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Anticancer and antiproliferative activity of natural brassinosteroids

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Abstract

Brassinosteroids (BRs) are steroid plant hormones that are essential for many plant growth and developmental processes, including cell expansion, vascular differentiation and stress responses. Up to now the inhibitory effects of BRs on cell division of mammalian cells are unknown. To determine basic anticancer structure–activity relationships of natural BRs on human cells, several normal and cancer cell lines have been used. Several of the tested BRs were found to have high cytotoxic activity. Therefore, in our next series of experiments, we tested the effects of the most promising and readily available BR analogues with interesting anticancer properties, 28-homocastasterone (**1**) and 24-epibrassinolide (**2**), on the viability, proliferation, and cycling of hormone-sensitive/insensitive (MCF-7/MDA-MB-468) breast and (LNCaP/DU-145) prostate cancer cell lines to determine whether the discovered cytotoxic activity of BRs could be, at least partially, related to brassinosteroid-nuclear receptor interactions. Both BRs inhibited cell growth in a dose-dependent manner in the cancer cell lines. Flow cytometry analysis showed that BR treatment arrested MCF-7, MDA-MB-468 and LNCaP cells in G_1 phase of the cell cycle and induced apoptosis in MDA-MB-468, LNCaP, and slightly in the DU-145 cells. Our results provide the first evidence that natural BRs can inhibit the growth, at micromolar concentrations, of several human cancer cell lines without affecting the growth of normal cells. Therefore, these plant hormones are promising leads for potential anticancer drugs.

Keywords: Brassinosteroids; Anticancer activity; Cell cycle; Hormone-dependent/independent cancers

1. Introduction

Brassinosteroids (BRs) are steroid plant hormones that play important regulatory roles in various physiological processes, including growth, differentiation, root and stem elongation, disease resistance, stress tolerance and senescence (Clouse and Sasse, 1998; Nemhauser and Chory, 2004). This group of plant steroids includes more than 70 compounds that seem to be ubiquitously distributed throughout the plant kingdom. BRs have been detected and isolated from seeds, fruits, leaves, galls and pollen (Sasse, 1991; Khripach et al., 1999; Sakurai, 1999). They are the most structurally similar of the plant hormones to animal steroid hormones and, like their animal counterparts, BRs regulate the expression of numerous genes, affect the activity of complex metabolic pathways, and contribute to the regulation of cell division and differentiation. They are also involved in the regulation of various processes, including photomorphogenesis and cell expansion in the presence of a potentially growth-limiting cell wall (Clouse, 2002).

Brassinolide, the most biologically active BR, was initially isolated in an ambitious experiment in which more than 200 kg of *Brassica napus* pollen was used as the starting material (Grove et al., 1979). Subsequently, brassinolide has been identified in all plant species examined to date (Bajguz and Tretyn, 2003; Zullo and Adam, 2002;

Abbreviations: BrdU, bromodeoxyuridine; BRs, brassinosteroids; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.

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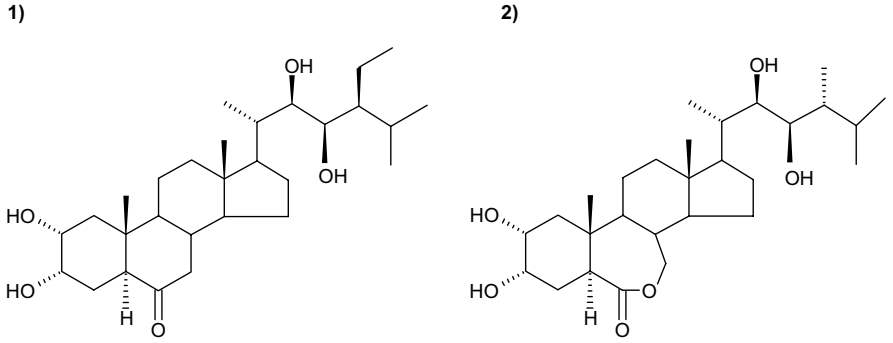

Fig. 1. Structures of representative natural brassinosteroids. 28-homocastasterone **(1)** and 24-epibrassinolide **(2)**.

Swaczynová et al., 2006). However, it is present at very low levels in plant tissues (pmol/g fr. wt) and it is very difficult to synthesize chemically. Therefore, efforts are being made to develop analogues that are easier to synthesize but have similar or greater, activity, than the natural BRs (Litvinovskaya et al., 2006; Šíša et al., 2005). A number of brassinosteroids have already been synthesized and, interestingly, some of them were later found to occur in nature (Soeno et al., 2000; Clouse and Sasse, 1998; Thompson et al., 1981).

