Anticancer Activities of Brassinosteroids

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Abstract: Molecular and cellular effects of two groups of antiproliferative agents, natural brassinosteroids (BRs) and their synthetic derivatives, were examined in different human cancer cell lines and in primary endothelial cells in vitro. Natural and synthetic BRs caused growth inhibition, cell cycle arrest and initiation of apoptosis in many different cancer cell lines. The inhibition of proliferation and migration of human endothelial cells by BRs was demonstrated and evidences were obtained that BRs initiate cell death by apoptosis. And, analogues of BRs were found to be more effective than natural BRs. Observed inhibition of migration and tube formation demonstrated the antiangiogenic activity of BRs. These findings indicate a potential use of BRs in the prevention of metastasis development. Investigation of the mechanisms of action of BRs in human cancer and endothelial cells using cellular and molecular techniques indicated the possible involvement of steroid receptors in BR action. However, BRs were shown not to bind directly to steroid receptors which demonstrate that BRs act via steroid receptor-independent pathway(s). Concluding, BRs and their derivatives are capable to inhibit growth of several human cancer cell lines and to inhibit angiogenesis-like behaviour of primary endothelial cells in vitro, as well.

Keywords: Antiangiogenic activity, anticancer drugs, apoptosis, breast cancer, metastasis development, cell cycle arrest.

1. BRASSINOSTEROIDS

The phytohormones known as brassinosteroids (BRs) are low-molecular weight steroid compounds occurring in plants [1]. The first brassinosteroid (22R,23R,24S)-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. [2]. Up to date, more than 70 of these phytohormones have been discovered [3]. 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 [4, 5].

BRs play an important role in hormone signalling in plants and in the physiological responses of plants to environmental stimuli in processes such as 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 [5-8].

The BRs are essential for many growth and development processes in plants. Contradictory reports on the effects of BRs and steroids on cell division in different plant species and cultured cell lines have been previously reported [1, 9, 10]. However, the effect of BRs on cell division in plants has been lately shown to be mainly promotive. BRs can mimic the effect of cytokinins on plant cell division: both BRs and cytokinins induce cycD3 gene expression and promote cell division during the early phases in plant cell cultures, suggesting that BRs are rate-limiting factors in the induction of the cell cycle [11].
2. ANTIPROLIFERATIVE ACTIVITIES OF BRASSINOSTEROIDS AND THEIR DERIVATIVES

Information about the effects of BRs and their synthetic analogues on animal and/or human cells is still very limited. However, recently, the first medical applications of BRs were published. These studies reported that some natural BRs, such as 28-homocastasterone (28-homoCS) or 28-homobrassinolide (Fig. 1) and their synthetic analogues, have \textit{in vitro} antiviral activity against several pathogenic viruses [12-14]. There is also a report describing the effects of 24-epibrassinolide (24-epiBL) on cultured hybridoma mouse cells [15]. Typical effects of 24-epiBL were: (I) increase in mitochondrial membrane potential; (II) reduction in intracellular antibody level; (III) increase in number of cells in G\textsubscript{0}/G\textsubscript{1} phase; (IV) and decrease the proportion of cells in S phase. Furthermore, the density of viable cells was significantly higher at 24-epiBL concentrations of 10\textsuperscript{-13} and 10\textsuperscript{-12} mol/l [15].

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Structures of 24-epibrassinolide and 28-homocastasterone.}
\end{figure}

