444 (NH), 4380 (NH/OH), 1742 (ester CO), 1714 (carbamate CO), 1498 (amide), 1250, 1234, 1201, 1165 (C-O) $cm^{-1}$. MS: m/e 522 $(M+H)^+$, 504 $(M-H_2O)$, 486 $(M-2xH_2O)$, 466 (522-*tert*Bu), 422 (522-Boc). For $C_{29}H_{47}O_7N$ (522.7) calculated: 66.47 % C, 9.08 % H, 2.68 % N, found: 66.44 % C, 8.93 % H, 2.87 % N.

*4.4. Endothelial cell cultures*

Human microvascular endothelial cells (HMEC-1) were a kind gift of Dr. F.J. Candal from the CDC, Atlanta, GA, USA. These cells were cultured in endothelial cell growth medium (Provitro, Berlin, FRG) supplemented with 10% serum. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by collagenase digestion and used at passage two or three as described previously (McGregor et al., 1994).

*4.5. Proliferation assay*

Proliferation of endothelial cells was examined using crystal violet. Briefly, HMEC-1 cells were seeded at a density of 1,500 per well in 96 well plates. After 24 h one plate was stained with crystal violet (0.5% [w/v] crystal violet, 20% [v/v] methanol) for 10 minutes, washed and air dried (day 0). The other plates were incubated with the tested compounds for 72 h. After this time, the plates with test compounds were also stained and dried, the stain was dissolved in ethanol and citrate, and absorbance measured at 540 nm in a photometer (SPECTRAFluor Plus TECAN, Crailsheim, Germany). Absorption linearly correlates with cell number. Each experiment was performed in hexaplicates and independently at least three times.

*4.6. Flow cytometry*

HMEC-1 cells were trypsinized, seeded in 6 well dishes, and immediately incubated with the respective compounds. After 48 h, the cells were again detached with trypsin, washed

and stained overnight at 4°C in 0.1% [m/v] sodium citrate, 0.1% [v/v] Triton X-100, and 50 µg/ml propidium iodide in PBS. DNA content was assessed with a flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany). In a histogram analysis, distribution of cells into the sub$G_1$ (apoptotic cells), the $G_0/G_1$, S and the $G_2$/M peak was quantified using FlowJo software (Tree Star, Oregon, USA).

*4.7. Migration assay*

Confluent HUVEC monolayers were scratched and immediately treated either with starvation medium M199 (serum free, negative control; PAN Biotech, Aidenbach, Germany) or full endothelial cell growth medium containing different substances. After 16 h, images were taken with a TILLvisiON-system (TILL Photonics, Lochham, Germany) connected to an Axiovert 200 microscope (Zeiss, Germany). Migration was expressed as the ratio of pixels covered by cells and the total number of pixels in the wound area (S.CO LifeScience, Garching, Germany).

*4.8. Tube Formation*

Ibidi µ-slides (18-well, Ibidi GmbH, Munich, Germany) were coated with Matrigel® (Schubert & Weiss-OMNILAB, Munich, Germany). Medium containing $1\times10^4$ HMECs and the compound to be tested were added to the wells. After 16 h images were taken using a TILLvisiON-system connected to an Axiovert 200 microscope. Formation of tubes was expressed as the number of tubes and number of nodes on treated cells compared with untreated control cells using specific software (S.CO LifeScience, Garching, Germany).

*4.9. SDS–polyacrylamide gel electrophoresis and immunoblotting*

For immunoblotting, harvested cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 mM PMSF, 1 mM DTT, 10 mg/ml of aprotinin and leupeptin). Proteins in lysates were quantified by the Bradford method and then diluted with Laemmli electrophoresis buffer. Proteins were then separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, CA, USA) and stained with Ponceau S to check equal protein loading. The membranes were blocked with 5% (w/v) BSA and 0.1% Tween-20 in TBS for 2 h and probed with the specific primary antibodies overnight. After washing in TBS and TBS containing 0.1% Tween-20, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies and visualized with chemiluminescent detection reagent West Pico Supersignal (Thermo Fisher Scientific, Rockford, USA). To confirm equal protein loading, immunodetection was performed with the anti-PCNA monoclonal antibody. The experiments were repeated three times. The protein expressions in treated cells were compared to untreated controls.

*4.10. Reporter assay*

The generated U2OS stable reporter cell lines for ERα-LBD, ERβ-LBD, GR-LBD, MR-LBD, PR-LBD and full-length AR were maintained in a monolayer in DMEM supplemented with 10% FBS, 2 mM glutamine and penicillin/streptomycin and incubated in a 5% $CO_2$ humidified atmosphere at 37°C. The culture medium was changed 48 h preceding the experiment to phenol red-free DMEM supplemented with 4% C/D FBS and 4 mM glutamine. After 48 h cells were trypsinized, counted and seeded at a density of $10^4$ cells/well in white

opaque cell culture 384-well plates (Corning Inc., NY, USA). Each compound tested was serially diluted in DMSO and stored in 384-well plates before being transferred by the JANUS® Automated Workstation (PerkinElmer, Inc.) equipped with a Pin Tool (V&P Scientific, Inc., San Diego, CA, USA). 5 µl of culture medium containing 6-fold concentrated agonist was added to each well on the plate in the antagonist mode. After further 18 h incubation, luciferase activity was determined using the One-Glo™ Luciferase Assay System according to the manufacturer´s protocol (Promega Corp.). Luminescence was recorded on the EnVision™ plate reader using 1s integration and data were analyzed by GraphPad Prism 5.0 statistical software. $EC_{50}$ values were calculated from the regression function (dose response, variable slope).

*4.11. Fluorescence polarization-based competitive binding assay*

The binding affinity of each compound for both ERs was performed according to the manufacturer´s protocol. The experiment was carried out in black 384-well plates (Corning Inc., NY, USA) in a total volume of 30 µl. Fluorescent data were collected on the EnVision™ (PerkinElmer, Inc., Waltham, MA, USA) plate reader after a 3 h incubation at room temperature using optimized BODIPY TMR FP Label consisting of 531 nm excitation filter, 595 nm S polarized emission filter, 595 nm P polarized emission filter and BODIPY TMR FP optical module. Collected data were subsequently analyzed by GraphPad Prism 5.0 statistical software and $IC_{50}$ values were calculated from the regression function (dose response, variable slope).

*4.12. Statistical Analysis*

All experiments were performed in triplicates in at least three independent experiments. All quantitative data are presented as mean ± standard error of the mean (SEM) or as mean ± standard deviation of the mean (SDM) using SigmaPlot 10 sofware. For comparisons with the control, a one way ANOVA was used.

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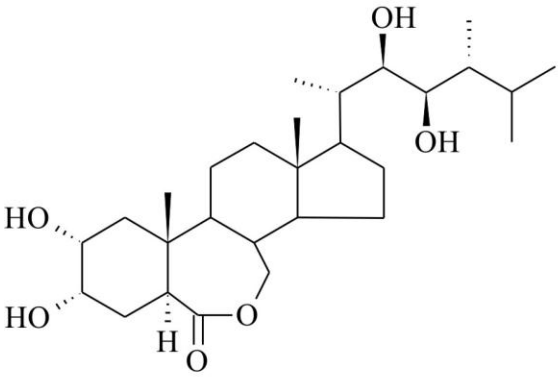
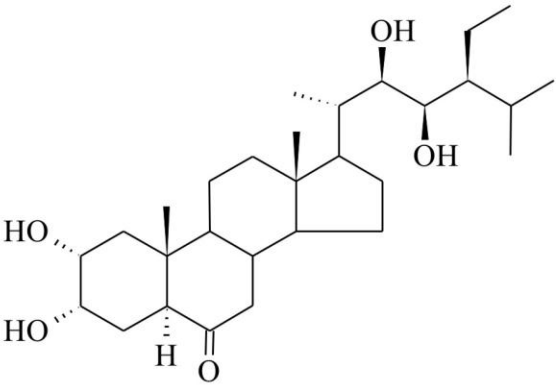
**Figures**

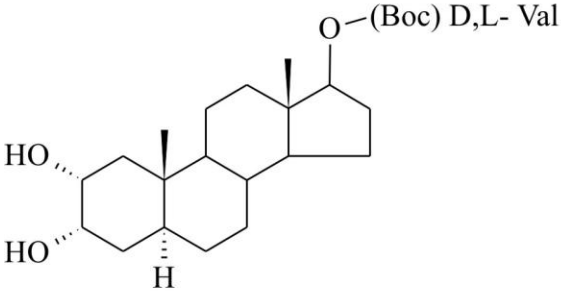
**Figure 1**



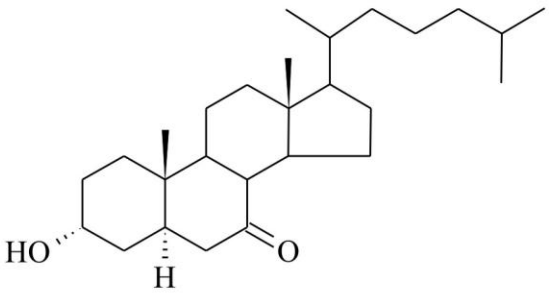

24-Epibrassinolide (24-epiBL)




28-Homocastasterone (28-homoCS)




BR4848




cholestanon

**Figure 2**

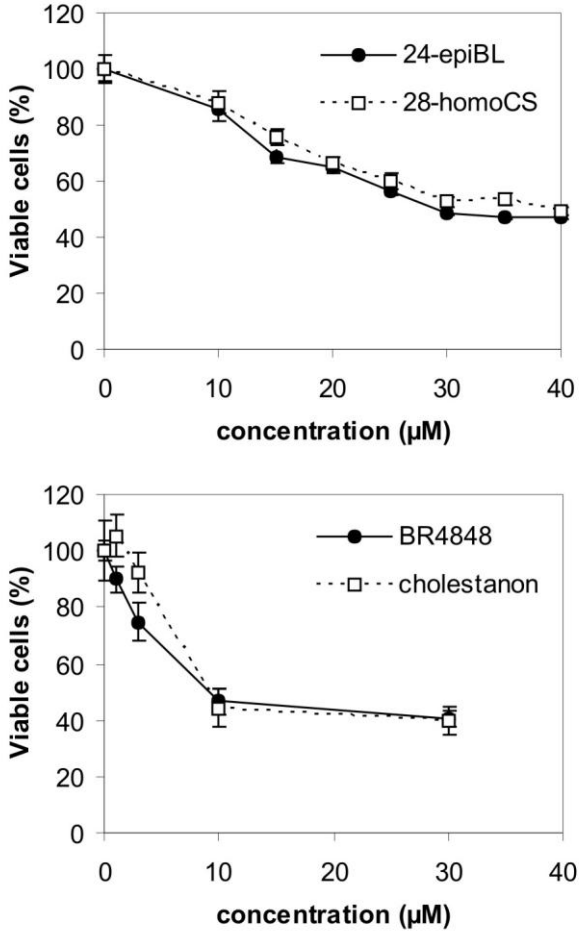

120
100
80
60
40
20
0
Viable cells (%)
24-epiBL
28-homoCS
0
10
20
30
40
concentration (µM)
120
100
80
60
40
20
0
Viable cells (%)
BR4848
cholestanon
0
10
20
30
40
concentration (µM)

**Figure 3**

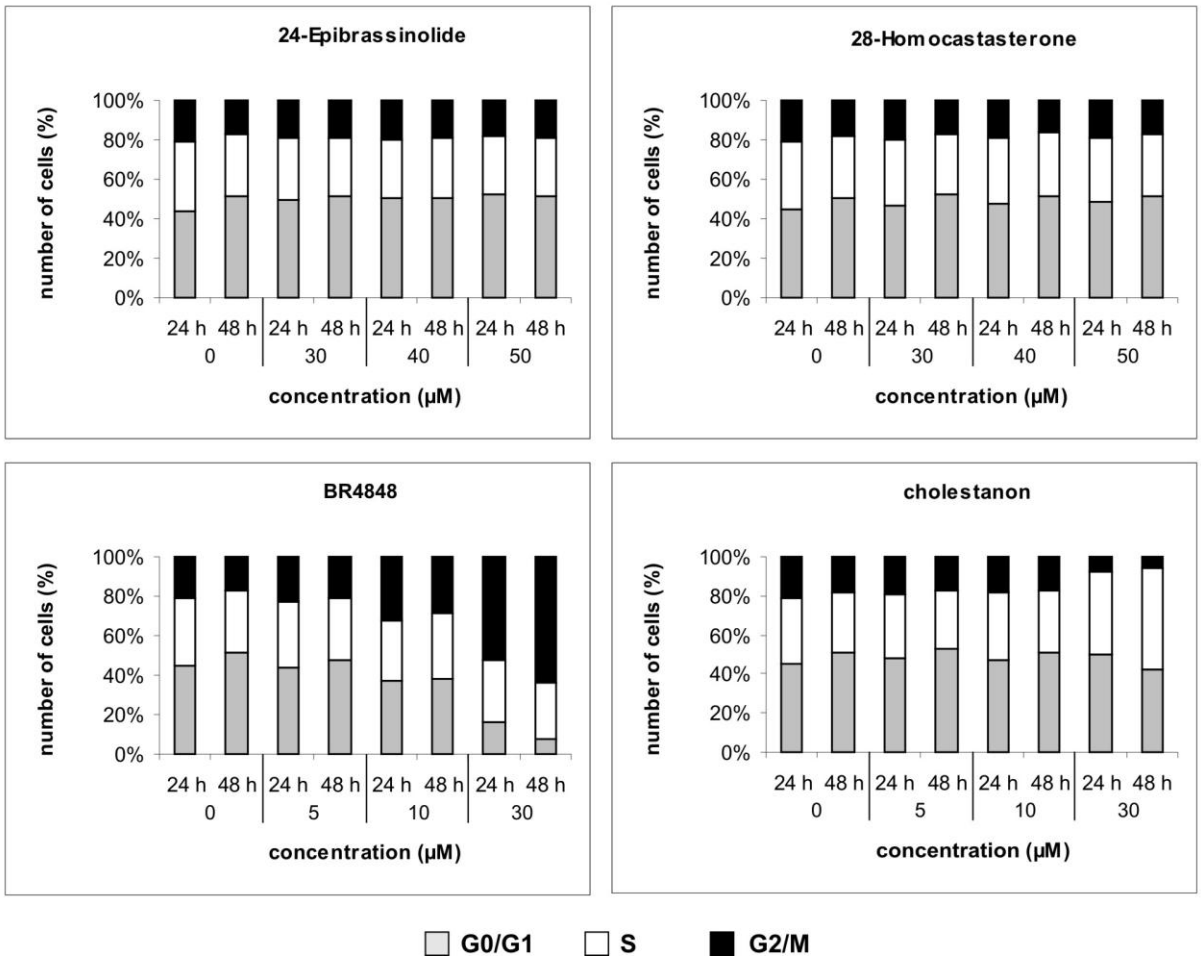

24-Epibrassinolide
28-Homocastasterone
BR4848
cholestanon
number of cells (%)
100%
80%
60%
40%
20%
0%
24 h 48 h
0
30
40
50
5
10
concentration (µM)
G0/G1
S
G2/M

**Figure 4**

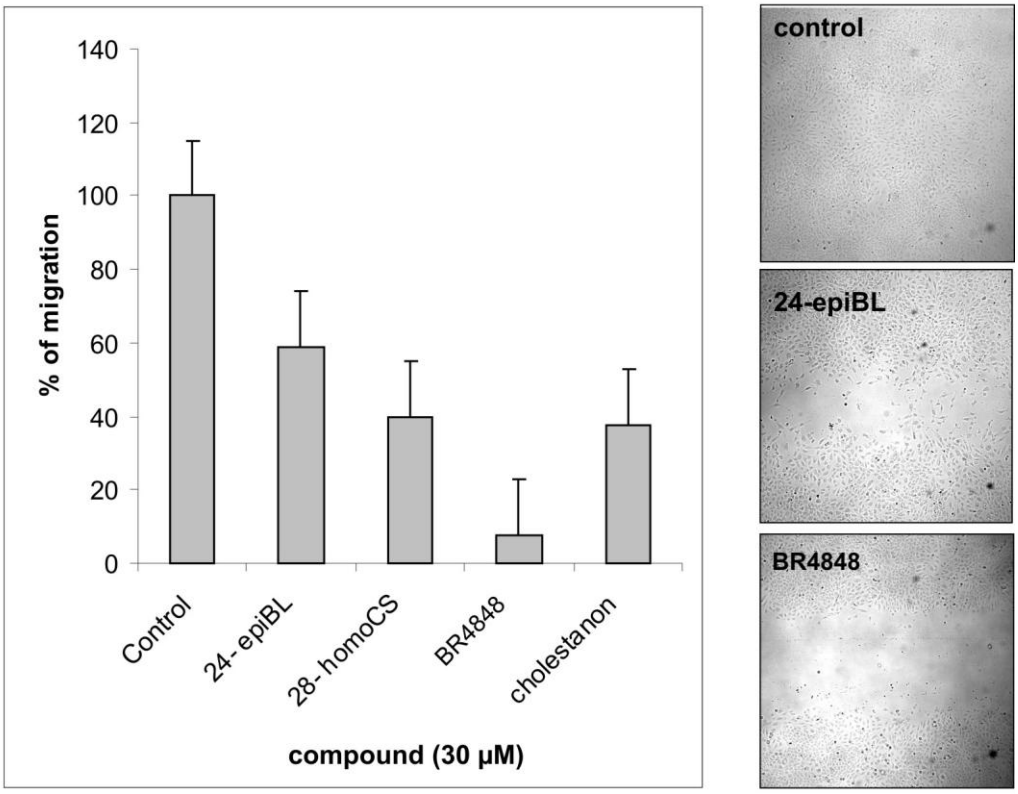

140
120
100
80
60
40
20
0
% of migration
Control
24- epiBL
28- homoCS
BR4848
cholestanon
compound (30 µM)
control
24-epiBL
BR4848