An example of such a compound is the 24-epimer of brassinolide, **2** (Back et al., 1997; Brosa et al., 1997). This brassinosteroid has been intensively investigated, partly because it appears to be the best currently available brassinosteroid for practical applications, and the potential agricultural utility of various formulations has been evaluated (Ikekawa and Zhao, 1991).

Brassinosteroids have been reported to have conflicting, but mainly promotive, effects on cell division in various plant species and cultured cell lines (Roth et al., 1989; Nakajima et al., 1996; Oh and Clouse, 1998; Kauschmann et al., 1996; Gaudinová et al., 1995). Notably, for instance, they can functionally substitute for cytokinins (e.g. both BRs and cytokinins induce *cycD3* gene expression) (Hu et al., 2000) and promote cell division during early cell culture phases, suggesting that BRs are rate-limiting factors in cell cycle induction (Miyazawa et al., 2003).

Some potential medical applications of BRs have also been reported (Wachsman et al., 2000, 2002, 2004). Wachsman et al. (2000, 2002) found that two natural BRs (**1** and 28-homobrassinolide) and synthetic analogues of these compounds have substantial *in vitro* antiviral activity against various pathogenic viruses, including herpes simplex virus type 1 (HSV-1), arenaviruses and measles virus (Wachsman et al., 2000, 2002). Indeed, several of the BR analogues were 10- to 18-fold more active than ribavirin (used as reference drug) towards HSV-1 and the arenaviruses. However, further studies are needed to elucidate the precise *in vitro* antiviral mechanism(s) of these BR analogues and structural features that are associated with their bioactivity. In addition, it has been reported that **2** can: (i) increase the mitochondrial membrane potential, (ii) reduce intracellular antibody levels, (iii) increase the proportions of cells in G_0/G_1 phase, (iv) reduce the proportions of cells in S-phase, and increase the proportions of viable hybridoma mouse cells at subnanomolar concentrations (Franěk et al., 2003).

The findings outlined above indicate that BRs may have a number of medical effects and possible effects on hybridoma mouse cells. Therefore, we hypothesized that they may have inhibitory effects on the cycling of mammalian cells, and/or antiproliferative properties. The aim of the study presented here was to test this hypothesis by assessing the effects of selected natural brassinosteroids and related analogues on a range of human normal and cancer cell lines. The results show (for the first time to our knowledge) that natural BRs can inhibit the growth of various cancer cell lines at micromolar concentrations, despite having minimal effects on normal cells. Information on the mechanisms of action of BRs at a molecular level is currently scarce, but they may involve interactions with steroid receptors. To explore this possibility, we also assessed the effects of two natural BRs, **1** and **2** (Fig. 1), on the viability, proliferation and cycling of hormone-sensitive/insensitive cancer cell lines. The findings indicate that BRs are promising leads for the development of a new generation of anticancer drugs.

2. Results and discussion

Brassinosteroids are steroid plant hormones that are essential for many plant growth and development processes (Clouse and Sasse, 1998; Haubrick and Assmann, 2006; Asami et al., 2005; Bishop and Koncz, 2002) and their structure–activity relationships (SAR) in plant bioassays have been appearing in many publications (Zullo and Adam, 2002; Baron et al., 1998; Back and Pharis, 2003; Bajguz and Czerpak, 1998; Adam and Marquardt, 1986).

For reasons outlined in Section 1, in this study we initially investigated the effects of several selected BRs and related steroids on the viability (in Calcein AM assays) of BJ fibroblasts (as an example of a "normal" cell line) and human cancer cell lines of various histopathological ori-

gins, including: 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. Cells of all of these lines were exposed to six serial 4-fold dilutions of each drug for 72 h, the proportions of surviving cells were then estimated and IC_{50} values (concentrations leading to 50% inhibition of viability) were calculated (Table 1). Treatments with **1** and **2** resulted in potent, dose-dependent reductions in the viability of CEM and RPMI 8226 cells, albeit at different levels (Fig. 2). **1** was the most potent compound towards CEM cells (IC_{50} 13 μM) while its 22*S*,23*S*-isomer was the most potent towards RPMI 8226 cells (IC_{50} 25 μM). In addition to **1**, high cytotoxicity was also observed after application of castasterone and its artificial SS-homologue. The brassinolides, which are usually the most active compounds in plant bioassays, were however inactive or exhibited almost zero cytotoxic activity with artificial 22*S*,23*S*-28-homobrassinolide being the most effective (IC_{50} 31–35 μM). All of the tested non-brassinosteroid plant sterols, including cholesterol, stigmasterol, brassicasterol, 5α-cholestane, β-ecdysone, β-sitosterol and related compounds had extremely weak or no detectable activity, even when tested in concentrations up to 50 μM, although some of them (including cholesterol, stigmasterol, and β-sitosterol) showed some cytotoxity ($IC_{50} > 30$ μM) towards the RPMI 8226 cell line. None of the tested compounds showed any cytotoxicity towards the K 562, A 549, HeLa, and HOS cancer cell lines. A striking observation from these data was that no brassinosteroid-mediated loss of viability was observed in the BJ fibroblasts, suggesting that BRs induce different responses in cancer and normal cells. 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 (Tang and Porter, 1997; Matsuya et al., 2005; Shi et al., 2005). This study also provides the first evidence of BRs as anticancer compounds with antiproliferative properties. Previously, the only known effects of BRs on the growth rates of mammalian cells was that **2** stimulates the growth of mouse hybridoma cells at 10^{-12}–10^{-13} M concentrations (Franěk et al., 2003).