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 recently reported [16]. Using Calcein AM assay, 28-homoCS and 24-epiBL were shown to affect the viability 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 A549, 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 [16, 17]. Cytotoxicity of natural brassinosteroids against various human cancer lines is shown in Table 1. The results provide several indications regarding structural features that are associated with cytotoxic activity. The most active compound against CEM cells was 28-homoCS, which induced approximately three times stronger responses than 28-homobrassinolide (28-homoBL), indicating that transformation of 6-oxo-7-oxalactone to 6-oxo functionality substantially increases the growth inhibitory activity of BRs; the presence of a 24R side chain strongly reduces BR cytotoxicity; the 24R side chain in 24-epicastasterone also reduces anticancer activity compared to castasterone. However, 28-homoCS and 28-homoBL, both of which have an ethyl group in their side chains at C24, are somewhat more effective than the corresponding analogues with C24 methyl groups. Since β-ecdysone, which contains 2β,3β,22α–functionality, showed no detectable activity, a 3α-hydroxy group, 2α,3α-vicinal diol or 3α,4α-vicinal diol may be important for cytotoxic activity [16]. Further testing of natural BRs (see Table 1) such as typhasterol and teasterone, however, showed that these 3α-hydroxy analogues are inactive when tested on different cancer cell lines. Dolicholide type BRs also showed marginal anticancer activity. Many natural products were described as a source of new anticancer drugs. Up to date, more than 70 anticancer molecules are derived from natural products [18].

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 treatment with BRs. This finding may point to possible modulation of steroid receptor-mediated responses by natural BRs. Furthermore, 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 are promising candidates for development as potential anticancer drugs [16].

Brassinosteroids are also able to disturb cell cycling in breast and prostate cancer cell lines. Using flow cytometry, it was shown that treatment of breast and prostate cell lines with 28-homoCS and 24-epiBL blocked the cell cycle in the G1 phase, with concomitant reductions in the percentages of cells in the S phase [16]. In the MCF-7 breast cancer cell model, the most widely used experimental system to study breast cancer, the typical growth inhibitory response of antiestrogens is manifested by similar reductions in the proportions of cells synthesizing DNA (S phase) after the antiestrogen treatment, and a corresponding increase in the proportions of cells in G0/G1 phase [19].

Table 1: Cytotoxicity of natural BRs against human cancer cell lines and human fibroblasts.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CEM</th>
<th>MCF-7</th>
<th>HeLa</th>
<th>A549</th>
<th>BJ</th>
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<tr>
<td>Cholesterol</td>
<td>&gt;50</td>
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<tr>
<td>Castasterone</td>
<td>16.6±5.3</td>
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<td>brassinolide</td>
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<tr>
<td>28-Homobrassinolide</td>
<td>48.1±1.3</td>
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<td>28-Homocastasterone</td>
<td>13.2±2.8</td>
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<td>24-Epicastasterone</td>
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<td>24-Epibrassinolide</td>
<td>44.0±2.2</td>
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<td>brassicasterol</td>
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<td>49.0±1.8</td>
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<td>35.3±2.2</td>
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<td>S,S-Homocastasterone</td>
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<td>45.0±3.1</td>
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<td>Typhasterol</td>
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<td>37.9±13.0</td>
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To determine the biological effects of synthetic cholestane derivatives of BRs (Fig. 2) in cancer cells, various cell-based assays were used. In order to evaluate the cytotoxic properties of several cholestane derivatives and a related steroid, cholesterol, on the viability of normal and cancer cell lines of different histopathological origin, 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, normal human skin fibroblast cells, BJ. We observed a potent and dose-dependent decrease in the viability of CEM, RPMI 8226 and HeLa cells, albeit at different concentrations. The highest cytotoxicity was observed after application of cholestanon and compound 1966 (3beta-hydroxy-7a-homo-cholest-5-en-7a-one) [20]. Cholesterol, which is a non-cholesterol derived plant and animal sterol, was, however, inactive or exhibited almost null cytotoxic activity. The 24R side chain was also shown to be a decisive group to increase the cytotoxicity of cholestane derivatives. Changing the 6-oxo-7-oxalactone to a 6-oxo functionality dramatically increased the growth inhibitory activity of the cholestane derivatives. 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 (manuscript in preparation). At present, only a few natural agents are known to possess the potential ability to selectively/preferentially eliminate cancer cells without affecting growth of normal cells. Results point to the potential use cholestane derivatives as pharmaceuticals for inhibition of hyperproliferation in tumors [21].