**Figure 5**

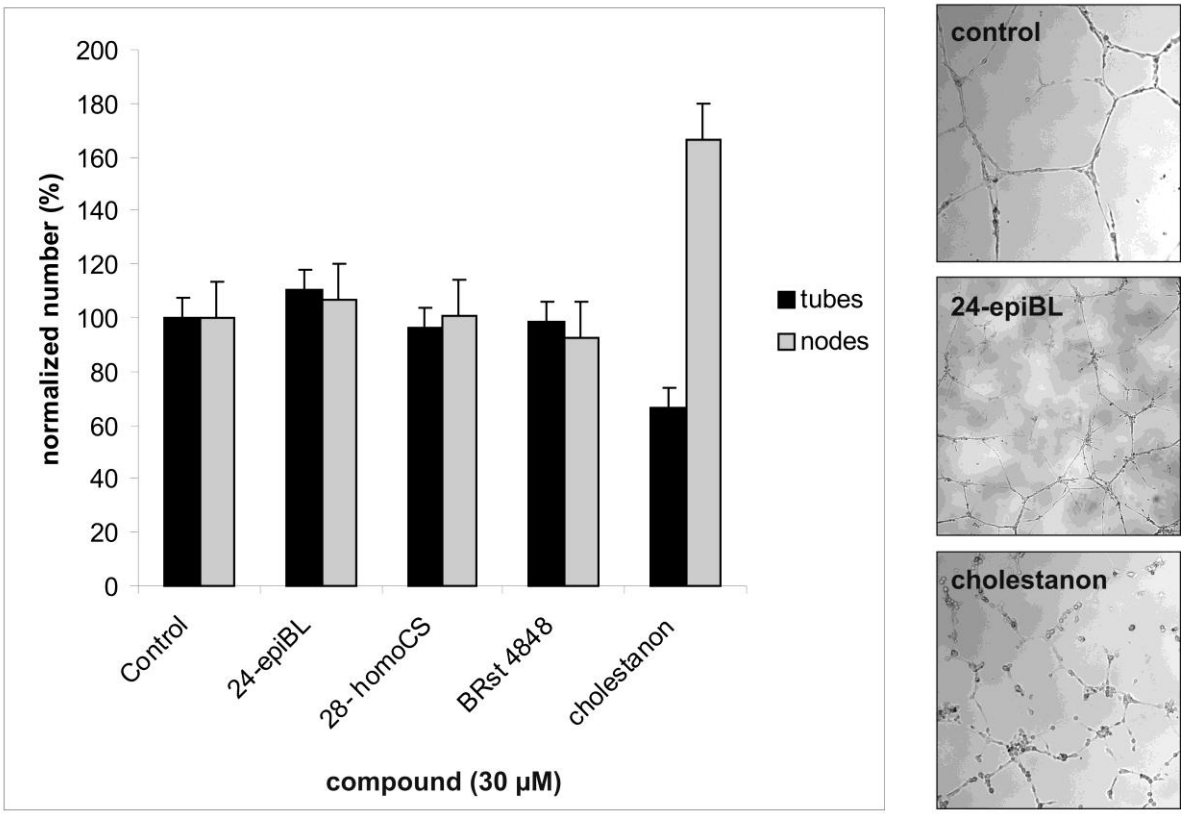

200
180
160
140
120
100
80
60
40
20
0
normalized number (%)
tubes
nodes
Control
24-epiBL
28- homoCS
BRst 4848
cholestanon
compound (30 µM)
control
24-epiBL
cholestanon

**Figure 6**

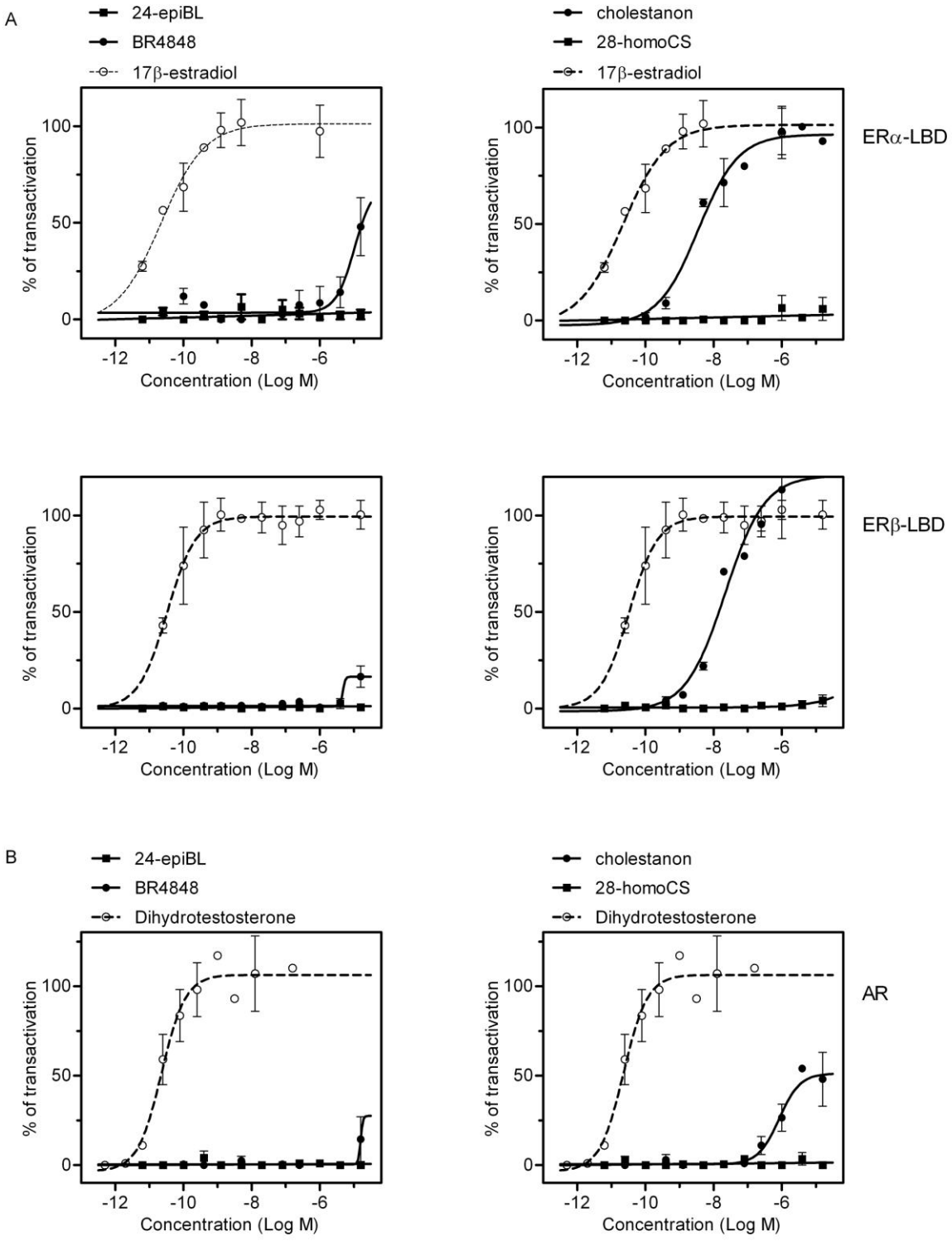

A
24-epiBL
BR4848
17β-estradiol
cholestanon
28-homoCS
17β-estradiol
ERα-LBD
ERβ-LBD
% of transactivation
Concentration (Log M)
B
24-epiBL
BR4848
Dihydrotestosterone
cholestanon
28-homoCS
Dihydrotestosterone
AR

**Figure 7**

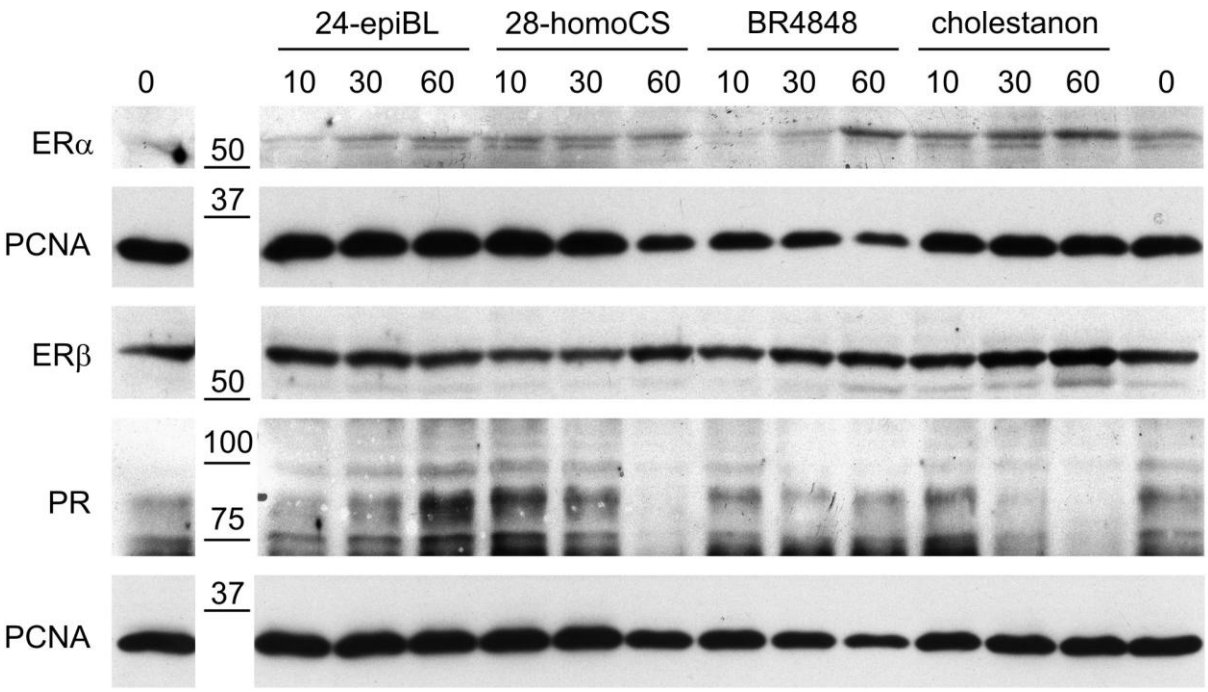

24-epiBL
28-homoCS
BR4848
cholestanon
0
10 30 60 10 30 60 10 30 60 10 30 60 0
ERα
50
37
PCNA
ERβ
50
100
PR
75
37
PCNA

**Figure Legends**

**Figure 1.** The structures of tested brassinosteroids and their synthetic analogues

**Figure 2.** The inhibitory effect of 24-epiBL or 28-homoCS and BR4848 or cholestanon on the viability of HMEC-1 cells. Cytotoxicity was determined by the Crystal violet assay. The cell viability is presented as the percentage of viable cells after 72 h (untreated control cells 100%). The data shown are means ± SD obtained from three independent experiments in hexaplicates.

**Figure 3.** Flow cytometric analysis of the cell cycle in HMEC-1 cells treated with 24-epiBL, 28-homoCS, BR4848 and cholestanon for 24 and 48 h compared with untreated cells. The graphs present the proportions of cells in $G_0/G_1$, S and $G_2$/M fractions. The data shown are means from three independent experiments performed in triplicate.

**Figure 4.** The inhibition of migration of HUVEC cells treated with 30 µM 24-epiBL, 28-homoCS, BR4848 and cholestanon. The data shown are means ± SE obtained from at least three independent experiments performed in triplicate. Figures from the scratch assays show untreated cells, cells treated with 30 µM 24-epiBL and 30 µM BR4848.

**Figure 5.** The effect of 30 µM 24-epiBL, 28-homoCS, BR4848 and cholestanon on formation of tubes by HMEC-1 cells. The data shown are means ± SE obtained from three independent experiments performed in triplicate. Untreated control cells, cells treated with 30 µM 24-epiBL and 30 µM cholestanon are presented in figures from the tube formation assay.

**Figure 6.** A) Transactivation of luciferase reporter by Gal4 DBD fused either to ERα LBD or ERβ LBD in response to increasing concentrations of tested compounds (●, ■) and 17β-estradiol (ο) in U2OS stable reporter cell lines. The highest stimulation of the receptor in response to 17β-estradiol was arbitrarily set as 100%. Each value is reported as a mean ± standard error of the mean (SEM) of 2 determinations. B) Transactivation of luciferase reporter by full-length AR in response to increasing concentrations of tested compounds (●, ■) and dihydrotestosterone (ο) in U2OS stable reporter cell line. The highest stimulation of the receptor in response to dihydrotestosterone was arbitrarily set as 100%. Each value is reported as a mean ± standard error of the mean (SEM) of three determinations.

**Figure 7.** Immunoblotting of steroid receptors (ER-α, ER-β, PR) in HUVEC cells. Cells were treated with 24-epiBL, 28-homoCS, BR4848 and cholestanon for 20 h at concentrations of 10, 30 and 60 μM and protein expressions were compared with those of untreated control cells. Proliferating cell nuclear antigen (PCNA) was probed as a protein loading marker.

## 9.2. Supplement II

Steigerová, J., Oklešťková, J., Levková, M., Hoffmannová, L., Kolář, Z., Strnad, M. Brassinosteroids cause cell cycle arrest and apoptosis of human breast cancer cells via cell cycle machinery. Chem Biol Interact., submitted.

**Brassinosteroids cause cell cycle arrest and apoptosis of human breast cancer cells via interactions with the cell cycle machinery**

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*Keywords:* Brassinosteroids; Apoptosis; Cell cycle; Hormone-sensitive/insensitive breast cancer cells

*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.

**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, DNA ladder assays and immunofluorescence analyses. The studied BRs inhibited cell growth and induced blocks in the $G_1$ cell cycle phase, accompanied by reductions in cyclin $D_1$ and CDK4/6 expression and pRb phosphorylation, together with up-regulation of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. 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). Conversely, 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 MDA-MB-468 cells resulted in $G_1$ phase arrest and increases in the sub$G_1$ fraction, which represents apoptotic bodies. Furthermore, TUNEL staining confirmed 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.

## 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 and 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 contain similar DNA- and hormone-binding domains, they both have two transcription-activating domains [4], their transcriptional activities are modulated in very similar fashions by certain ligands, they are both expressed in the same types of cells and their expression is generally induced by similar signals [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 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 herbs and 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, including the induction of apoptosis in cancer cells.

## 2. Materials and methods

### *2.1. Chemicals and antibodies*

28-homoCS and 24-epiBL (Fig. 1) were obtained from either SciTech or Olchemim Ltd. (Czech Republic) then stock solutions (10 mM) were prepared by dissolving appropriate quantities of each substance 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), mcm-7 (clone DCS 141.2), caspase-6 (clone H-90), caspase-9 (clone F-7), CDK2 (clone D-12), CDK4 (clone DCS-35), CDK6 (clone C-21), and ER-β (H-150) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Bcl-2 (clone 100) from Biogenex (San Ramon, CA, USA); Bcl-$X_L$ (clone H-62), and ER-α (clone 6F11) from Novocastra (Newcastle upon Tyne, UK); Bid and caspase-3 (clone Asp175) from Cell Signaling Technology (Danvers, MA, USA); 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 DakoCytomation (Glostrup, Denmark), respectively. All other chemicals used were commercially available.

### *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 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 $1.4\times10^4$cells/cm$^2$ (MCF-7) or $1.6\times10^4$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. Quantification of apoptosis using propidium iodide and acridine orange double staining*

Apoptotic morphology was investigated by double staining with propidium iodide (PI) and acridine orange (AO). The cells were plated at densities of either $1.4\times10^4$cells/cm$^2$ (MCF-7) or $1.6\times10^4$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, 48h. 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 into a glass slide and covered by coverslip. Slides were observed under UV-fluorescence microscope (Olympus, Japan) within 30 minutes 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. 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 1.4×104cells/cm2 (MCF-7) or 1.6×104cells/cm2 (MDA-MB-468) in 6-well plates. They were allowed to grow at 37°C in a humidified CO2 incubator until they were 70–80% confluent. Then the cells were treated with BRs (IC50) for 24, 48h. 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 into a glass slide and covered by coverslip. Slides were observed under UV-fluorescence microscope (Olympus, Japan) within 30 minutes 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.

*2.6. 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.

*2.7. Preparation of cells and Western blot analysis*

The cells were seeded at densities of $1.4\times10^4$cells/cm$^2$ (MCF-7) and $1.6\times10^4$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 the cells were scraped into an ice-cold protein extraction ion 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 a 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 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 immunohistochemical staining 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.

*2.8. Flow cytometry analysis*

Flow cytometry was used to evaluate the number of cells in specific phases of the cell cycle, and the proportions with sub$G_1$ DNA levels. The cells were seeded at densities of $1.4\times10^4$cells/cm$^2$ (MCF-7) and $1.6\times10^4$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 ($1\times10^{-6}$) 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, and RNA was cleaved by RNAse (50 μg/ml). Propidium iodide was used to stain the cells' DNA, which was then quantified with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

*2.9. 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, B). Strong, uniform ER-α immuno-nuclear 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). No significant changes in the localization of ER-β were found following BR treatment in MDA-MB-468 cells (data not shown).