As well as providing evidence that BRs have cytotoxic properties, the results of these assays provide several indications regarding structural features that are associated with their activity. The most active compound was **1**, which induced approximately three times stronger responses than 28-homobrassinolide, indicating that transformation of 6-oxo-7-oxalactone to 6-oxo functionality substantially increases the growth inhibitory activity of BRs. In addition, the presence of a 24*R* side chain strongly reduces the cytotoxicity of BRs, in accordance with previous findings that BRs with a 24*S*-methyl or -ethyl group have ca.

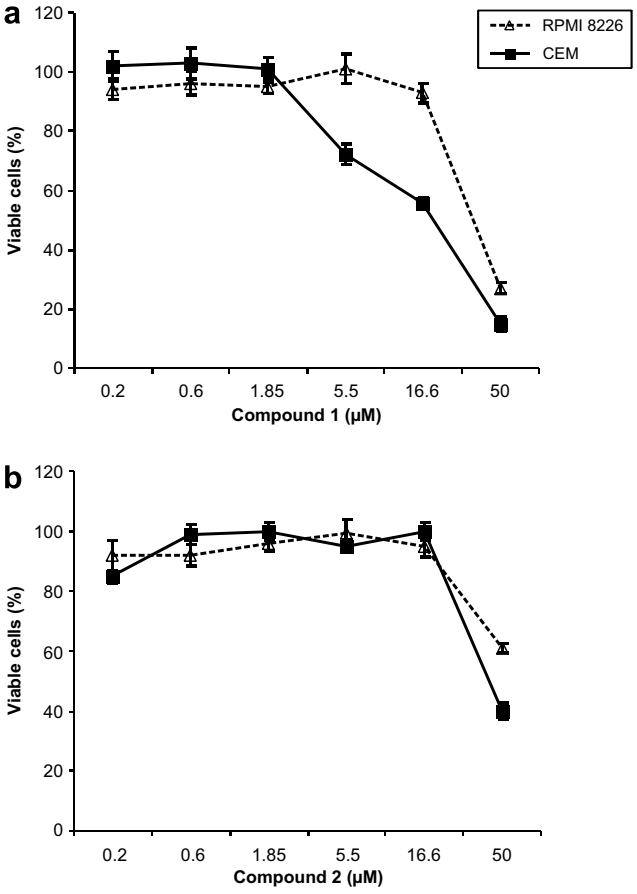

Fig. 2. Inhibitory effects of **1** (a) and **2** (b) on the viability of T-lymphoblastic leukaemia (CEM) and multiple myeloma (RPMI 8226) cells. Cytotoxicity was determined by the Calcein AM assay. The data shown are means obtained from three independent experiments ± SD.

Table 1
IC_{50} (μM) values obtained from the Calcein AM assays with the tested cancer and normal cell lines; means ± SD obtained from three independent experiments performed in triplicate

Compound (IC_{50}, μM)	Cell line			
	CEM	RPMI 8226	G 361	BJ h-TERT
Cholesterol	>50	38 ± 2.9	>50	>50
β-Ecdysone	>50	>50	>50	>50
5α-Cholestane	>50	>50	>50	>50
Brassicasterol	>50	>50	>50	>50
Stigmasterol	>50	32 ± 0.6	>50	>50
Sitosterol	>50	32 ± 1.6	>50	>50
Brassinolide	>50	>50	>50	>50
28-Homobrassinolide	48 ± 1.3	>50	>50	>50
22*S*,23*S*-28-Homobrassinolide	35 ± 2.2	31 ± 7.3	>50	>50
2	44 ± 2.2	>50	>50	>50
22*S*,23*S*-24-Epibrassinolide	>50	>50	>50	>50
Castasterone	16 ± 5.3	33 ± 1.3	>50	>50
1	13 ± 2.8	26 ± 1.4	>50	>50
22*S*,23*S*-28-Homocastasterone	24 ± 1.5	25 ± 4.7	45 ± 2.2	>50
24-Epicastasterone	>50	>50	>50	>50
22*S*,23*S*-24-Epicastasterone	49 ± 1.8	>50	>50	>50