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Because of the strong anticancer activities of the cholestane derivatives cholestanon and 1966, we further examined their antiproliferative properties. Flow cytometry analysis showed an increase in the proportion of cells in the subG1 phase of the cell cycle (apoptotic cells) in MCF-7 and MDA-MB-468 cell lines after treatment with cholestanon or 1966. Cholestanon treatment increased the proportion of cells in the subG1 and G0/G1 phase and decreased the proportion in the S phase. Treatment with 1966 caused a strong increase in the proportion of subG1 phase cells (apoptotic cells) and a concomitant decrease in other cell phases. Treatment with cholestane derivatives resulted in decreased percentage of cells in the S phase in both, MCF-7 and MDA-MB-468 cell lines, being the effect stronger in MCF-7 estrogen-sensitive breast cancer cells [21].

Using a fluorogenic substrate Ac-DEVD-AMC and/or the caspase 3 and -7 inhibitor Ac-DEVD-DHO, the activity of caspases 3 and -7 in MDA-MB-468 cells exposed to cholestanon or 1966 was determined. Caspases 3 and -7 start the apoptotic cascade in cells. Cells treated with cholestanon presented a strong five-fold increase in the activity of effector caspases 3 and -7, when compared to untreated cells. However, derivative 1966 only weakly affected the activity of caspase 3 and -7.

To detect changes in apoptosis-related protein expression in breast cancer cell lines, cells treated with cholestane derivatives were used for Western blot immunodetection. Expression of a tumor suppressor protein, p53, was observed in controls of breast cancer cell lines, and, cholestanon and compound 1966 enhanced its expression. Enhanced expression of p53 correlated with a decreased expression of the antiapoptotic protein Mcl-1 in cells treated with cholestanon and compound 1966. These results confirm that the cholestane derivatives cholestanon and compound 1966 induce, in a dose-dependent way, apoptosis through caspase 3 and 7 activation [21].

3. ANTIANGIOGENIC PROPERTIES OF BRASSINOSTEROIDS AND THEIR ANALOGUES

Angiogenesis (Fig. 3), the growth of new blood vessels in animals, is essential for organ growth [22-25] as well as for growth of solid tumors and for metastasis [25-27]. Endothelial cells are the main players in angiogenesis [27] and these cells could be a target for antiangiogenic therapy because they are non-transformed, and easily accessible to antiangiogenic agents. Endothelial cells are also unlikely to acquire drug resistance, because these cells are genetically stable, homogenous and have a low mutation rate [26]. The vascularization of tumors plays a crucial role in cell nutrition and oxygen distribution. Targeting tumor angiogenesis using novel drugs could potentially be achieved by the inhibition of proteolytic enzymes, which break down the extracellular matrix surrounding existing capillaries, and by 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 [28-31].

Many natural products that inhibit angiogenesis have been reported, including compounds with steroid structure. These compounds include, among others, an active component of chilli peppers, capsaicin [32]; a low molecular weight natural product isolated from Dendrobium chrysotoxum Lindl, erianin [33]; a small molecule from extracts of the seed cone of Magnolia grandiflora L., honokiol [34]; a natural product from marine sponges, laulimalide [35]; the plant alkaloid, sanguinarine [36]; and a sesquiterpene purified from fruits of...
Torilis japonica (Houtt.) DC., torilin [37]. Recently, several steroids, including 2-methoxyestradiol, progestin, medroxyprogesterone acetate, and glucocorticoids such as dexamethasone and cortisone (Fig. 4),

**Figure 3:** Angiogenesis in tumors. Angiogenesis in tumors is the creation of a network of blood vessels that penetrates into tumors. New blood vessels supply nutrients and oxygen and remove waste products. Starting molecules activate cancer cells which send signals to surrounding normal tissue to initiate tumor angiogenesis. This signaling activates certain genes in the normal endothelial cells (EC) to make proteins which stimulate growth of new blood vessels.