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. However, treatment with either BR led to downregulation 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 (Table 2). 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. 2). 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, but no pRb expression occurred in the latter line, MDA-MB-468, that is pRb negative. The time-dependent reductions in the expression of both phosphorylated and dephosphorylated forms of pRb were observed 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. 2). 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. 2).

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 attended 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. 2). 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 (SER 15; SER392) but no alterations were found in both cell lines after BR treatment (data not shown).

The expression of cyclins $D_1$ and E was decreased in both cell lines after 24-epiBL treatment for 6, 12 or 24 h, and there were reductions in CDK2, CDK4, and CDK6 expression in both cell lines after 24 h of each BR treatment (cyclin $D_1$ shown only in Fig. 2). These findings suggest that 28-homoCS and 24-epiBL can 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 appoptotic 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 $IC_{50}$ levels resulted in increased numbers of TUNEL-positive cells at all tested timepoints (6, 12 and 24 h), and substantial increases in the percentages of such cells in the 24-epiBL-treated MDA-MB-468 cultures (18.3%) after 24 h and 28-homoCS treated MCF-7 cultures (13.7%) after 12 and 24 h (Table 1).

A distinguishing feature of apoptosis, generally, is the appearance of fragmented, low molecular weight DNA. However, there was no pronounced internucleosomal DNA fragmentation in any of the cells, regardless of BR treatment (data not shown).

Representative results obtained from the acridine orange–propidium iodide double staining show viable cells with intact DNA and nucleus give a round and green nuclei. Early apoptotic cells will have fragmented DNA which gives several green colored nuclei. Late apoptotic and necrotic cells DNA 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 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 analyze 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. 3. 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. 3). 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 at each timepoint (Fig. 3). 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 and 24 h), while Bcl-$X_L$ expression was not affected by exposure to either BR. However, MDA-MB-468 cells subjected to 6 h of either BR treatment showed increased levels of Bax expression and reductions in uncleaved Bid expression (Fig. 3). To test the possibility that BRs could induce the recruitment of the adaptor protein FADD to Fas receptor, another detections were performed. But Western blot analysis revealed no changes in the Fas and Fas-L expression after the treatment in both type of cell lines.

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 the latter line, due to its mutation. Expression of caspase-6 and -9 was also differently modulated by BR treatments according to each cell line. Caspase-6 showed a pattern of up-regulation in BR-treated MCF-7 cells (12 and 24 h) and in 24-epiBL-treated MDA-MB-468 cells (24 h). Caspase-9 was up-regulated in both cell lines after BR treatments (12 and 24 h). Furthermore, increases in poly-(ADP-ribose) polymerase (PARP) expression were observed in the breast cancer cell line controls, but neither BR had any effect on either its expression or degradation over a 24 h period (data not shown).

## 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 [19]. We found that low micromolar concentrations of 28-homoCS significantly inhibited MCF-7 cell proliferation, whereas MDA-MB-468 cells were more resistant. The natural BRs 28-homoCS and 24-epiBL were also found to induce significant arrest of the cell cycle at the $G_1$ phase and the appearance of a sub$G_1$ peak in the hypodiploid region of cell cycle DNA histograms, presumably representing apoptotic bodies. 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.

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] and thus only express ER-β. Changes in ER-α and ER-β localization patterns in MCF-7 cells subjected to 24 h treatment with the studied BRs are shown in Fig. 1. 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. 2). 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 time-dependent reductions following BR treatment in CDK2 and CDK6 in MCF-7 and MDA-MB-468 cells, respectively. Western blot analysis also showed up-regulation of the CKIs $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ following BR treatment. 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 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 D1 promoter, which leads to increases in cyclin D1 mRNA levels [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 faslodex renders them inactive [39]. Nevertheless, 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 [40,41], 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 [42]. In turn, p53 and MDM-2 regulate the estrogen-dependent down-regulation of ER-α and, possibly, susceptibility to antiestrogens [42]. Previous reports have suggested that one of the mechanisms underlying the pro-apoptotic effect of antiestrogens could be the relieve of 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 [41]. 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 staining confirmed that apoptosis occurred in both cell lines following BR treatment, although gel electrophoresis did not detect any fragmented, low molecular weight DNA, which is generally a distinguishing feature of apoptosis. The reason for this discrepancy may be the short duration of the BR treatments or low sensitivity to the DNA ladder assay.

Tamoxifen has been shown to increase both caspase-3 activity in ER-insensitive breast cancer cells [43] and the expression of caspase-6, -7, and -9 in MCF-7 cells [44]. In 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. (2000) [45]. 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 caspases 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. Accordingly, other studies have revealed that Bcl-2 forms a heterodimer with Bax, thereby potentially neutralizing its pro-apoptotic effects [46], and Bcl-2 is known to prevent the release of caspases [47]. However, MDA-MB-468 cells treated with studied BRs showed up-regulation of caspase-3, -6 and -9, and identical changes were observed in the MCF-7 cells, albeit without caspase-3 expression due to the deletion mutation in exon 3 of the MCF-7 cells' caspase-3 gene [48].

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 [45], while antiestrogens counteract this effect [49]. 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 [44]. However,

other studies have reported no (anti)estrogenic effects on either Bax or Bak protein levels [45]. Therefore, there is no clear consensus regarding the roles of these molecules in the induction of apoptotosis. 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 [50,51]. 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 main death pathway activated by Fas receptor involves the adaptor protein FADD (for Fas-associated deathdomain) that connects Fas receptor to the caspase cascade. Anticancer drugs have been shown to enhance both Fas receptor and Fas ligand expression on tumor cells (Micheau et al, 1999). In the case of BR treatment of breast cancer cell line, we found no changes in the expression of Fas and Fas-L. We suppose that cell death induced by tested compounds is mediated by mitochondrial pathway.

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, resulting in apoptotic changes in breast cancer cells. 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.

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**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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**Tables and figures**

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

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 $p < 0.05$.

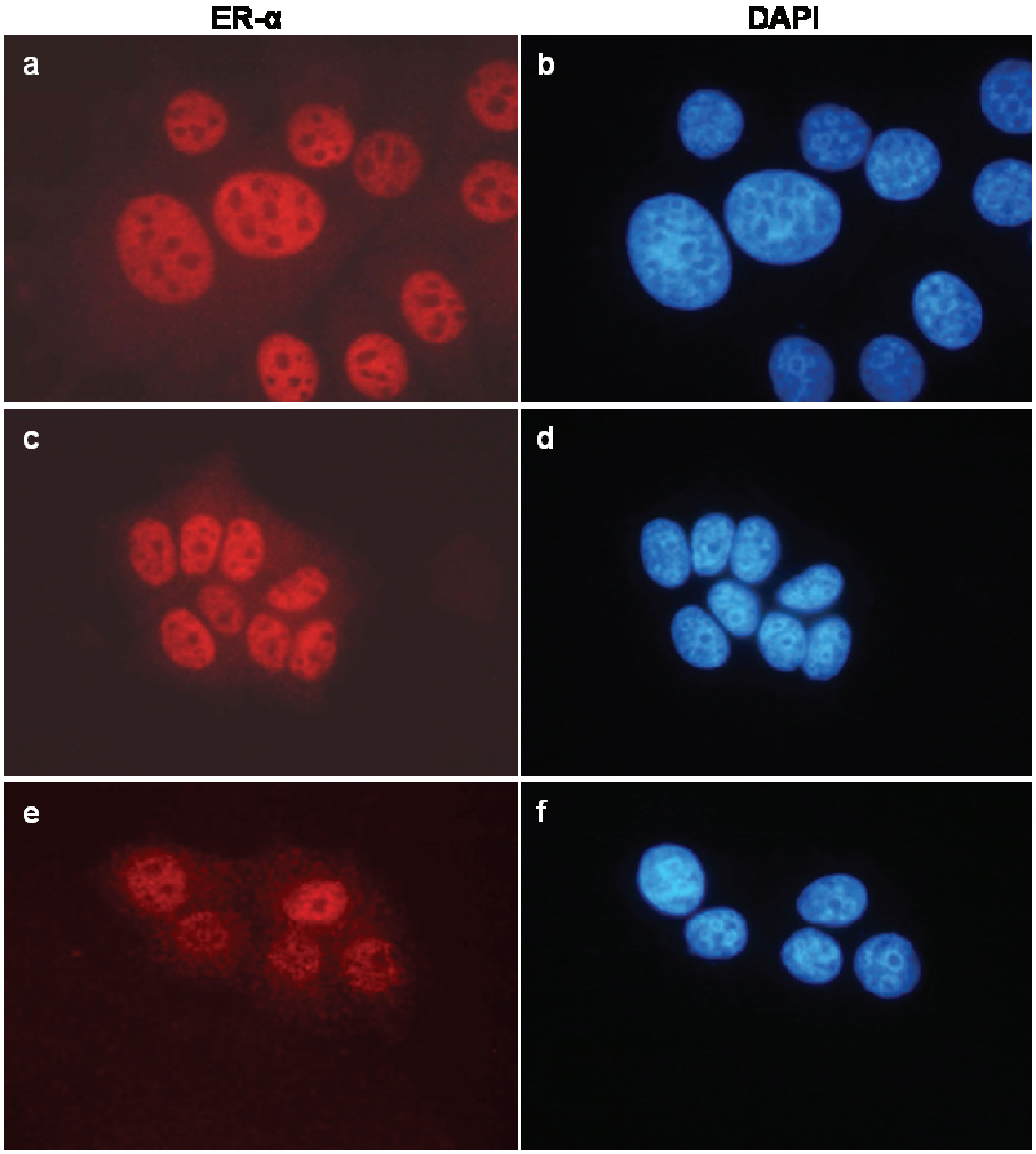
| **Control / BRs ($IC_{50}$)** | **MCF-7** | **MDA-MB-468** |
|---|---|---|
| Ctrl 6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 28-homoCS 6 | 10.6 ± 1.5* | 1.7 ± 0.6 |
| 24-epiBL 6 | 2.7 ± 1.2* | 4.7 ± 0.6* |
| Ctrl 12 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 28-homoCS 12 | 13.7 ± 3.8* | 2.3 ± 0.6 |
| 24-epiBL 12 | 8.3 ± 3.1* | 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* |

**Table 2.** Effect of BRs on cell cycle distribution. MCF-7 and MDA-MB-468 cells were treated with 28-homoCS or 24-epiBL ($IC_{50}$) for 24 h and analyzed by flow cytometry. The percentages of cells in each cell cycle phase ($G_0/G_1$, S and $G_2/M$) are indicated.

| | *Control / compound* | *Cell cycle distribution* | | |
|---|---|---|---|---|
| *Cell line* | *($IC_{50}$; 24 h)* | *$G_0/G_1$* | *S* | *$G_2/M$* |
| MCF-7 | **Control** | 60% | 14% | 26% |
| | **28-homoCS** | 80% | 11% | 9% |
| | **24-epiBL** | 81% | 7% | 12% |
| MDA-MB-468 | **Control** | 52% | 31% | 17% |
| | **28-homoCS** | 74% | 17% | 9% |
| | **24-epiBL** | 88% | 10% | 2% |

**Fig. 1**

**A**



**B**

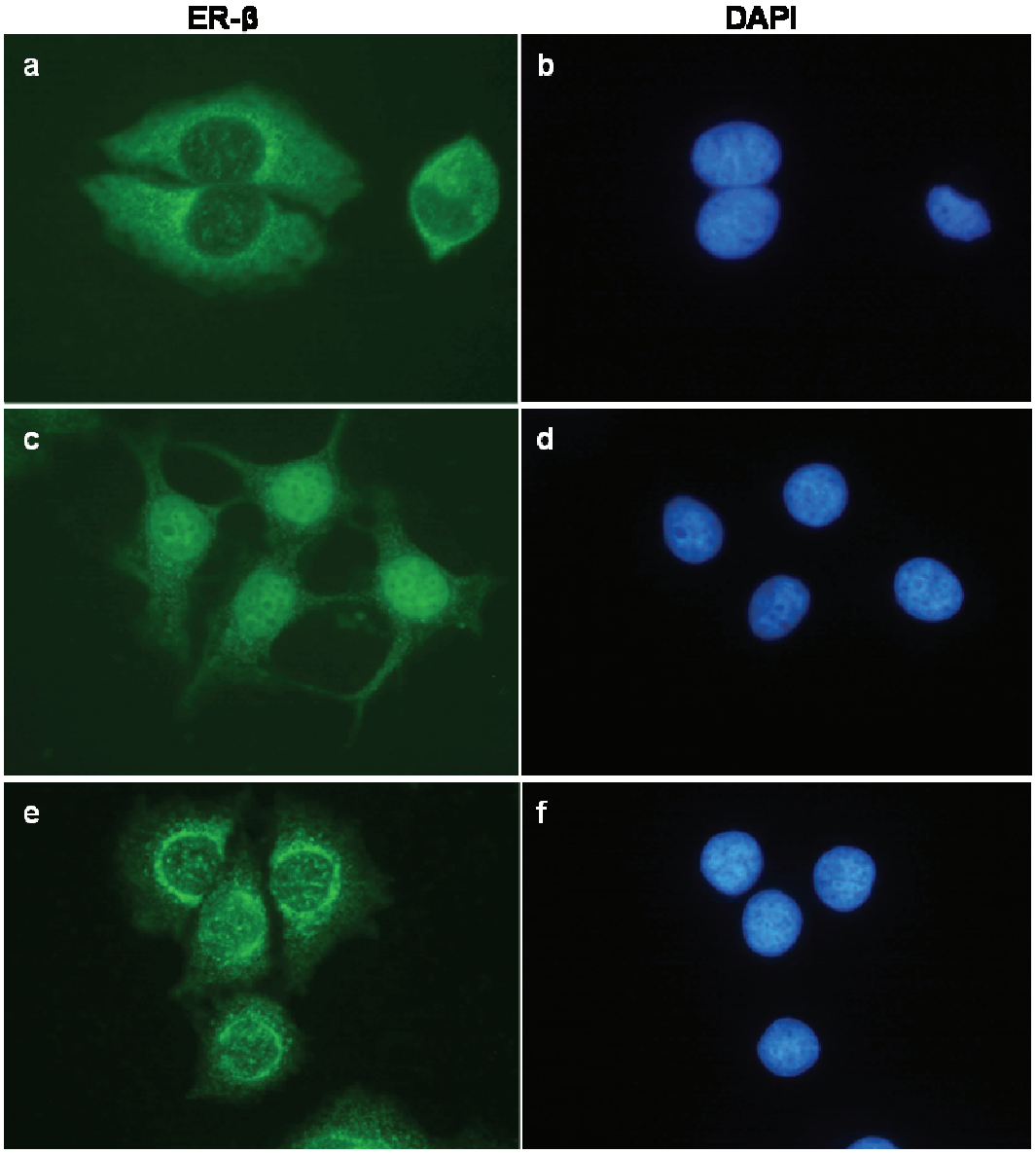


**Fig.2**

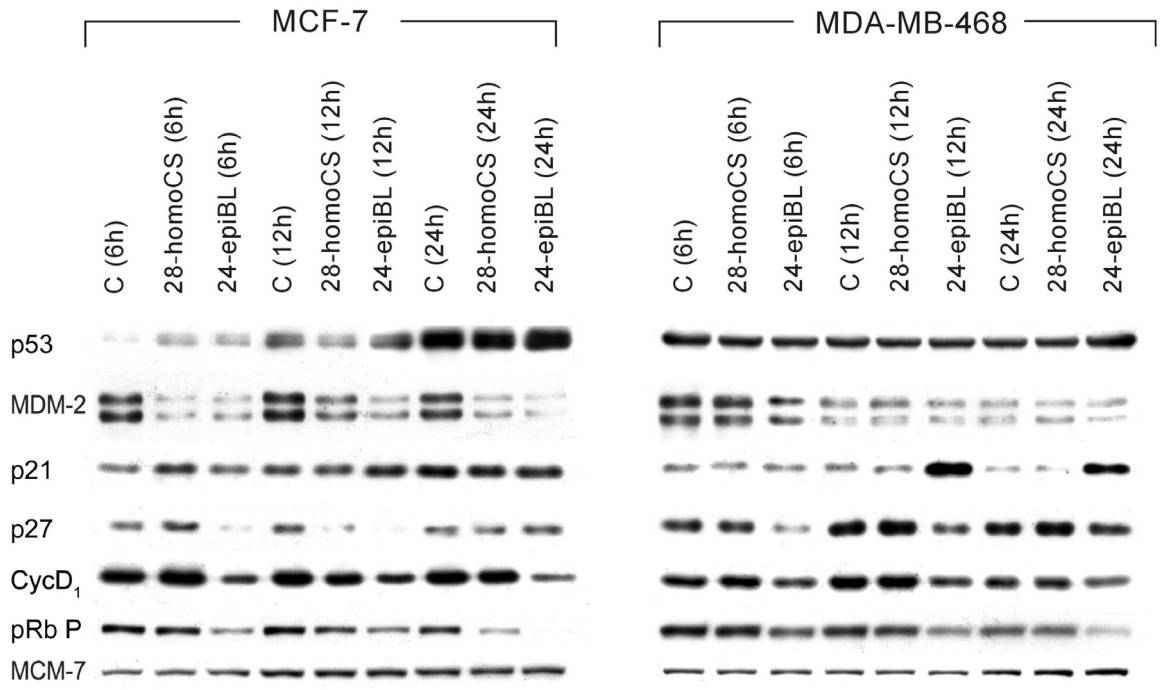

MCF-7
MDA-MB-468
C (6h)
28-homoCS (6h)
24-epiBL (6h)
C (12h)
28-homoCS (12h)
24-epiBL (12h)
C (24h)
28-homoCS (24h)
24-epiBL (24h)
p53
MDM-2
p21
p27
CycD1
pRb P
MCM-7