10-fold higher activity in plant bioassays than corresponding hormones with a 24*R*-alkyl function. The more flexible conformational behavior of the 24*R* side chain in 24-epicastasterone compared with castasterone, as determined by NMR and modeling studies (Drosihn et al., 2001; Stoldt et al., 1997), may be a critical property that reduces its anticancer activity. However, **1** and 28-homobrassinolide, both of which have an ethyl group in their side chains at C24, are somewhat more effective than corresponding analogues with C24 methyl groups. From the data presented here we cannot determine whether 2α,3α- and 22,23-*cis*-vicinal diols are essential for the cytotoxic activity of BRs, but 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 their activity. Cytotoxicity testing of BR analogues with these features is currently in progress.

The most promising and readily available BR analogues with interesting anticancer properties (**1** and **2**) were further evaluated in assays with the breast cancer cell lines MCF-7 (estrogen receptor-α-positive) and MDA-MB-468 (estrogen receptor-α-negative), and the prostate cancer cell lines LNCaP (androgen-sensitive) and DU-145 (androgen-insensitive). It should be noted here that the physiology and responses of prostate cancers in humans are very complex since they progress from an androgen-responsive to an androgen-unresponsive state, and mixtures of both androgen-dependent and androgen-independent cells are present in most clinically diagnosed prostate cancers (Gleave et al., 1998). Mortality from prostate cancer is generally due to the proliferation and invasion of androgen-unresponsive cells, which fail to undergo apoptosis, culminating in hormone-refractory prostate cancer for which no cure, only palliative treatment, is currently available (Denmeade et al., 1996). Androgen-sensitive cells undergo rapid apoptosis on androgen ablation, but the apoptosis pathways are by-passed in androgen-insensitive cells during androgen withdrawal, although they retain the molecular machinery for apoptosis. There are similar patterns in the hormone responsiveness of estrogen-sensitive and estrogen-insensitive breast cancers. Therefore, eliminating both cell types seem to be an intensifying approach for treatment of these cancers (Weisburger, 1998).

To evaluate the cytotoxic effects of the selected brassinosteroids (**1** and **2**) on the hormone-sensitive and -insensitive cancer cell lines (MCF-7, MDA-MB-468, LNCaP and DU-145), we analyzed their viability after incubation with BRs in MTT assays (Mosmann, 1983; Carmichael et al., 1987). Both of the analogues inhibited the growth of all of the cancer cell lines in a dose-dependent manner. MCF-7 breast cancer cells were the most sensitive to **1** treatment than the other three cell lines. **2** showed the cytotoxic effects at lower tested concentrations (Fig. 3). IC_{50} values (concentrations leading to 50% inhibition of cell viability after 24 h) are presented in Table 2. Interestingly, there was a little difference between the potency of **1** and **2** on different cell lines as IC_{50} values moved consistently

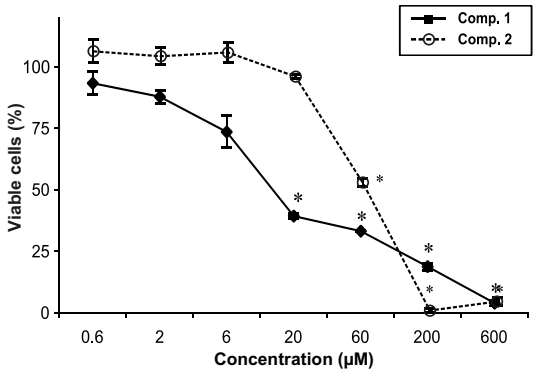

Fig. 3. The effect of **1** or **2** on cell viability of MCF-7 cell line determined by MTT test. The cell viability is presented as percentage of viable cells (the cell viability of control cells was regarded as 100%) after 24 h. The data shown are means ± SD obtained from three independent experiments in triplicate. Asterisks (*) denote values that are significantly different from control values at $p < 0.05$.