have been shown to present antiangiogenic activity [38, 39]. However, to date, there is no information on the effect of natural BRs on endothelial cells, including potential antiangiogenic activity. Therefore, we decided to investigate the effects of naturally occurring BRs and some of their synthetic analogues on cell proliferation and cycling in human microvascular endothelial (HMEC-1) and umbilical vein endothelial cells (HUVEC), more specifically on the migration and formation of tubes by these cells (submitted manuscript). All of the tested compounds, two natural brassinosteroids (24-epiBL, 28-homoCS) and two synthetic analogues of BRs (BR4848, cholestanon) (submitted manuscript), inhibited growth of HMEC-1 cells in a dose-dependent manner. 24-epiBL and 28-homoCS reduced the number of viable cells, while both synthetic analogues were similarly effective, but at a three-fold lower concentration. Both, BR4848 and cholestanon, decreased the number of cells adhering to a plastic surface more strongly than it did to a plastic surface coated with collagen. This effect may contribute to the antiproliferative activity observed for the tested compounds once different cells types require adhesion to a solid surface in order to proliferate (submitted manuscript).

**Figure 4:** Structures of 2-methoxyestradiol (a), progestin (b), cortisone (c) and medroxyprogesterone acetate (d).

Flow cytometric analysis showed that treatment with 24-epiBL or 28-homoCS only slightly increased the proportion of cells in the subG₁ (apoptotic) fraction in HMEC-1 cells, when compared to the untreated controls. In contrast, treatment with synthetic derivatives enhanced the number of subG₁ cells, compared to the untreated controls. Moreover, BR4848 blocked the G₂/M phase of the cell cycle, similarly to what had been previously reported for 2-methoxyestradiol [40]. The tested steroids were more effective towards cell cycle arrest and induction of apoptosis than were the natural BRs, which only had cytostatic effects.

The two natural BRs tested, 24-epiBL and 28-homoCS, reduced migration of HUVEC cells, however, BR analogues presented a stronger inhibitory effect. Natural BRs caused only a slight inhibition of tube formation. Treatment with 24-epiBL or 28-homoCS slightly reduced the number of tubes, as well as the number of nodes, one of the parameters usually used to access antiangiogenic activity. Synthetic analogues also decreased the number of tubes compared to the control treatments (submitted manuscript). This antiangiogenic activity of BRs, along with their antiproliferative activity, suggests that these plant hormones might become important for the development of new anticancer drugs.
Figure 5: Actions of brassinosteroids in the plant cell (a) and possible action mechanism in animal cells (b). (a) BRs bind to the extracellular domain of brassinosteroid-insensitive 1 (BRI1), a leucine-rich repeat receptor kinase (LRR-RK), localized in the plasma-membrane. 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. BES1 ( bri1-EMS-suppressor 1 ) and BZR1 ( brassinazole-resistant 1 ) are phosphorylated by BIN2 and are closely related transcriptional activators of BR-induced genes. BSU1 ( bri1 suppressor 1 ) counteracts the effects of BIN2 [41]. (b) Steroid receptors (such as those for estrogen, androgen, progesterone, mineralcorticoid and glucocorticoid) are ligand-activated transcription factors that belong to the nuclear hormone receptor super-family. Lipophilic steroids that diffuse through the plasma membrane bind to the steroid receptors located in the cytosol or nucleus. Ligand binding induces a conformational change and dimerization with another receptor that allows the ligand/receptor complex to bind to the DNA and directly modify gene expression and protein synthesis [42].

4. MOLECULAR MECHANISM OF ACTION OF BRs

Signalling by BRs, and the resulting genomic responses in plants, are initiated by the binding of a BR molecule to a receptor kinase, brassinosteroid-insensitive 1 (BR11), localized in the plasma-membrane [41, 42]. There are however differences between the action of steroids in animal cells and in plant cells (Fig. 5). In the classic animal model, lipophilic steroids bind to steroid receptors located either in the cytosol or in the nucleus 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 to the DNA and directly modify gene expression over a time period of hours or even days [43].