**Fig.3**

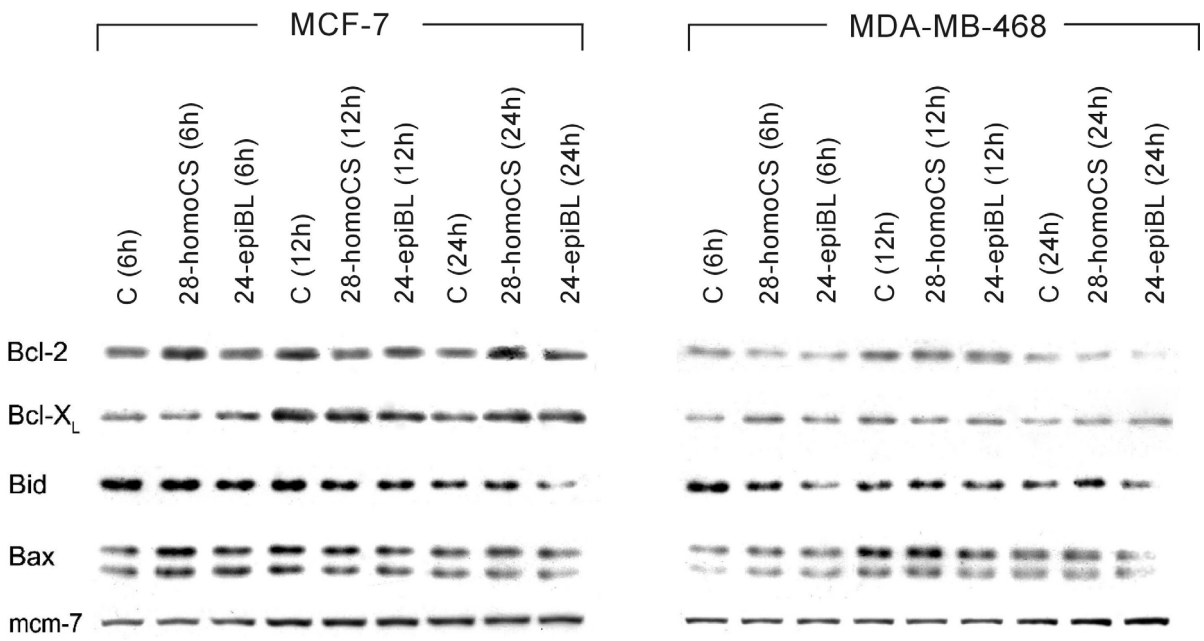

MCF-7
MDA-MB-468
C (6h)
28-homoCS (6h)
24-epiBL (6h)
C (12h)
28-homoCS (12h)
24-epiBL (12h)
C (24h)
28-homoCS (24h)
24-epiBL (24h)
Bcl-2
Bcl-X$_L$
Bid
Bax
mcm-7

**Figure legends**

**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-b) Control cells showing ER-α labeling restricted to nuclei. (c-d) Cells treated with 28-homoCS ($IC_{50}$) for 24 h. Note the appearance of bright cytoplasmic speckles of ER-α immunofluorescence. (e-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-b) Control cells. Note the specific cytoplasmic labeling of ER-β immunofluorescence. (c-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-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.

**Fig. 2.** Western blot comparison of levels in cell cycle-related proteins (p53, MDM-2, p21, p27, cyclin $D_1$, pRb and pRb-P) 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.

**Fig. 3.** Western blot comparison of apoptosis-related proteins Bcl-2, Bcl-$X_L$, Bax, Bid 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.

### 9.3. Supplement III

Hoffmannová, L., Steigerová, J., Oklešťková, J., Kohout, L., Kolář, Z., Strnad, M. Anticancer structure-activity relationships of cholestane brassinosteroid derivatives. Manuscript in prep.

**Anticancer structure-activity relationships of cholestane brassinosteroid derivatives**

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**Key words:** anticancer activity, brassinosteroid, structure-activity relationships

**Abstract**: A number of novel brassinosteroid analogues, mostly cholestane type brassinosteroid derivatives and closely related substances, were synthesized and tested to determine relationships between chemical structure and anticancer activity. Several human normal and cancer cell lines have been used for determination of anticancer activity. The study was also performed on hormone-sensitive and hormone-insensitive (MCF-7/MDA-MB-468) breast cancer cell lines. Most of the tested cholestane type brassinosteroid derivatives inhibited cell growth in all cancer cell lines examined, without affecting the growth of normal cells.

## Introduction

The brassinosteroids (BRs) represent a class of endogenous plant growth regulators of ubiquitous occurrence in the plant kingdom. They possess strong growth-promoting activity, and have been evaluated for use in improving crop yield and quality and tolerance to stress factors (Cutler et al., 1991; Nemhauser and Chory, 2004). In the few last years medical applications of BRs have also already been published. These studies reported that some natural BRs, such as 28-homocastasterone (28-homoCS) or 28-homobrassinolide and their synthetic analogues, have *in vitro* antiviral activity against

several pathogenic viruses (Wachsman et al., 2000; Wachsman et al., 2002). The effects of natural BRs and related analogues on a range of human cancer cell lines have also been described and it has been shown that natural BRs can inhibit the proliferation of various cancer cell lines at micromolar concentration (Malíková et al., 2008).

Much research has been directed towards understanding the physiological, biochemical and molecular properties of BRs, and to the synthesis of new BR analogues. There is a paucity of information on the structure-activity relationships (SAR) of BRs. Such information would provide a better understanding of the bioactivity and mode of action of these interesting compounds (Brosa, 1999). Early SAR studies established that several key structural features were required for strong bioactivity in brassinosteroids (Wada and Marumo, 1981; Thompson et al., 1982; Kohout et al., 1991; Brosa, 1997, 1999; Kripach et al., 1999; Back and Pharis, 2003). Among the modifications to the BR molecule which affect its biological activity are: in ring A, variation in configuration, position and number of hydroxyl groups and 2,3-seco; in ring B, substitutions with 6-keto, lactones, lactams and sulfo derivatives, exo- and endo cyclic double bonds, unsubstituted, and 5,6-seco; whether the junction of AB rings is *trans* or *cis;* and the structure of the side chain, viz. the number of carbon atoms it contains, the number, position and configuration of the hydroxyl groups, the nature of other substituents (such as alkyl, carboxyl, ether, ester, amide, phenyl and some other groups) and the presence and the position of double bonds (Kripach et al. 1999).

Some general conclusions regarding the SAR of brassinosteroids are possible. The presence of a 2α, 3α-diol moiety is necessary for optimum biological activity. The absence of either A-ring hydroxyl group or a change in their configurations is accompanied by a significant loss of bioactivity. The seven-membered B-ring lactone is needed for the highest level of activity, although some modifications to the B-ring are tolerated. For example, castasterone, with a 6-keto function, is strongly active, but less so than brassinolide, while typhasterol, which contains only one hydroxyl group in the ring-A, as well as a 6-keto moiety, is the least active of the three compounds. The complete absence of an oxygen function in the B-ring results in very low or insignificant activity. The side chain hydroxyl groups also play an important role in the brassinosteroid bioactivity. In general, a vicinal diol moiety with 22R,23R configuration affords optimum activity. Considerable variation is possible in the side-chain alkyl substituents. Thus, 24-epibrassinolide and 28-homobrassinolide display relatively strong bioactivity, although generally lower than that of brassinolide, but they are cheaper to

synthesize than brassinolide and so have seen frequent use in field trials (Back and Pharis, 2003).

In this study, we describe the anticancer structure-activity relationship between synthetic cholestane brassinosteroid derivatives and their anticancer activity in the Calcein AM cytotoxic test. We also present the effect of these compounds on the hormone-sensitive and hormone-insensitive (MCF-7/MDA-MB-468) breast cancer cell lines.

## Results and discussion

*Cytotoxicity of synthetic derivatives of brassinosteroids*

We investigated the effects of cholestane derivatives on the viability of normal and cancer cell lines of different histopathological origin. To evaluate the cytotoxic properties of several derivatives and some related steroids (cholesterol), we used the T-lymphoblastic leukemia cell line, CEM, the breast carcinoma cell line, MCF-7, the myeloma cell line, RPMI 8226, the cervical carcinoma cell line, HeLa, the human glioblastoma cell line, T98, and the normal human skin fibroblast cells, BJ. The cells were exposed to six 4-fold dilutions of each drug for 72 h prior to determination of cell survival. The $IC_{50}$ (concentration leading to 50% inhibition of viability) values obtained from Calcein AM cytotoxicity assay are presented in Table 1.

**Table 1**: $IC_{50}$ (μmol/l) assessed by Calcein AM assay of surviving cells. The results are means of three independent experiments performed in triplicate.

| Cholestane derivative No. | Compound | Cell line | | | | | |
|---|---|---|---|---|---|---|---|
| | | CEM | MCF-7 | RPMI 8226 | T98 | HeLa | BJ |
| | Cholesterol | >50 | >50 | 38,4 | >50 | >50 | >50 |
| 1 | 4176 | 8,4 | 37,0 | | | | 24,8 |
| 2 | Hel 176 | 15,5 | 50,0 | | | | >50 |
| 3 | Hel 175 | 13,3 | 43,0 | | | | 43,0 |
| 4 | 2096 | 19,7 | 50,0 | | | | >50 |
| 5 | KAR 2251 | 9,7 | 31,6 | | | | 42,3 |
| 6 | 2558 | 10,4 | 19,0 | 9,0 | 38,3 | 21,5 | 44,6 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 7 | 2015 | 13,9 | 45,0 | | | | 48,0 |
| 8 | KAR 2294 | 14,7 | 25,0 | | | | 47,5 |
| 9 | 2532 | 14,4 | 50,0 | | | | >50 |
| 10 | 2927 | 12,5 | 47,0 | | | | |
| 11 | 2535 | 11,0 | 40,0 | | | | 47,0 |
| 12 | KAR 4238 | 16,2 | 49,0 | | | | 32,2 |
| 13 | KAR 3090 | 11,8 | 13,7 | | | | 10,7 |
| 14 | II A | 9,2 | 50,0 | | | | |
| 15 | 4190/B | 7,7 | 26,0 | 16,0 | 44,1 | 35,0 | 45,4 |
| 16 | 4192 | 8,4 | 22,0 | | | | 42,7 |
| 17 | 1933 | 4,5 | 18,0 | 4,7 | 46,3 | 9,0 | 42,8 |
| 18 | KAR 2938 | 12,8 | 43,0 | | | | 48,2 |
| 19 | KAR 3377 | 20,5 | 41,0 | 5,2 | 41,1 | | 41,2 |
| 20 | KAR 2696 | 10,2 | 46,0 | | | | 42,8 |
| 21 | 2827 | 44,5 | 50,0 | | | | >50 |
| 22 | 2088 | 18,5 | 43,2 | | | | 41,0 |
| 23 | 1825 | 17,6 | 47,0 | | | | >50 |
| 24 | 211/B | 19,1 | 50,0 | | | | >50 |
| 25 | 1682 | 15,5 | 50,0 | | | | >50 |
| 26 | 1609 | 8,8 | 25,0 | 3,9 | 44,9 | 19,9 | 35,9 |
| 27 | M20 | 15,0 | 50,0 | | | >50 | >50 |
| 26 | M16 | 6,3 | 12,0 | 6,4 | 16,0 | 8,7 | 8,2 |
| 29 | SK 189 | 9,9 | 44,0 | | | | 12,7 |
| 30 | SK 182 | 12,1 | 14,0 | 15,0 | 46,6 | 15,9 | 12,7 |
| 31 | M21 | 13,1 | 47,0 | 28,0 | 47,9 | 40,6 | 22,0 |
| 32 | 2292 | 10,0 | 37,0 | 19,7 | 46,6 | 34,4 | 44,8 |
| 33 | MP5 | 18,1 | 38,0 | 24,5 | 42,2 | 40,7 | 28,5 |
| 34 | M51 | 15,3 | 24,0 | 8,6 | 44,7 | 19,0 | 44,4 |
| 35 | M4 | 31,4 | 50,0 | | | | 36,3 |
| 36 | M22 | 17,5 | 40,0 | 16,1 | 45,1 | 33,1 | 31,5 |
| 37 | M11/C | 6,9 | 10,0 | 6,7 | 46,2 | 8,7 | 41,4 |
| 38 | M11/B | 27,4 | 35,0 | 8,8 | 42,9 | 8,5 | 7,1 |
| 39 | 1966 | 6,8 | 16,0 | 8,5 | 21,4 | 13,2 | 44,6 |
| 40 | SK 188 | 8,3 | 21,5 | 12,5 | 43,0 | 9,8 | 10,5 |
| 41 | SK 327 | 11,2 | 10,0 | | | | |
| 42 | KAR 6299 | 5,2 | 19,0 | 10,5 | 15,2 | 11,9 | 18,4 |
| 43 | KAR 2001 | 9,6 | 50,0 | 12,9 | 49,9 | | >50 |
| 44 | C4 | 11,6 | 50,0 | 14,4 | | | >50 |
| 45 | C6 | 17,8 | 13,0 | 16,7 | | | >50 |
| 46 | SK 468 | 4,3 | 25,0 | 1,7 | 17,2 | 14,7 | >50 |

The treatment with cholestane derivatives resulted in a potent and dose- dependent decrease in the viability of CEM, RPMI 8226 and HeLa cells, albeit at different concentrations. Only effective cholestane derivatives are shown here. Inhibitory effects of KAR 6299 and 1966 on breast cancer cell lines MCF-7 and MDA-MB-468 are shown in Figure 2. Cholestane derivative SK468 was the most potent compound on CEM cells ($IC_{50}$: 4.3 μmol/l) and on RPMI 8226 cells ($IC_{50}$ 1.7 μmol/l). In addition to SK468, a high cytotoxicity was also observed after application of 1933, KAR 6299 and 1966. Cholesterol, which is non-cholestane derived plant and animal sterol, is however inactive or exhibited almost zero cytotoxic activity. Cholesterol also showed only minimal anticancer activity ($IC_{50}$ >35 μmol/l) on the RPMI 8226 cell line. A striking observation from this data was that in the BJ human fibroblasts a cholestane derivative-mediated loss of viability was not observed. These results suggest that cancer cells and normal cells respond differentially to cholestane derivatives. At present, only few natural agents are known to posses the potential ability for selective/preferential elimination of cancer cells without affecting growth of normal cells. This study also provides the first evidence of cholestane derivatives as anticancer compounds with antiproliferative properties.

To conclude the most active compound from the SAR study of cholestane derivative cytotoxicity on cancer cells was KAR 6299, 1966 and SK468. Changing 6-oxo-7-oxalactone to 6-oxo functionality dramatically increases the growth inhibitory activity of the cholestane derivatives. Thus, KAR 6299 produced about a 3-times stronger response than M22. The 24R side chain is also a decisive group which increases the cytotoxicity of cholestane derivatives. Results in Table 1 point to the use cholestane derivatives as pharmaceuticals for inhibition of hyperproliferation in tumors.

To evaluate the effect of the selected cholestane derivatives on the viability of hormone-sensitive and hormone-insensitive cancer cell lines (MCF-7, MDA-MB-468, LNCaP and DU-145), we analyzed cell viability after cholestane treatment by MTT assay. The cytotoxic concentrations were reached with all of the analogues in all four cancer cell lines.

**Table 2**

$IC_{50}$ (μmol/l) concentrations of selected cholestane derivatives were determined by MTT assay as described above. The results are means of three independent experiments performed in triplicate.

| Compound | Cell line: $IC_{50}$ (μmol/l) | | | |
|---|---|---|---|---|
| | MCF-7 | MDA-MB-468 | LNCaP | DU-145 |
| **M16** | 27.8 | 27.8 | 13.0 | 26.0 |
| **M11/C** | 27.4 | 17.3 | 16.5 | 26.0 |
| **1966** | 25.9 | 18.6 | 29.0 | 45.0 |
| **KAR 6299** | 29.5 | 14.8 | 27.6 | 24.0 |

All cholestane derivatives inhibited cell growth in a dose-dependent manner in all cancer cell lines. Derivative KAR 6299 exhibited cytotoxic effects at lower concentration tested (Figure 3). Employing the MTT assay, we also determined the concentrations of cholestane derivatives leading to 50% inhibition of cell viability ($IC_{50}$) after 24 h (Table 2). Interestingly, there was little difference between the potency of cholestane derivatives on different cell lines as $IC_{50}$ values were consistently in the micromolar range. The only exceptions were MDA-MB-468 estrogen-insensitive breast cancer cell line and DU-145 androgen-insensitive prostate cancer cell: these cells were more resistant than the others to the application of cholestane derivatives.

*Activity of caspases-3/7after cholestane derivative treatment*

We determined the activity of caspase-3/7 in MDA-MB-468 cells exposed to KAR 6299 or 1966 using a fluorogenic substrate Ac-DEVD-AMC and/or caspase-3/7 inhibitor Ac-DEVD-DHO. Cells were treated in a time- and dose-dependent manner with drug concentrations exceeding their $IC_{50}$ values (5, 15, 30, 40 μM) for 24 and 48 h. KAR 6299 strongly induced the activity of caspase-3/7; after treatment for 24 h at a concentration of 30 μM, a fivefold increase in the effector caspases was observed compared to the untreated control. This decreased to a threefold increase after 40 μM treatment and/or after 48 h (Figure 7). Unlike KAR 6299, derivative 1966 only weakly affected the activity of caspases-3/7; a twofold enhancement of the activity was detected after 24 h and after longer treatment and higher concentrations the caspase-3/7 activity decreased (data not shown).