Table 2
IC_{50} (μM) values for **1** and **2** determined by MTT assays on hormone-sensitive/insensitive breast (MCF-7/MDA-MB-468) and prostate (LNCaP/DU-145) cancer cell lines

Cell line	IC_{50} (μM)	
	1	**2**
MCF-7	40 ± 1.5	60 ± 1.8
MDA-MB-468	65 ± 2.8	68 ± 2.5
LNCaP	45 ± 2.3	60 ± 3.1
DU-145	45 ± 2.8	65 ± 0.9

The data shown are means ± SD obtained from three independent experiments performed in triplicate.

ca. 43.3 ± 2.4 μM and 61.7 ± 1.7 μM, respectively. There was only an exception in the case of MDA-MB-468 estrogen-insensitive breast cancer cell line, which was more resistant to **1** and **2**, with IC_{50} values of 65 ± 2.8 and 68 ± 2.5 μM, respectively. Previous findings of correlations between the estrogen receptor status of tumors responses to endocrine therapy have already led to the development of estrogen antagonists that are currently used in cancer therapies. Tamoxifen, an antiestrogen with estrogen agonistic as well as antagonistic activities, is currently the most widely used substance in the treatment of hormone-sensitive breast cancer since it significantly decreases rates of both disease recurrence and death. However, tamoxifen therapy is hindered by the frequent development of cellular resistance. For this reason, more potent antiestrogens have been developed, which do not have agonistic properties, but have more prolonged efficacy and are effective against cancers that have developed resistance to nonsteroidal antiestrogens (Picard, 1999). However, further development of such agents (and treatments) is required, and the findings presented here suggest that BRs, or compounds derived from them, could play a valuable role.

To evaluate whether the observed reductions in proportions of viable cells were due to reductions in cell prolifer-

ation, we obtained estimates of rates of DNA replication in the cancer cells incubated in the presence of **1** and **2** at their IC_{50} concentrations for 6, 12, and 24 h, relative to controls incubated in the absence of the BRs, in BrdU incorporation assays. Both BRs resulted in reduced percentages of BrdU-positive cells, and thus inhibited proliferation in a dose- and time-dependent manner in all cell lines tested (Table 3). The most potent effects (reductions in the proportions of viable cells from $69 \pm 4.5\%$ in controls to $29 \pm 4.0\%$) were observed when MCF-7 cells were incubated for 24 h in the presence of **1**.

In recent years, many chemotherapeutic and chemopreventive agents have been shown to have antiproliferative effects by arresting cells at certain checkpoints of the cell cycle (Tang and Porter, 1997; Weerasinghe et al., 2001a,b,c). Similarly, the concept of cell cycle-mediated apoptosis is also gaining increasing attention, and the anticancer properties of certain compounds, are believed to function via this pathway (Tang and Porter, 1997; Buolamwini, 2000; Collins et al., 1997). Therefore, we explored the possibility that BRs may perturb cell cycling in breast and prostate cancer cell lines by flow cytometry, and the results showed that treatment with either **1** and **2** induced blocks in the G_1 phase of the cell cycle in the MCF-7, MDA-MB-468 and LNCaP cell lines (Table 4), with concomitant reductions in the percentages of cells in the S phase of the cell cycle. However, in DU-145 cells increases in the percentages in the G_2/M phase accompanied by reductions in the other cell cycle phases following the BR treatments were observed (Table 4). 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; Chawnshang, 2002).

Table 3
Cell proliferation measured by the BrdU incorporation assay

Control/BRs (IC_{50})	Cell line			
	MCF-7	MDA-MB-468	LNCaP	DU-145
Ctrl (6 h)	50 ± 3.6	64 ± 1.5	44 ± 6.0	51 ± 3.8
1 (6 h)	46 ± 2.5	55 ± 5.0	$25 \pm 3.0^*$	41 ± 4.5
2 (6 h)	42 ± 3.2	$53 \pm 4.4^*$	36 ± 5.5	$32 \pm 3.8^*$
Ctrl (12 h)	53 ± 3.5	58 ± 3.0	52 ± 7.6	54 ± 4.2
1 (12 h)	$31 \pm 3.8^*$	48 ± 4.6	$28 \pm 4.6^*$	$36 \pm 4.4^*$
2 (12 h)	40 ± 7.1	55 ± 3.5	$31 \pm 2.0^*$	$27 \pm 5.3^*$
Ctrl (24 h)	69 ± 4.5	64 ± 5.6	59 ± 8.0	56 ± 6.5
1 (24 h)	$29 \pm 4.0^*$	$28 \pm 4.6^*$	$27 \pm 2.1^*$	$38 \pm 4.0^*$
2 (24 h)	$36 \pm 5.5^*$	$43 \pm 3.6^*$	$29 \pm 4.7^*$	16 ± 1.5

Data indicate percentages (%) of proliferating BrdU-positive cells. Data shown are means ± SD obtained from three independent experiments. MCF-7, MDA-MB-468, LNCaP and DU-145 cancer cells were treated with **1** or **2** at IC_{50} concentration for 6/12/24 h and compared with untreated control cells.