Steroid hormone receptors exert their influence in embryonic development and adult homeostasis, as hormone-activated transcriptional regulators. Their modular structure, consisting of a DNA binding domain (DBD), nuclear localization signals, a ligand-binding domain (LBD) and several transcriptional activation functions, are conserved among 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 such as glucocorticoid receptor [44]. Steroid receptors interact directly, in vitro, with components of the transcription initiation complex. The binding of ligands is influenced by co-activators that would act as bridging factors between steroid receptors and the transcription initiation complex. Steroid receptors not only stimulate gene activity, but they are also able to repress transcription by competition for the DNA-binding site, by competition for common mediators of the transcription initiation complex, or by sequestration of the transcription factors into inactive forms [45].

Because of the similarity between BRs and 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-epiBL was a weak antagonist of estrogen-receptor-α (ER-α); the synthetic BRs, cholestanon 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 cholestanon, which showed weak binding to both, ER-α and ER-β. 24-epiBL, 28-homoCS and BR4848 did not bind to estrogen receptors. Summarizing, all of the tested compounds, in one or another way, showed effects in our angiogenesis assays in vitro, at micromolar concentrations. Furthermore, the ability of the tested compounds to modulate steroid responses in reporter cell lines was significantly strong. On the other hand, direct binding of the tested compounds, at least to steroid receptors ER-α and ER-β, is weak. Therefore, we can only speculate about the action mechanism of natural BRs and synthetic derivatives on angiogenesis. It seems possible that there are multiple effects, both steroid receptor-dependent and independent, similarly to what is considered for 2-methoxyestradiol, which binds to estrogen receptors. However, the action mechanism of the antiangiogenic response is probably unrelated to the receptor pathway (submitted manuscript).

On the other hand, changes in pattern of ER-α and ER-β localization were observed in MCF-7 cells, after BR treatment, using immunofluorescence detection. Strong, uniform ER-α immuno-nuclear labeling was detected in untreated MCF-7 cells, while cytoplasmic speckles of ER-α immunofluorescence were observed in MCF-7 cells treated with 28-homoCS or 24-epiBL. In contrast to ER-α, ER-β was predominantly found in the cytoplasm of untreated MCF-7 cells. However, ER-β was notably re-located 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.
Anticancer Activities of Brassinosteroids

Our work has shown 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. In breast cancer cells, BRs inhibited cell growth and blocked the G1 phase of the cell cycle, with concomitant reductions in the percentages of cells in the S phase. Cell cycle arrest was accompanied by reductions in cyclin-dependent kinases (CDKs) 2/4/6, cyclin D1 and E expression and pRb phosphorylation, together with up-regulation of the cyclin-dependent kinase inhibitors p21Waf1/Cip1 and p27Kip1, which inhibit cyclin/CDK complexes. In addition, 28-homoCS and 24-epiBL treatments induced expression of the anti-apoptotic Bcl-2 and Bcl-XL proteins in MCF-7 cells. The levels of p53 and MDM-2 proteins were slightly affected by BR treatments. In MDA-MB-468 cells it was found that caspase-3 was cleaved into fragments (part of the apoptotic cascade) 24 h after the BR treatment. Furthermore, BR application to MDA-MB-468 cells resulted in G1 phase arrest and increases in the subG1 fraction, which represents apoptotic bodies. It was confirmed that the BR-mediated apoptosis occurred in both cell lines, although changes in the expression of apoptosis-related proteins were modulated differently by the BRs in each cell line [46]. When seen together, these data indicate that a wide range of carcinomas are likely to be targets for the antiangiogenic effects of BRs and their synthetic derivatives. The action mechanisms of BRs in animal cells are still largely unknown, but it seems possible that BRs may interact with one or more of the numerous steroid-binding proteins. It also seems possible that BRs induce multiple effects, both, steroid receptor-dependent and independent. These properties may lead to the development of novel natural product-derived anticancer drugs.

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