*Flow cytometry analysis of the cell cycle and apoptosis of cholestane derivative treated cells*

The flow cytometry analysis showed an increase in the proportion of the cells in $subG_1$ phase of the cell cycle (apoptotic cells) in MCF-7 and MDA-MB-468 cell lines after treatment with cholestane derivatives KAR 6299 or 1966 (Table 3). In MCF-7 cells, KAR 6299 treatment increased the proportion of cells in $subG_1$ and $G_0/G_1$ phase and decreased the proportion in S phase. Treatment with 1966 caused a strong increase of $subG_1$ phase (apoptotic cells) and a concomitant decrease in other cell phases (Figure 4 A). In the MDA-MB-468 breast cancer cell line treatment with KAR 6299 enhanced the proportion of cells in $subG_1$ and decreased the proportion in S phase. Treatment with 1966 caused only a slight increase in the proportion of cells in $subG_1$ in MDA-MB-468 (Figure 4 B). Cholestane derivatives caused a decrease in the percentage of cells of both cell lines found in the S phase of the cell cycle, but a stronger effect was detected in MCF-7 estrogen-sensitive breast cancer cells (Figure 4). It seems that the actions of cholestane derivatives are connected with steroid receptors.

**Table 3**

Distribution of cell cycle phases in MCF-7 and MDA-MB-468 cells after flow cytometry analysis. Histograms of the treated cells were compared with control untreated cells. The percentages indicate proportion of cells in $subG_1$ fraction and $G_0/G_1$, S, $G_2/M$ phases of the cell cycle.

| *Cell line* | *Control / compound (40 μM; 24 h)* | *Apoptosis* | *Cell cycle distribution* | | |
|---|---|---|---|---|---|
| | | *$subG_1$* | *$G_0/G_1$* | *S* | *$G_2/M$* |
| MCF-7 | **Ctrl** | 2% | 54% | 17% | 27% |
| | **KAR 6299** | 2% | 64% | 11% | 22% |
| | **1966** | 20% | 41% | 13% | 27% |
| MDA-MB-468 | **Ctrl** | 3% | 68% | 15% | 13% |
| | **KAR 6299** | 11% | 60% | 13% | 16% |
| | **1966** | 15% | 57% | 9% | 10% |

*Effect of cholestane derivatives on the steroid receptors*

MCF-7 control cells exhibited high expression of both ER-α and ER-β. MDA-MB-468 cells expressed relatively low levels of ER-β and, as expected, no ER-α. Treatment with 1966 led to downregulation of ER-α expression in MCF-7 cells in a concentration- and

time-dependent manner, while ER-β expression was reduced only after 48 h of treatment (40 and 30 μM). In the MDA-MB-468 cells, ER-β expression was on the threshold of the detection limit. AR expression decreased in MCF-7 cells after 48 h incubation with 40 μM.

A concentration- and time-dependent decrease was found in ER-α expression after treatment with KAR 6299. (Figure 6 A)

*Western blot analysis of pro- and anti-apoptotic proteins in breast cancer cells*

Western blot analysis was used to detect changes in apoptosis-related protein expression in breast cancer cell lines. To monitor changes, we collected the cells after 24 and 48 h treatment with cholestane derivatives at 5, 15, 30 and 40 μM. Changes in apoptosis-related protein expression after treatment with cholestane derivatives are shown in Figure 5. Expression of a tumor suppressor protein p53 was observed in controls of breast cancer cell lines and KAR 6299 and 1966 caused its enhanced expression after 24 and 48 h. Expression of the protein increased strongly following treatment with 30 μM KAR 6299 and 1966**,** but at 40 μM protein expression slightly decreased. Enhanced expression of p53 correlated with a decreased expression of antiapoptotic Mcl-1 protein in 30 and 40 μM treatments with KAR 6299 and 1966. At the same concentrations and time of treatment, a decrease in phosphorylation of pRb S780 was observed, indicating the commencement of apoptosis. Antiapoptotic Bcl-2 protein increased after 30 μM KAR 6299 and 1966 treatment (24, 48 h). (Figure 5 A, B)

It is known that the mechanism of initiation of apoptosis is mediated by caspase cascade activation (Budihardjo et al., 1999). Caspase-3 is an executioner protease that results in the cleavage of PARP and subsequent DNA degradation and apoptotic death (Allen et al., 1998; Cain et al., 2002). These results confirm that cholestane derivatives KAR 6299 and 1966 can support apoptosis with caspase-3 activation (Figure 7) and modulation of protein Bcl-2 (Figure 5).

## Experimental

*Materials*

The synthetic analogues of brassinosteroids and related compounds (Figure 1) tested in these studies were synthesised at the Institute of Organic Chemistry and Biochemistry, AVCR, Prague. Stock solutions (10mmol/l) were prepared by dissolving the appropriate

quantity of substance in DMSO. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, F-12 medium, fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from Sigma (MO, USA) and 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Serva Electrophoresis (Heidelberg, Germany).

*Cell Cultures*

The following cell lines used for screening were obtained from the American Type Culture Collection (Manassas, VA, USA): T-lymphoblastic leukaemia CEM; breast carcinoma MCF-7 (estrogen-sensitive); breast adenocarcinoma MDA-MB-468 (estrogen-insensitive); prostate carcinoma DU-145 (androgen-insensitive); prostate carcinoma cell line LNCaP (androgen-sensitive); and human foreskin fibroblasts BJ (Ramirez et al., 2004). MCF-7 cells were cultured in F-12 medium (Sigma, MO, USA) and all others cells were cultured in DMEM medium (Sigma, MO, USA). All media used were supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. The cell lines were maintained under standard cell culture conditions at 37°C and 5% $CO_2$ in a humid environment. Cells were subcultured twice or three times a week using the standard trypsinization procedure.

*Calcein AM Cytotoxicity Assay*

Suspensions with approximately $1.25 \times 10^5$ cells/ml were distributed in 96-well microtiter plates and after 12 h of stabilization the BRs to be tested were added at the desired concentrations in DMSO. Control cultures were treated with DMSO alone, and the final concentration of DMSO in the reaction mixture never exceeded 0.6%. In most cases six serial 4-fold dilutions of the test substances were added at time zero in 20 µl aliquots to the microtiter plate wells and the highest final concentration in the wells was 50 µM. After incubation for 72 h, Calcein AM solution (2 µM, Molecular Probes) was added and the cells were incubated for a further hour. The fluorescence from viable cells was then quantified using a Fluoroskan Ascent fluorometer (Microsystems). Each compound was tested in triplicate and the entire test was repeated at least three times. The $IC_{50}$ value of each tested substance (the concentration reducing the number of

viable cells to 50%), was calculated from the obtained dose response curves (Kryštof et al., 2005).

*Cell Viability Assay (MTT)*

Cell viability was assessed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to determine the $IC_{50}$ concentrations of the studied agents as described previously. The cells were seeded at $4.5\times10^3$ (MCF-7, DU-145) or $5\times10^3$ (MDA-MB-468, LNCaP) cells per well in 96-well plates in medium containing steroid-stripped serum. Cells were grown for 24 h (MCF-7, MDA-MB-468, DU-145) or 48 h (LNCaP). The cells (70% to 80% confluent) were treated with cholestane derivatives for 6, 12 and 24 h in cell culture medium. Cells that were used as controls were incubated with the maximum used amount of steroid diluent DMSO only. The concentration leading to 50% inhibition of viability ($IC_{50}$) after 24 h was determined by measuring MTT reductase activity. The absorbance was read at 570 nm using an ELISA reader Labsystem Multiscan RC. The viability of treated cells was related to the viability of control vehicle-treated cells, which was set at 100%. Each experiment was performed in triplicate and independently repeated at least four times.

*Caspases-3/7 activity assay*

Treated cells were harvested by centrifugations and homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, inhibitors of proteases, pH 7.4) on ice for 20 min. The homogenates were clarified by centrifugation at 10,000g for 20 min at 4ºC. The proteins were quantified by the Bradford method and diluted to the same concentration**.** Lysates were then incubated for 1 h with 100 mM Ac-DEVD-AMC as a substrate (Sigma–Aldrich) in an assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 mM Ac-DEVD-CHO as a caspase-3/7 inhibitor (Sigma–Aldrich). The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems) at 346/442 nm (excitation/emission wavelength).

*Flow cytometry analysis*

Flow cytometry was used to evaluate the proportion of cells in the particular phases of the cell cycle, including sub$G_1$ peak detection. After 24 and 48 h of incubation, control and treated cells were washed twice with PBS and centrifuged at 360 g for 10 min at 4°C. They were then washed in cold PBS twice, and fixed with chilled ethanol (70%; v/v) by low-speed vortexing. For detection of DNA content, propidium iodide staining was used. The cells were analyzed using a Cell Lab Quanta flow cytometer (Beckman Coulter, CA, USA).

*SDS–polyacrylamide gel electrophoresis and immunoblotting*

For immunoblotting, harvested cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 mM PMSF, 1 mM DTT, 10 mg/ml of aprotinin and leupeptin). Proteins in lysates were quantified by the Bradford method and then diluted with Laemmli electrophoresis buffer. Proteins were then separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad Laboratories, CA, USA) and stained with Ponceau S to check equal protein loading. The membranes were blocked with 5% (w/v) BSA and 0.1% Tween-20 in TBS for 2 h and probed with the specific primary antibodies overnight. After washing in TBS and TBS containing 0.1% Tween-20, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies and visualized with chemiluminescent detection reagent West Pico Supersignal (Thermo Fisher Scientific, Rockford, USA). To confirm equal protein loading, immunodetection was performed with the anti-PCNA monoclonal antibody. The experiments were repeated three times. Protein expression in treated cells was compared to that in untreated controls.

*Immunofluorescence labelling methods*

The cells were seeded at a density $1.4\times10^4$cells/cm$^2$ (MCF-7) or $1.6\times10^4$cells/cm$^2$ (MDA-MB-468) using the appropriate culture medium in 60-mm culture dishes with cover slips. Cells were grown for 24 h and then treated with 28-homoCS or 24-epiBL ($IC_{50}$) for 6, 12, and 24 h. After the given treatment period the cells were washed with PBS and fixed on the slides with cold acetone-methanol (1:1, v/v) for 10 min. Cells were labelled with antibodies to ER-α (clone 6F11, Novocastra; Newcastle, UK), ER-β (H-150, Santa Cruz; Santa Cruz, CA) and AR (Santa Cruz; Santa Cruz, CA) for 90 min at room temperature in the dark, washed with three changes of PBS, and incubated in

the appropriate fluorescently-conjugated secondary antibodies (goat-anti-mouse- or goat anti-rabbit-fluorescein or Texas Red, Jackson Immunoresearch). The cells were then washed three times in PBS and incubated with DAPI (50 μg/mL) for 10 min in the dark. The cover slips with cells were washed in deionized water and mounted on glass slides, using the hydrophilic medium Mowiol (Calbiochem; Fremont, CA) in glycerol-PBS (1:3, v/v) for fluorescence. Cells were visualized using a fluorescence microscope (BX50F, Olympus, Japan) and compared with untreated control cells.

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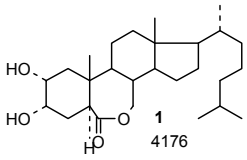
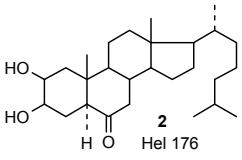
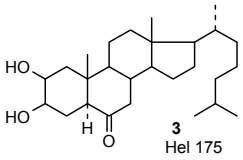
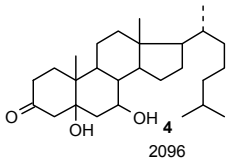
## Figures

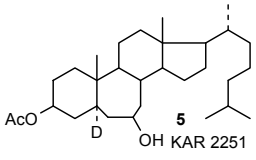
Figure 1

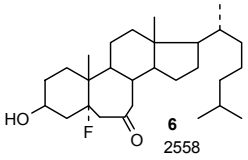


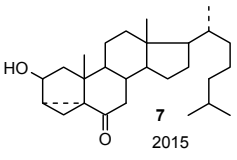



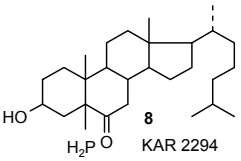



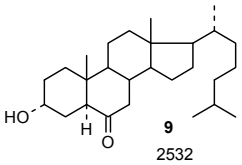



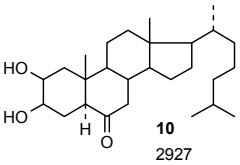



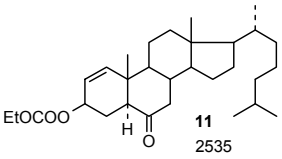



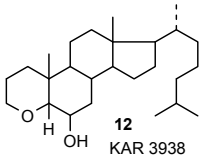



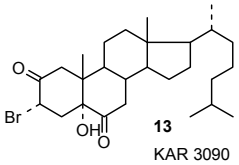



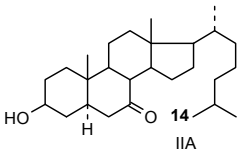



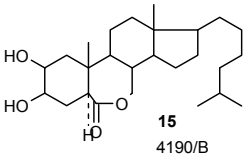



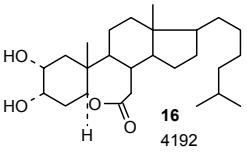



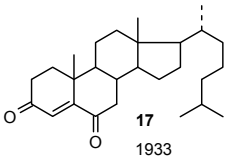



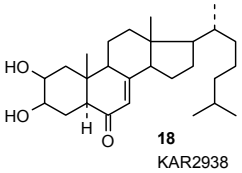



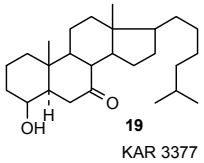



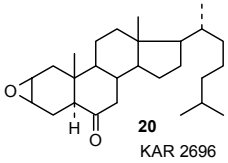



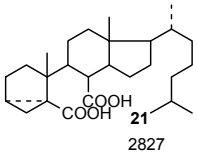



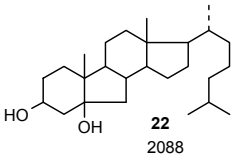



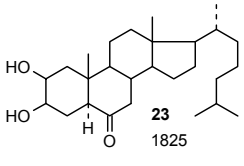



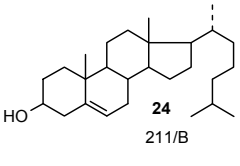



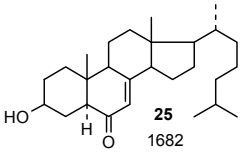



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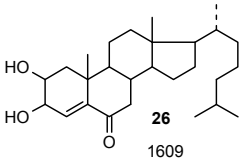


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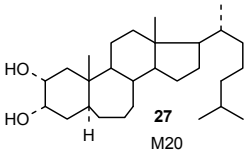


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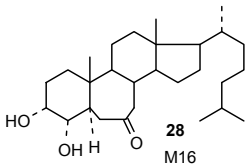


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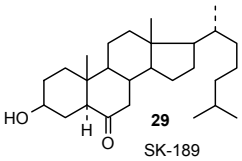


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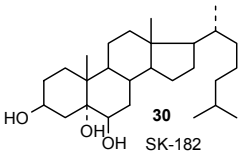


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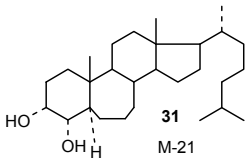


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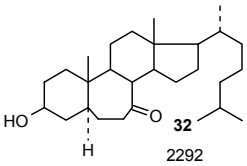


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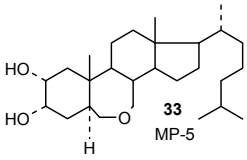


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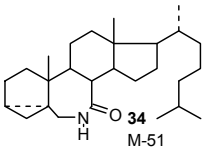


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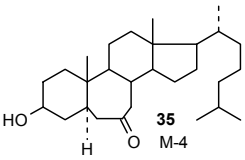
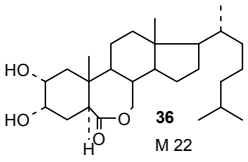
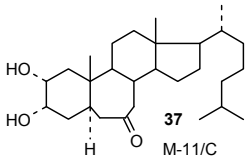