* Denote values that are significantly different from control value at $p < 0.05$.

Table 4
Cell cycle distribution of MCF-7, MDA-MB-468, LNCaP, and DU-145 cells analyzed by flow cytometry after 24 h treatment with **1** or **2** with IC_{50} concentrations

Cell line	Control/BRs (IC_{50}; 24 h)	Apoptosis $subG_1$ (%)	Cell cycle distribution (%)		
			G_1	S	G_2/M
MCF-7	Ctrl	17	60	14	26
	1	15	80	11	9
	2	13	81	7	12
MDA-MB-468	Ctrl	10	52	31	17
	1	35	74	17	9
	2	75	88	10	2
LNCaP	Ctrl	8	70	14	16
	1	18	94	2	4
	2	47	86	5	9
DU-145	Ctrl	20	62	15	23
	1	28	46	8	46
	2	24	53	8	39

Histograms of the treated cells were compared with those of untreated control cells. The percentages indicate number of cells in $subG_1$ fraction and G_1, S, G_2/M phases of the cell cycle.

Several authors have proposed that the induction of apoptosis may be cell cycle-dependent (Vermeulen et al., 2003; King and Cidlowski, 1998; Morgan and Kastan, 1997; Hartwell and Kastan, 1994). Therefore, in our next series of experiments, we tested the possibility that **1** and **2** may cause apoptosis of the breast and prostate cancer cells by blocking the cell cycle. For this purpose we carried out a flow cytometric DNA cell cycle analysis in which we sought evidence of endonucleolytic DNA degradation as manifested by losses of low molecular weight DNA moieties from the cells (Shankey et al., 1993; Gorczyca, 1999). The resulting cell cycle-related DNA histograms showed $subG_1$ peaks in the hypodiploid region that were substantially larger in the BR-treated MDA-MB-468 and LNCaP cells, and slightly larger in the DU-145 cells, relative to their respective controls (Table 4). However, no $subG_1$ peak containing apoptotic bodies was observed in MCF-7 cells. The representative pictures of untreated and BR-treated MDA-MB-468 cells are presented in Fig. 4. This evidence of apoptosis following brassinosteroid treatment is important because molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (Kastan et al., 1995; Molinari, 2000; McDonald and El-Diery, 2000). However, further evidence is required to confirm that BRs have apoptotic effects, and such evidence is currently being sought by other approaches.

In summary, the present study provides new data on cellular effects of BRs. The **1** and **2** are capable of arresting the cell cycle, resulting in apoptotic changes in human cancer cells. The results also reveal a new way in which the viability of human breast and prostate cancer could be therapeutically modified. **2**, one of the most widely studied compounds in this class, is probably the best candidate for practical applications at the moment, partly because it is relatively easy to synthesize, and partly because data

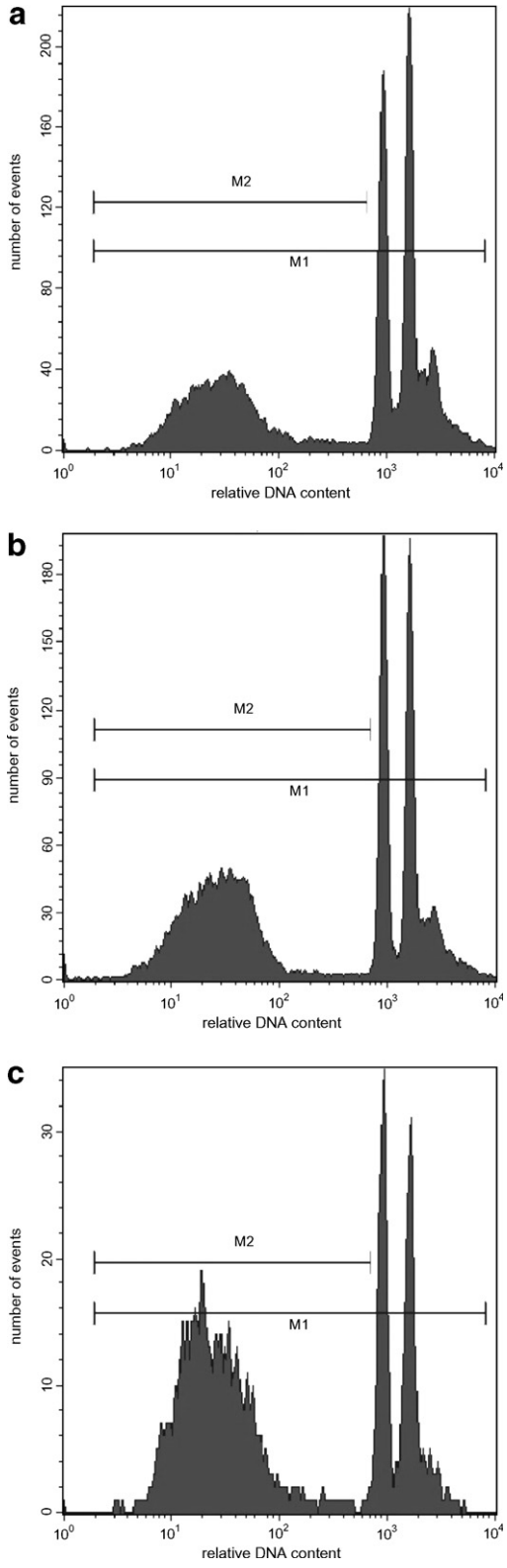

Fig. 4. Results of flow cytometric cell cycle analysis of untreated MDA-MB-468 control cells (a) and cells treated with **1** (b) and **2** (c) at IC_{50} concentrations for 24 h. The M_1 region represents signals from cells in the G_1, S, and G_2/M phases, and the M_2 region signals from apoptotic cells with reduced DNA contents (subG_1 peak). Histograms of the treated cells were compared with those of the untreated control cells. Horizontal and vertical axes indicate relative nuclear DNA contents and numbers of cells, respectively.

have already been collected (due to its agricultural use) showing that it has very low toxicity. Concentrations causing acute toxicity (LD_{50}) in mice (female, *per os*) have been found to be higher than 1000 mg/kg and the LD_{50} (*per os* and *cutaneously*) in rats higher than 2000 mg/kg; at a concentration of 0.01% **2** does not irritate mucous membranes of rabbit eyes; its toxicity to carp (TML48) is reportedly higher than 10 ppm; and in Ames mutagenicity tests it has shown negative results (Onatskiy et al., 1997; Kuzmitskii and Mizulo, 1991). Therefore, it seems highly probable that BRs does not have toxic effects in mammals and could be used in the treatment of various cancers without risks of strong side effects. If so, this new direction comprising medicinal applications may bring an important new stimulus to brassinosteroid research.

3. Concluding remarks

Brassinosteroids are steroid plant hormones that are essential for many plant growth and development processes. To our knowledge, this is the first study showing that natural BRs can inhibit the growth of various cancer cell lines at micromolar concentrations, despite having minimal effects on normal cells. Information on the mechanisms of action of BRs at a molecular level is currently scarce, but they may involve interactions with steroid receptors. To explore this possibility, we also assessed the effects of two natural BRs, **1** and **2**, on the viability, proliferation and cycling of hormone-sensitive/insensitive cancer cell lines. The findings indicate that BRs are promising leads for the development of a new generation of anticancer drugs. Additional studies are needed to explain the different response to **1** and **2** in cancer cells *versus* normal cells. The challenge for future studies will be to improve the understanding of the genetic and proteomic changes and specify regulatory pathways of BRs-induced apoptosis in disease states.

4. Experimental

4.1. Materials

The natural brassinosteroids and related compounds (cholesterol, β-ecdysone, brassicasterol, 5α-cholestane, stigmasterol, sitosterol, castasterone, brassinolide, 28-homobrassinolide, 28-homocastasterone, 24-epicastasterone, 24-epibrassinolide, 22*S*,23*S*-28-homocastasterone, 22*S*,23*S*-24-epicastasterone, 22*S*,23*S*-24-epibrassinolide, 22*S*,23*S*-28-homobrassinolide) tested in these studies were obtained either from SciTech or Olchemim Ltd., Czech Republic. Stock solutions (10 mM) were prepared by dissolving appropriate quantities of each 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). Monoclonal mouse anti-bromodeoxyuridine (BrdU) was obtained from Dako (Glostrup, Denmark) and calcein AM from PAA Laboratories GmbH (Pasching, Austria).

4.2. Cell cultures

The screening cell lines (T-lymphoblastic leukaemia CEM; breast carcinoma MCF-7, breast adenocarcinoma MDA-MB 468, prostate carcinoma DU-145 and LNCaP, lung carcinoma A-549, chronic myelogenous leukaemia K562, multiple myeloma RPMI 8226, cervical carcinoma HeLa, malignant melanoma G361, osteosarcoma HOS), and human foreskin fibroblasts immortalized with h-TERT BJ (Ramirez et al., 2004) were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were cultured in F-12 medium (Sigma, MO, USA), LNCaP cells in RPMI 1640 medium (GIBCO, USA) and all others cells were cultured in DMEM medium (Sigma, MO, USA). All media used were supplemented with 10% heat-inactivated fetal 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.