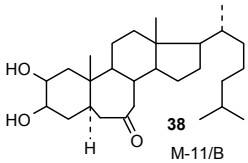

**35**
M-4



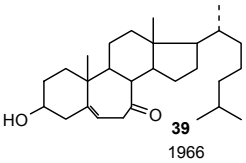

**36**
M 22



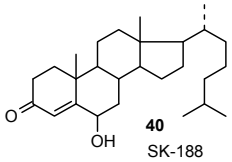

**37**
M-11/C



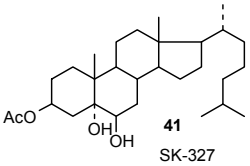

**38**
M-11/B



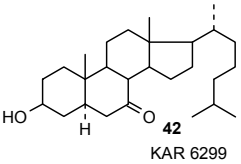

**39**
1966



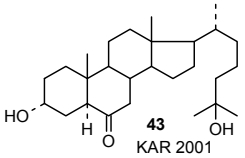

**40**
SK-188



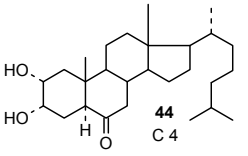

**41**
SK-327



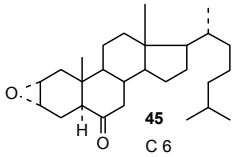

**42**
KAR 6299



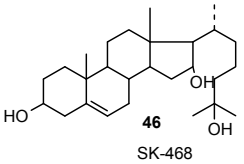

**43**
KAR 2001




**44**
C 4




**45**
C 6




**46**
SK-468

Figure 2

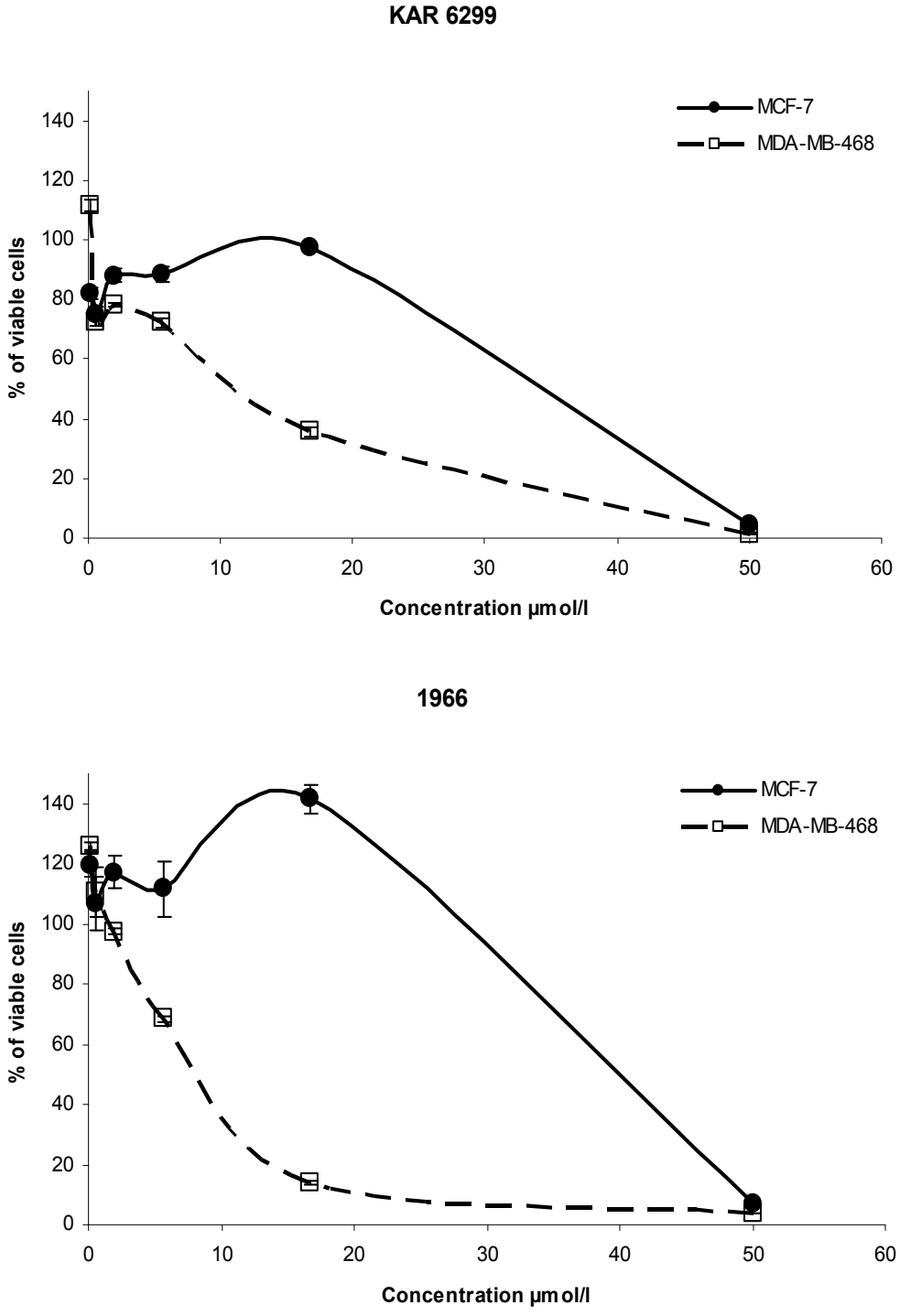
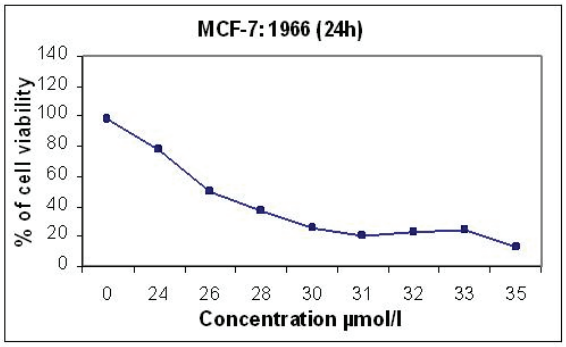
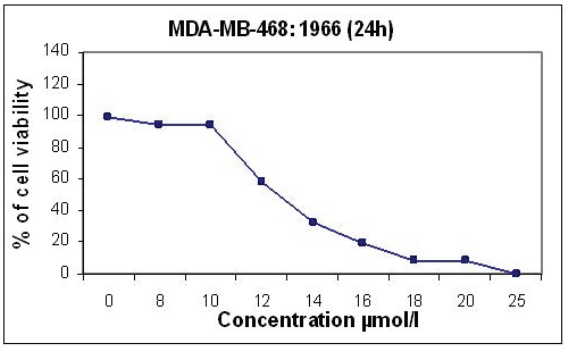
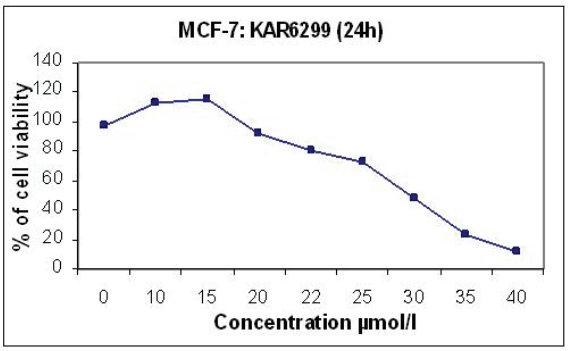

KAR 6299
MCF-7
MDA-MB-468
% of viable cells
Concentration µmol/l
0
20
40
60
80
100
120
140
0
10
20
30
40
50
60
1966
MCF-7
MDA-MB-468
% of viable cells
Concentration µmol/l

Figure 3


MCF-7: 1966 (24h)
% of cell viability
140
120
100
80
60
40
20
0
0 24 26 28 30 31 32 33 35
Concentration µmol/l


MDA-MB-468: 1966 (24h)
% of cell viability
140
120
100
80
60
40
20
0
0 8 10 12 14 16 18 20 25
Concentration µmol/l


MCF-7: KAR6299 (24h)
% of cell viability
140
120
100
80
60
40
20
0
0 10 15 20 22 25 30 35 40
Concentration µmol/l

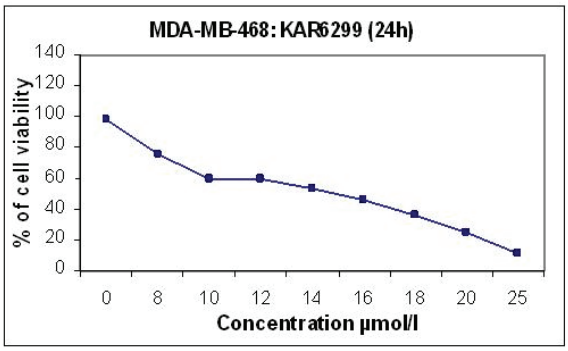

MDA-MB-468: KAR6299 (24h)
% of cell viability
140
120
100
80
60
40
20
0
0 8 10 12 14 16 18 20 25
Concentration µmol/l

Figure 4 **(A)**

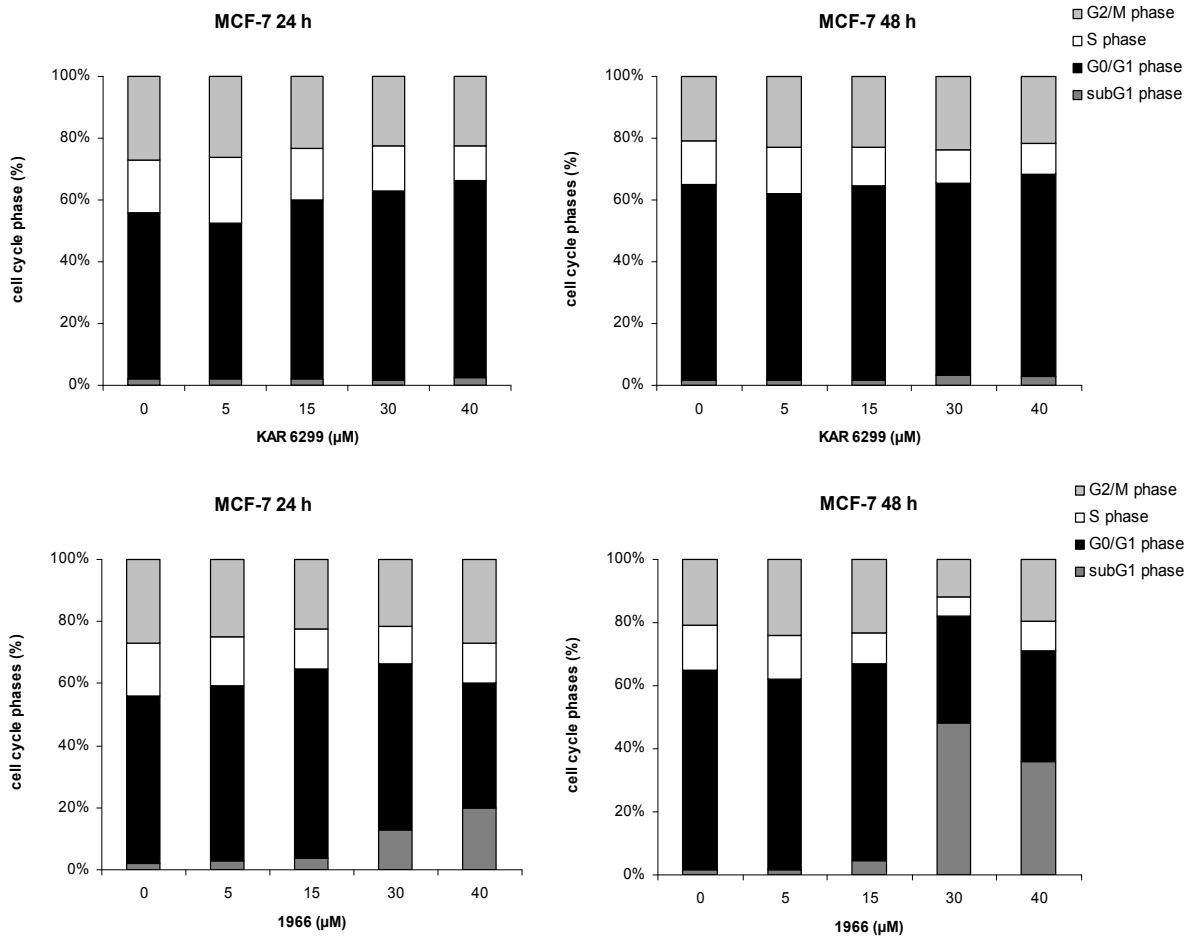

MCF-7 24 h
MCF-7 48 h
G2/M phase
S phase
G0/G1 phase
subG1 phase
cell cycle phase (%)
cell cycle phases (%)
100%
80%
60%
40%
20%
0%
0
5
15
30
40
KAR 6299 (µM)
1966 (µM)

Figure 4 **(B)**

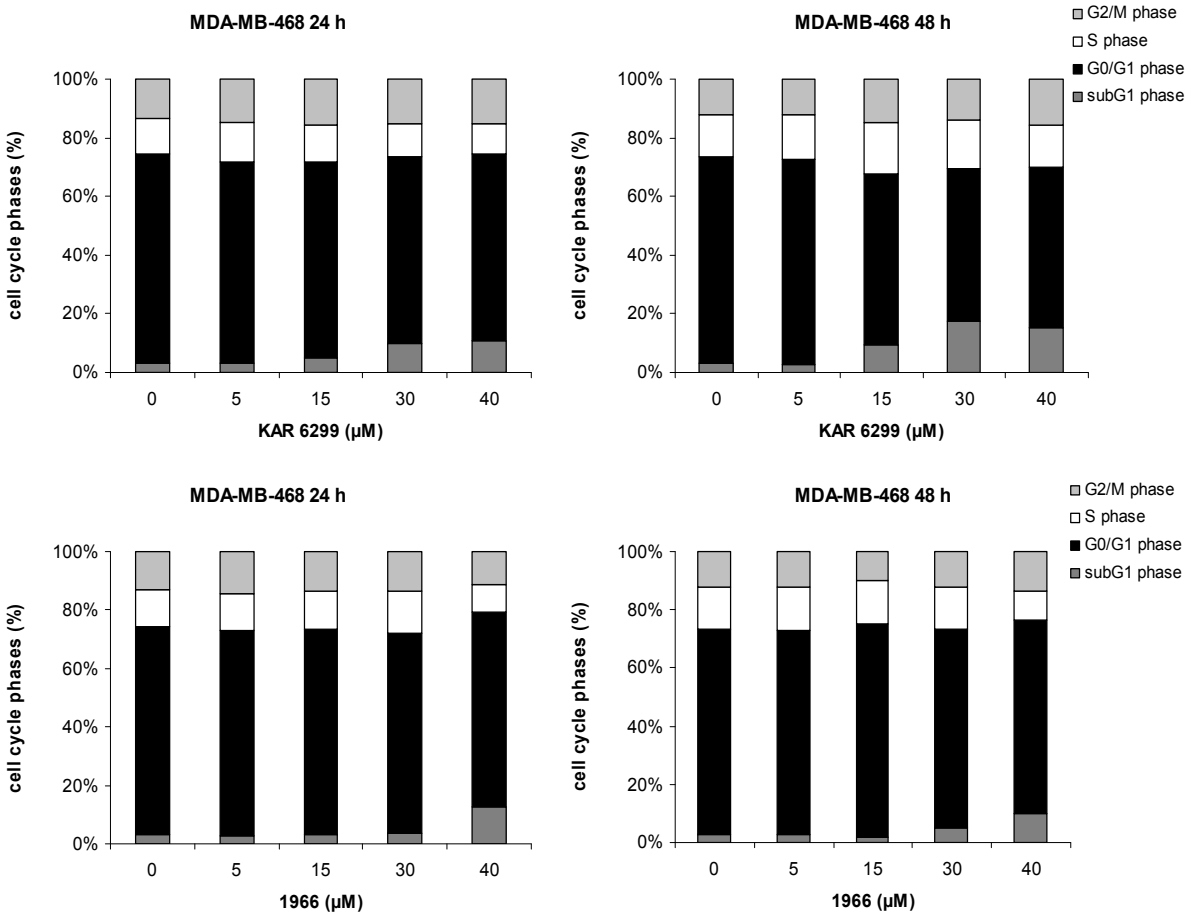
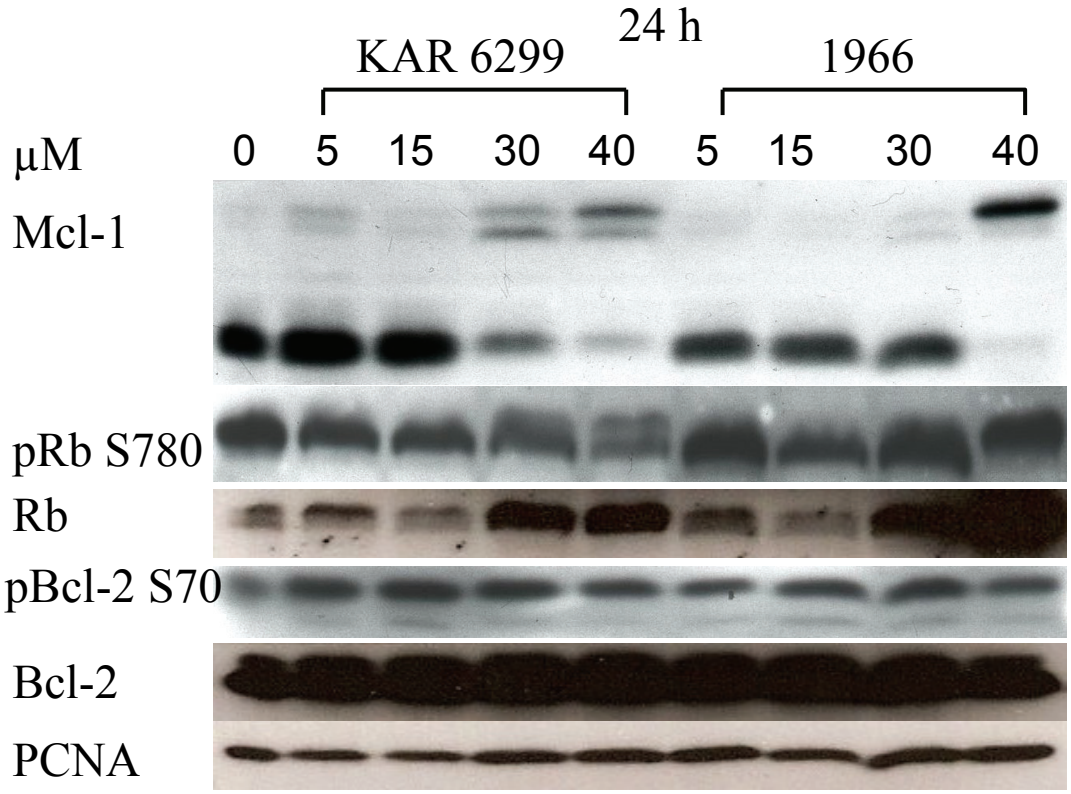

MDA-MB-468 24 h
MDA-MB-468 48 h
G2/M phase
S phase
G0/G1 phase
subG1 phase
cell cycle phases (%)
100%
80%
60%
40%
20%
0%
0
5
15
30
40
KAR 6299 (μM)
MDA-MB-468 24 h
MDA-MB-468 48 h
G2/M phase
S phase
G0/G1 phase
subG1 phase
cell cycle phases (%)
1966 (μM)

Figure 5 **(A)**


24 h
KAR 6299
1966
μM
0
5
15
30
40
5
15
30
40
Mcl-1
pRb S780
Rb
pBcl-2 S70
Bcl-2
PCNA

**(B)**

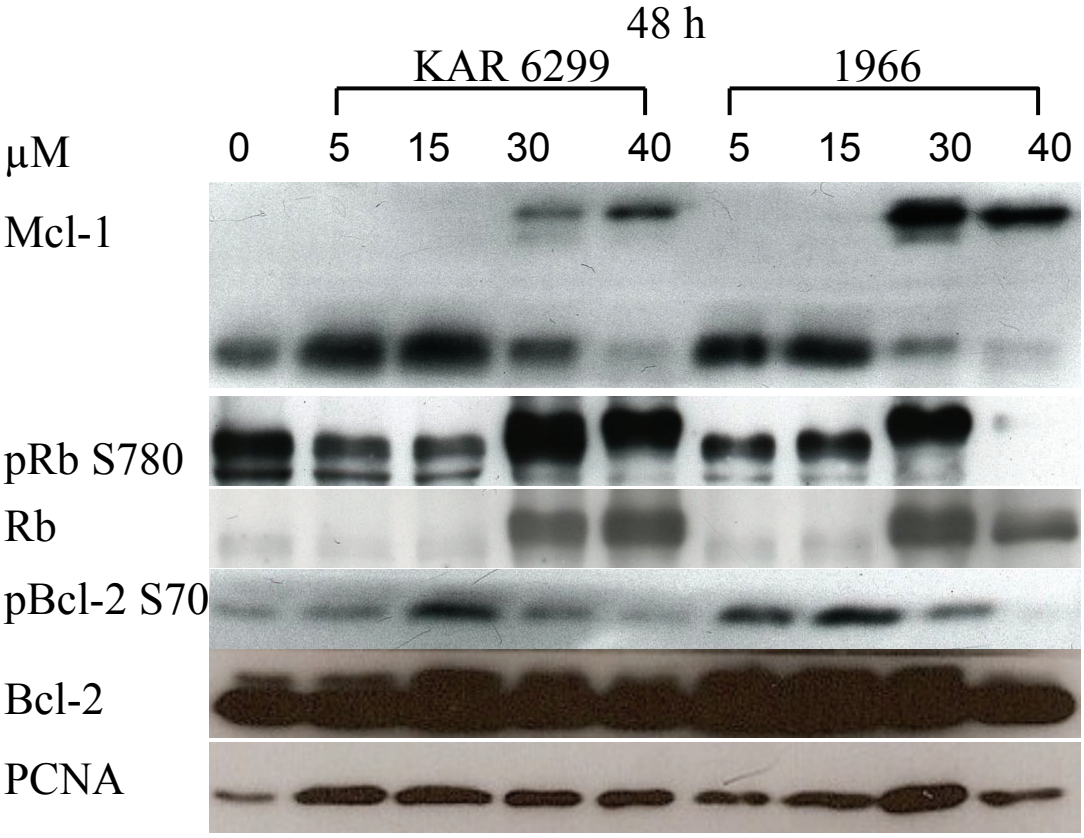
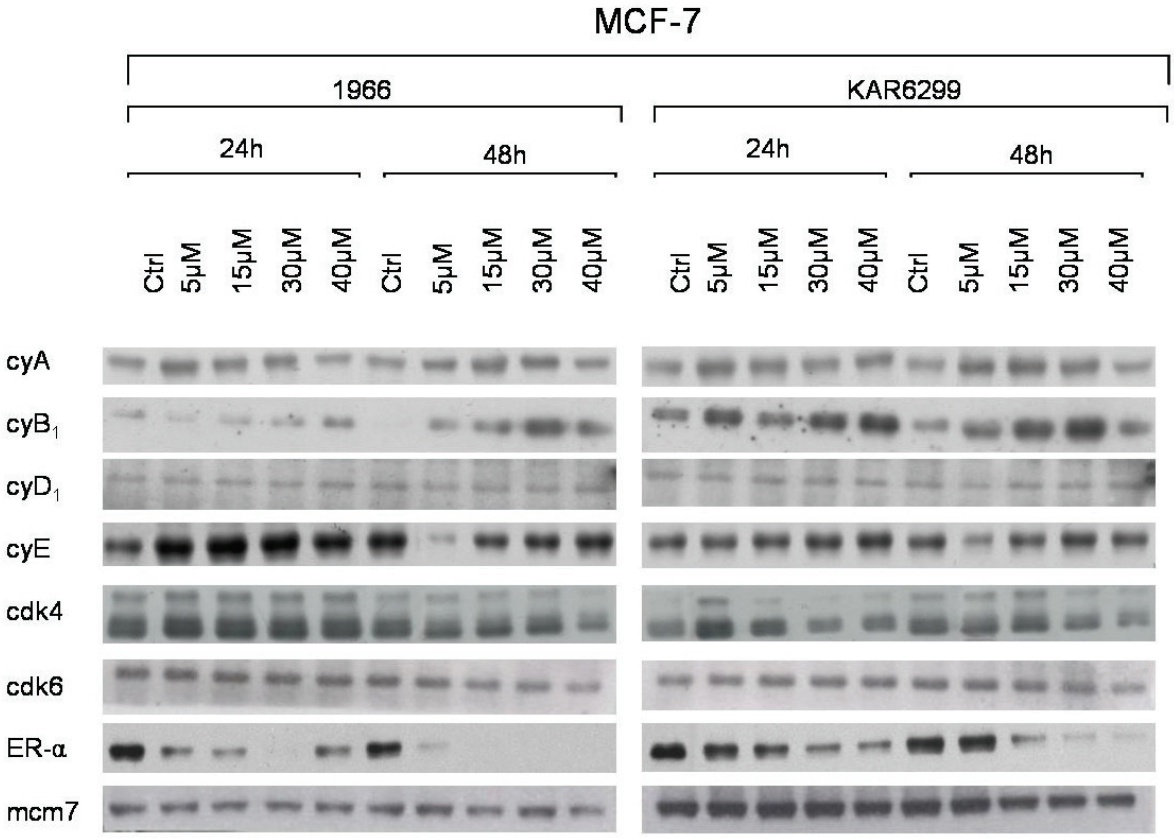

48 h
KAR 6299
1966
μM
0
5
15
30
40
5
15
30
40
Mcl-1
pRb S780
Rb
pBcl-2 S70
Bcl-2
PCNA

Figure 6 **(A)**


MCF-7
1966
KAR6299
24h
48h
24h
48h
Ctrl
5μM
15μM
30μM
40μM
cyA
cyB1
cyD1
cyE
cdk4
cdk6
ER-α
mcm7

**(B)**

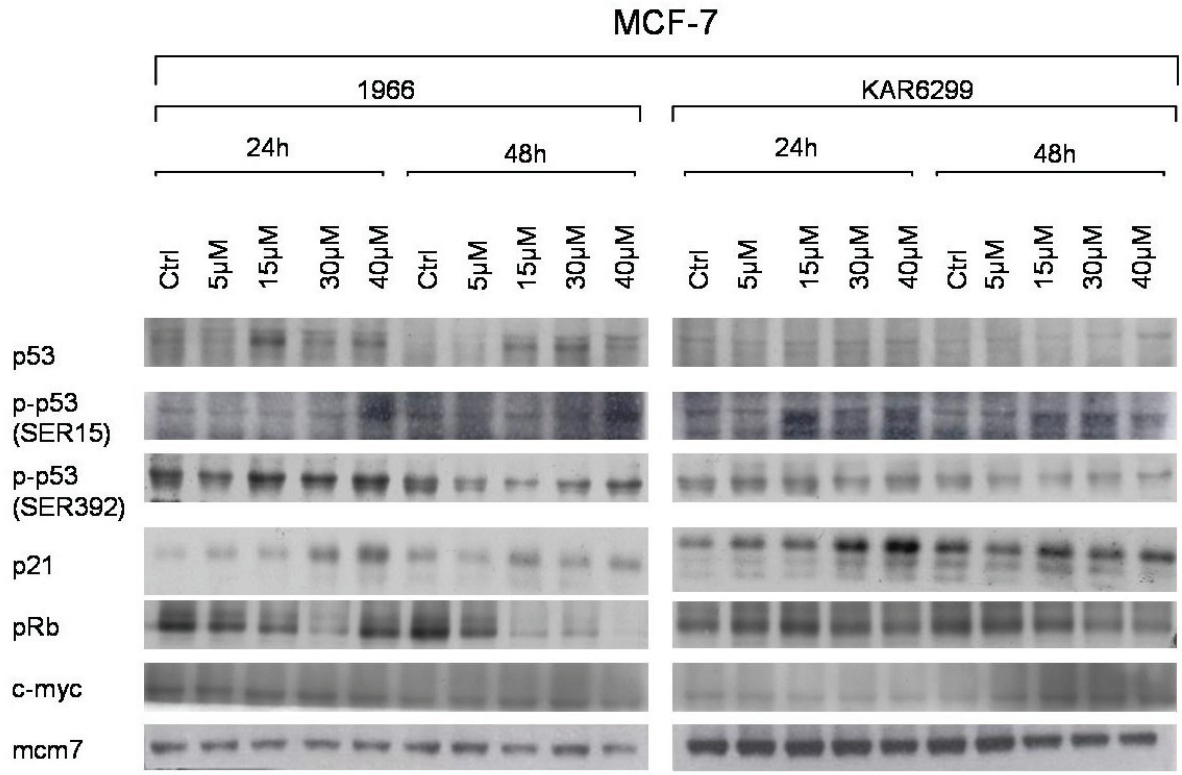

MCF-7
1966
KAR6299
24h
48h
24h
48h
Ctrl
5μM
15μM
30μM
40μM
p53
p-p53 (SER15)
p-p53 (SER392)
p21
pRb
c-myc
mcm7

Figure 7

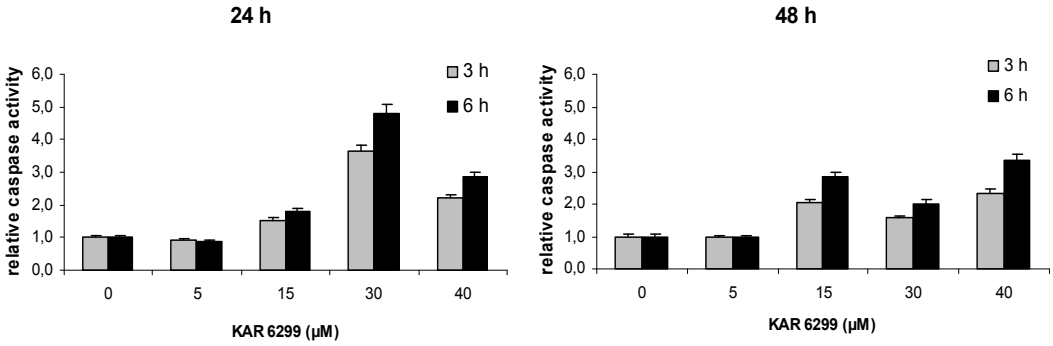
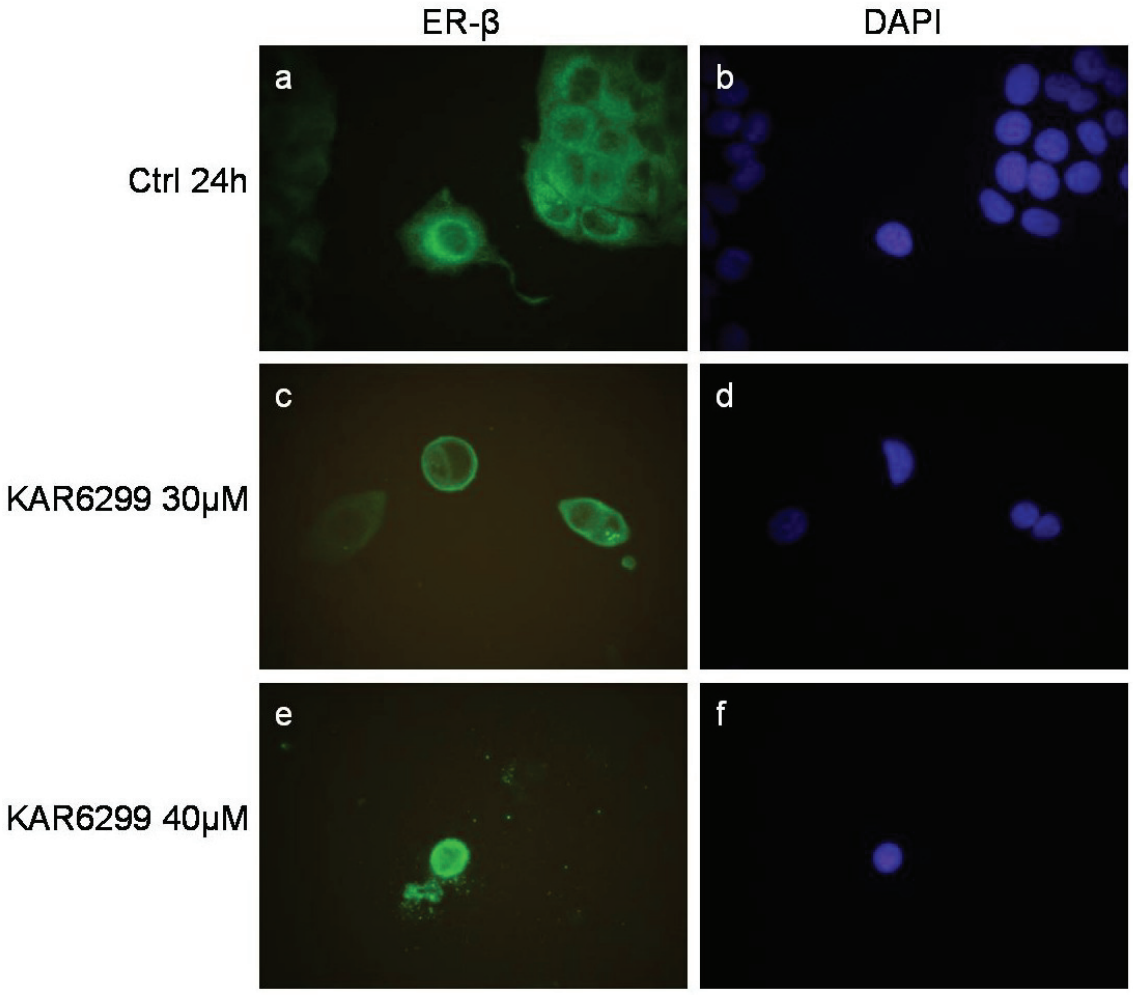