4.3. Calcein AM cytotoxicity assay

Suspensions with approximately 1.25×10^5 cells/ml were distributed in 96-well microtitre 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 Fluoroscan Ascent fluorometer (Microsystems). The percentage of surviving cells in each well was calculated from the equation $IC_{50} = (OD_{\text{drug exposed well}}/\text{mean } OD_{\text{control wells}}) \times 100\%$, each compound was tested in triplicate and the entire test was repeated at least three times. The IC_{50} value, the concentration lethal to 50% of the tumor cells, of each tested substance was calculated from the obtained dose–response curves (Kryštof et al., 2005).

4.4. Cell viability assay (MTT)

Cell viability was assessed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays to determine IC_{50} concentrations of the studied agents as described previously (Mosmann, 1983; Carmichael et al., 1987). In these assays 4.5×10^3 (MCF-7, DU-145) or 5×10^3 (MDA-MB-468, LNCaP) cells were added per well in culture medium to 96-well plates, and grown for 24 h (MCF-7, MDA-MB-468, and DU-145) or 48 h (LNCaP). The cells (70–80% confluent) were then treated with BRs for 6, 12 and 24 h in cell culture medium. Cells that were used as controls were incubated solely with the maximum used amount of the diluent DMSO. The concentration leading to 50% inhibition of viability (IC_{50}) after 24 h was determined by measuring absorbance at 570 nm, using a Labsystem Multiscan RC ELISA reader, as an indicator of MTT reductase activity. The viability of treated cells was expressed as a percentage relative to the viability of control vehicle-treated cells. Each experiment was performed in triplicate and independently repeated at least four times.

4.5. Bromodeoxyuridine incorporation assay

This immunostaining procedure is based on measuring the incorporation of bromodeoxyuridine (BrdU) into nuclear DNA in place of thymidine during the S-phase of the cell cycle using specific anti-BrdU antibodies (clone Bu20a, Dako, Denmark) following membrane permeabilization, as previously described (Gratzner, 1982). Cells (60–70% confluent) were cultured with brassinosteroids (IC_{50}) for 6, 12 and 24 h in 60-mm culture dishes with coverslips, as described above. The BrdU reagent (0.1 mM; Sigma, MO, USA) was added to the culture medium with cells for 4 h in a CO_2 incubator at 37 °C. After incubation the cells were washed three times with PBS and fixed with cold acetone–methanol (1:1, v/v) for 10 min. The cells were then denaturated by HCl (1:5; Lachema, Czech Republic) and incubated with the specific anti-BrdU antibodies (diluted 1:100 in PBS) for 60 min in the dark. The cells were then washed three times in PBS (10 mM, pH 7.4) and incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-labeled secondary antibody (Sigma, MO, USA) for 60 min in the dark. They were then washed three times in PBS and incubated with DAPI (50 μg/ml; Sigma, MO, USA) for 10 min in the dark. The coverslips with cells were washed in deionized water, mounted on glass slides in the hydrophilic medium Mowiol (Calbiochem, CA, USA) in glycerol-PBS (1:3, v/v) and examined under a fluorescence microscope (BX50F, Olympus, Japan). The FITC labeled cells incorporating BrdU (S phase cells) were counted in at least 25 fields and their numbers were compared with corresponding numbers of untreated control cells (Kao et al., 2001).

4.6. Apoptosis and cell cycle analysis

Flow cytometry was used to evaluate the number of cells in the particular phases of the cell cycle, including $subG_1$

peak detection. For this purpose the cells were seeded at densities of 1.6×10^4 cells/cm^2 (LNCaP) or 1.4×10^4 cells/cm^2 (DU-145, MDA-MB-468 and MCF-7) in their respective culture media in 100 mm culture dishes. After 24 h (DU-145, MDA-MB-468 and MCF-7) or 48 h (LNCaP) incubation the cells reached approximately 80% confluence. The cells were then treated with IC_{50} concentrations, as determined by the MTT assay described above, of **1** or **2**. Cells treated with DMSO alone were used as controls. After 24 h incubation, the cells were washed twice with cold PBS (10 mM, pH 7.4), centrifuged at 360*g* for 10 min at 4 C, and fixed in chilled ethanol (70%; v/v) with gentle vortex mixing. To determine their DNA contents, the cells were stained with propidium iodide and analyzed using a FACSCalibur flow cytometer following the manufacturer's recommendations (BD Biosciences, San Jose, CA) (Strakova et al., 2004).

4.7. Statistical analysis

The data presented are means ± standard deviations (SD) obtained from at least three independent experiments. Differences between the means were deemed to be significant if $p < 0.05$ according to Student's *t*-tests. All calculations were performed using MS Excel 2000.

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