Figure 8 **(A)**



**(B)**

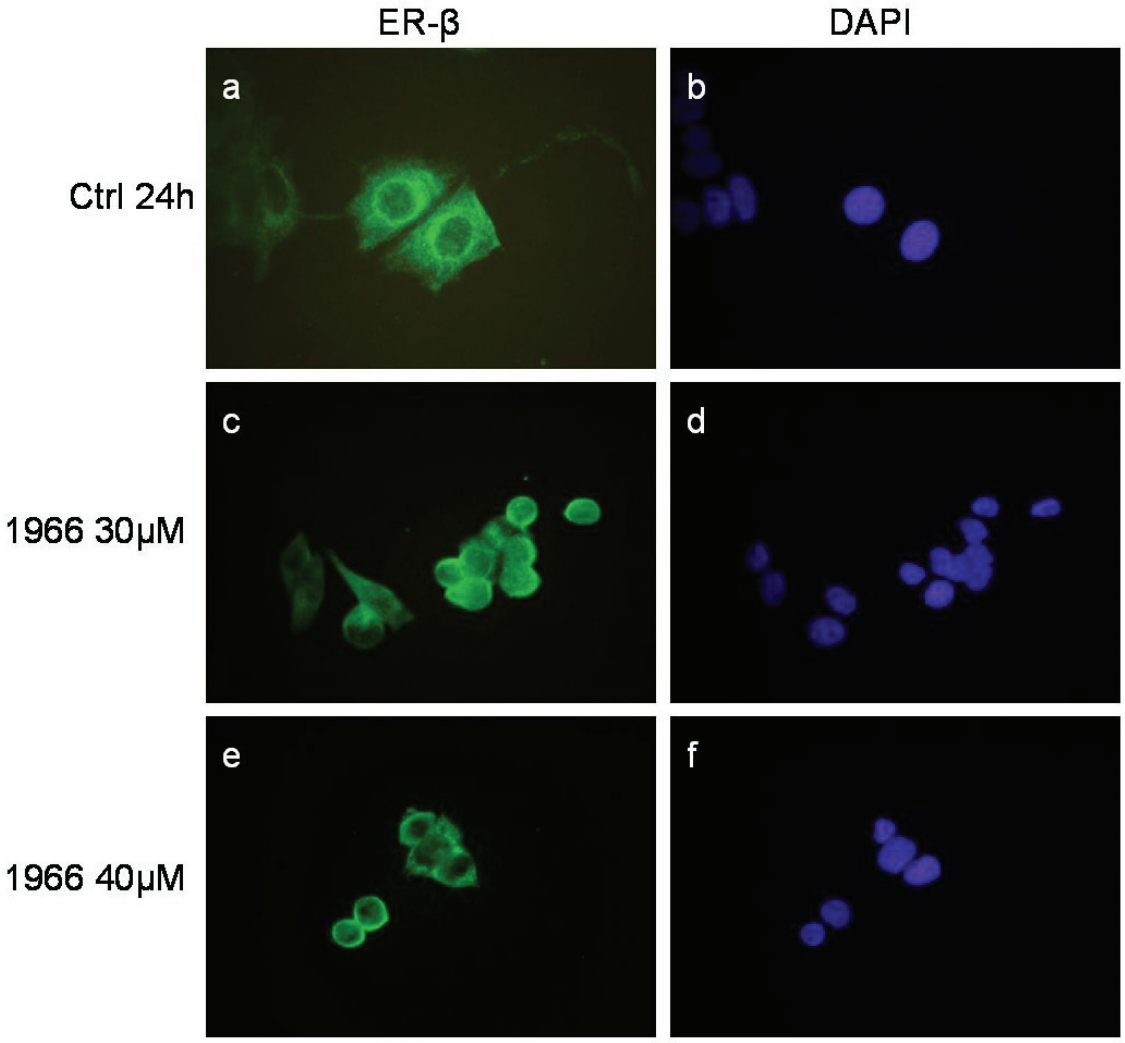


Figure 9

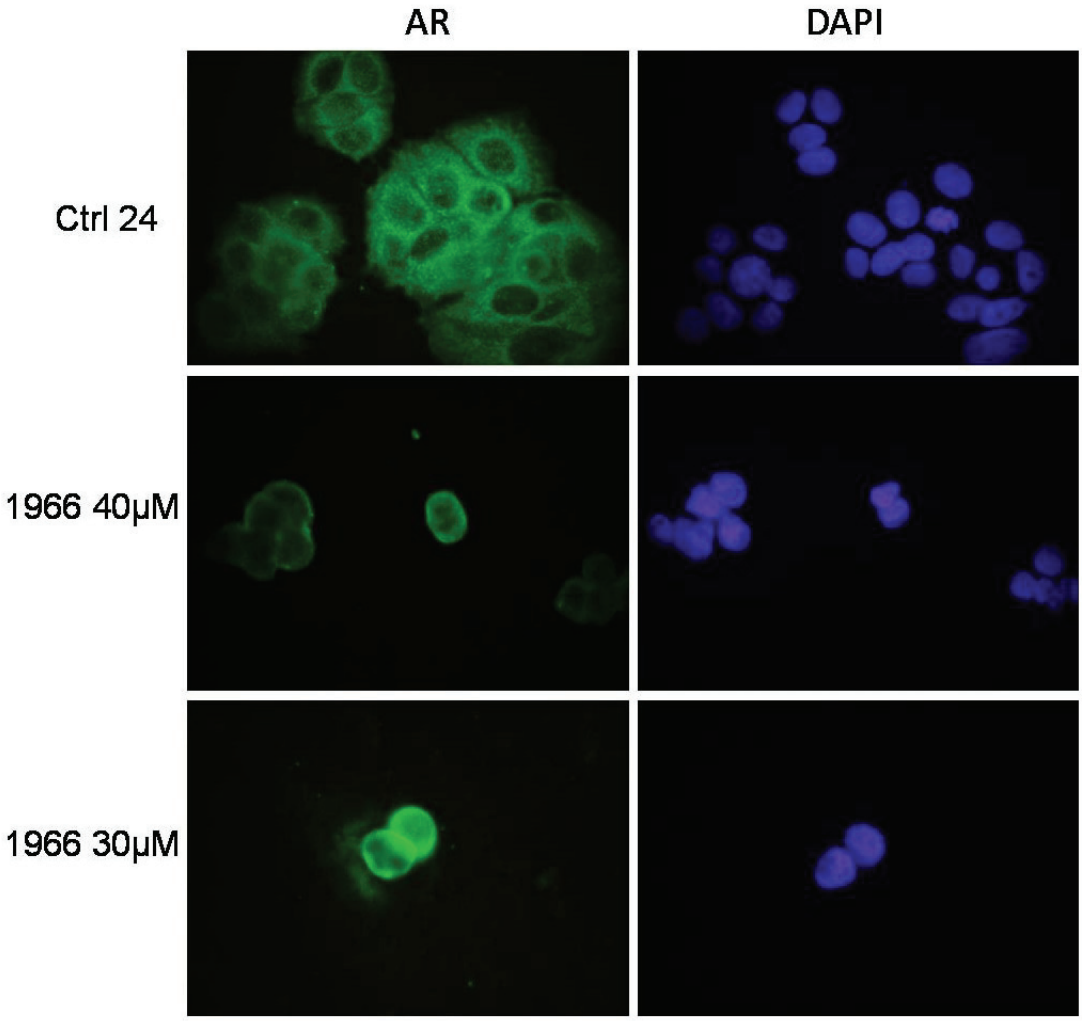


Figure 10

Fig.

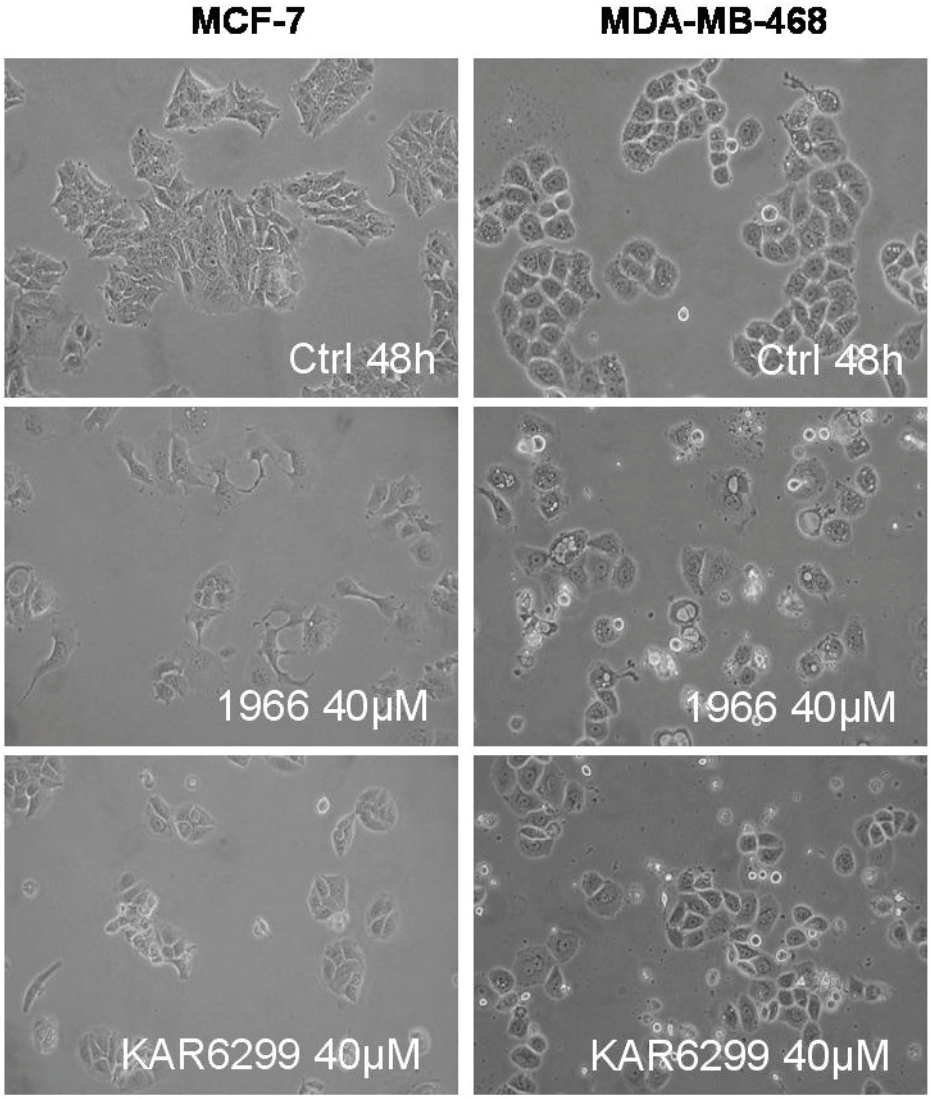


**Figure legends**

Figure 1: Structures of the cholestane derivatives of brassinosteroids.

Figure 2: The inhibitory effects of compounds KAR 6299 and 1966 on cell viability in MCF-7 and MDA-MB-468 cancer cell lines. The data represent the means of three experiments ± SD.

Figure 3: To evaluate the cytotoxic effects of the selected cholestane derivatives (KAR 6299 and 1966) on the hormone-sensitive and hormone-insensitive breast cancer cell lines (MCF-7 and MDA-MB-468), we analyzed cell viability after incubation with these compounds in MTT assays. Both analogues inhibited the growth of all of the cell lines in a dose-dependent manner. MDA-MB-468 cells were more sensitive than the MCF-7 cell lines to treatment with cholestane derivatives.

Figure 4: Analysis of the cell cycle and sub$G_1$ phase in MCF-7 **(A)** and MDA-MB-468 **(B)** cells: untreated controls are compared with cells treated with derivatives KAR 6299 and 1966 using a flow cytometer. The graphs represent cells in sub$G_1$ (apoptotic cells with a reduced DNA content), $G_1$, S, and $G_2$/M phases. Data are percentages (%) of the total number of cells in the respective phases.

Figure 5: Western blot analysis of apoptosis-related proteins (pRb, Rb, pBcl-2, Bcl-2, Mcl-1) in breast cancer cells (MDA-MB-468) treated with cholestane derivatives. The expression of proteins in cells treated with 5, 15, 30, 40 μM of derivatives KAR 6299 and 1966 for 24 h **(A)** and 48 h **(B)** were compared with the protein expression in untreated control cells. The expressions of PCNA or α-tubulin were used as protein loading markers.

Figure 6: Western blot analysis of steroid receptor-related proteins (ER-α, ER-β, cdk4) in breast cancer cells (MCF-7, MDA-MB-468) treated with cholestane derivatives. The expressions of proteins in cells treated with 5, 15, 30, 40 μM of derivatives KAR 6299 and 1966 for 24 and 48 h were compared with the protein expression of untreated control cells. The expressions of mcm-7 or α-tubulin were used as protein loading markers.

Figure 7: Activity of caspases-3/7. MDA-MB-468 breast cancer cells were treated with cholestane derivative KAR 6299 and compared with untreated control cells for 24 and 48 h. Data indicate the increase in relative activity of caspases-3/7.

Figure 8: Expression and localization of ER-β in MCF-7 cells as determined by immunofluorescence detection. Representative fluorescence results; magnification, 1000×. Cells were fixed and immunolabelled with an antibody to ER-β and counterstained with DAPI to visualize all nuclei (blue). (**A)** (a-b) Control cells 24 h. (c-d) Cells treated with 30 μM KAR 6299 for 24 h. (e-f) Cells treated with 40 μM KAR 6299 for 24 h. (**B)** (a-b) Control cells 24 h. (c-d) Cells treated with 30 μM 1966 for 24 h. (e-f) Cells treated with 40 μM 1966 for 24 h.

Figure 9: Expression and localization of AR in MCF-7 cells as determined by immunofluorescence detection. Representative fluorescence results; magnification, 1000×. Cells were fixed and immunolabelled with an antibody to AR and counterstained with DAPI to visualize all nuclei (blue). Control cells were compared with cells treated with 30 and 40 μM 1966 for 24 h.

Figure 10: Morphological changes of MCF-7 and MDA-MB-468 cells treated by 40 μM KAR 6299 and 1966 in comparison with control cells for 48 h.

## 9.4. Supplement IV

Oklešťková, J., Hoffmannová, L., Steigerová, J., Kohout, L., Kolář, Z., Strnad, M., Natural brassinosteroids for use for treating hyperproliferation, treating proliferative diseases and reducing adverse effects of steroid dysfunction in mammals, pharmaceutical composition and its use, World Intellectual Property Organization WO2008CZ00097 20080820, 2008.

Published patent application is available on www.espacenet.com

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(10) International Publication Number
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PV 2007-571 22 August 2007 (22.08.2007) CZ

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(75) Inventors/Applicants *(for US only)*: **OKLESTKOVA, Jana** [CZ/CZ]; Werichova 14, 778 00 Olomouc (CZ). **HOFFMANNOVÁ, Lucie** [CZ/CZ]; Prchalov 68, 742 58 Pribor (CZ). **STEIGEROVÁ,, Jana** [CZ/CZ]; Werichova 4, 779 00 Olomouc (CZ). **KOHOUT, Ladislav** [CZ/CZ]; Bazovského 1228/8, 163 00 Praha 6 (CZ). **KOLAR, Zdenek** [CZ/CZ]; Netusilova 1618/11, 796 01 Prostejov (CZ). **STRNAD, Miroslav** [CZ/CZ]; Západní 25, 779 00 Olomouc (CZ).

(74) Agent: **HERMAN, Václav**; Hlavní 43, 252 43 Průhonice (CZ).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

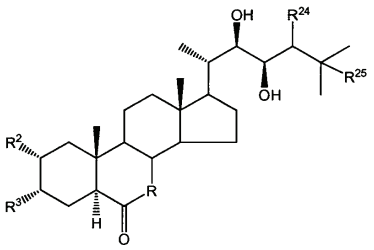
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17:**

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

*[Continued on next page]*

(54) **Title:** NATURAL BRASSINOSTEROIDS FOR USE FOR TREATING HYPERPROLIFERATION, TREATING PROLIFERATIVE DISEASES AND REDUCING ADVERSE EFFECTS OF STEROID DYSFUNCTION IN MAMMALS, PHARMACEUTICAL COMPOSITION AND ITS USE




(I)

(57) **Abstract:** The present invention relates to natural brassinosteroids of general formula (I), wherein R is $CH_2$ or O-$CH_2$ group, $R^2$ is hydrogen or hydroxyl, $R^3$ is hydroxyl, $R^{24}$ is alkyl or alkenyl, which are selected from the group consisting of methyl, ethyl, propyl, isopropyl, methylen, ethylen and propylen, and $R^{25}$ is alkyl selected from the group consisting of methyl and ethyl, and a pharmaceutically acceptable salt thereof, for use for treating hyperproliferation, treating proliferative diseases and reducing adverse effects of steroid dysfunction in mammals. The present invention also provides methods capable to arrest of the cell cycle by natural brassinosteroids resulting in apoptotic changes in cancer cells. More specifically, the present invention relates to use for treatment of the adverse effects of hyperproliferation on mammalian cells *in vitro* and *in vivo*, especially treatment of hyperproliferative diseases in mammals by administering compositions containing natural brassinosteroids. This invention also describes new use for treating consisting in a new therapeutic way for modifying cell viability of human breast and prostate cancer cells.

WO 2009/024103 A2

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

**Published:**
— *without international search report and to be republished upon receipt of that report*

## 9.5. Supplement V

Hoffmannová, L., Steigerová, J., Oklešťková, J., Kohout, L., Chodounská, H., Hniličková, J., Kasal, A., Černý, I., Kolář, Z., Strnad, M., Cholestane derivatives as an antiproliferative and antiangiogenic pharmaceuticals and pharmaceutic preparations containing these compounds, Czech Industrial Property Office CZ PV 2009-725, 2009.

**Cholestane derivatives as antiproliferative and antiangiogenic pharmaceuticals and pharmaceutic preparations containing these compounds**

Field of invention

This invention relates a method for inhibiting the hyperproliferation and angiogenesis in mammalian cells. It describes a method for treating proliferative and angiogenic diseases in mammals by means of cholestane derivatives. Especially, this invention relates to cholestane derivatives and to their use in suitable utilities, especially in anticancer therapies and for the prevention, inhibition and treatment of angiogenesis.

Background of the invention

This invention concerns cholestane derivatives, pharmaceutical preparations containing these compounds and their utilization, especially in anticancer therapy.
The growth of new blood vessels in animals (known as angiogenesis) is essential for organ growth (Folkman et al., 1974, Adv Cancer Res., 19(0):331-358; Folkman et al., 1992, J Biol Chem., 267(16):10931-10934; Carmeliet, 2005, Nature, 438 (7070): 932-936) as well as for growth of solid tumors and metastases (Bhat and Singh, 2008, Food Chem Toxicol, 46: 1334-1345; Kesisis et al., 2007, Curr Pharm Des., 13(27):2795-2809; Risau, 1997, Nature, 386(6626):671-674). Endothelial cells are the main participants in angiogenesis (Bagley et al., 2003, Cancer Res., 63(18):5866-5873) and could be a target for antiangiogenic therapy because they are non-transformed and easily accessible to achievable concentrations of antiangiogenic agents, and are also unlikely to acquire drug resistance (Bhat and Singh, 2008, Food Chem Toxicol, 46: 1334-1345). The vascularization of tumors plays a crucial role in cell nutrition and oxygen distribution. The targeting of tumor angiogenesis from the point of view of the discovery of novel drugs includes inhibition of proteolytic enzymes that break down the extracellular matrix surrounding existing capillaries, inhibition of endothelial cell proliferation and migration, and enhancement of tumor endothelial cell apoptosis. Potent angiogenic inhibitors capable of blocking tumour growth appear to have potential for the development of novel generations of anticancer drugs.