Steroids 117 (2017) 11–15

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Flow-cytometric analysis of reactive oxygen species in cancer cells under treatment with brassinosteroids

Pyotr A. Kisselev, Olesya V. Panibrat, Aliaksei R. Sysa, Marina V. Anisovich, Vladimir N. Zhabinskii *, Vladimir A. Khripach

Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Kuprevich str., 5/2, 220141 Minsk, Belarus

ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form 14 June 2016
Accepted 20 June 2016
Available online 23 June 2016

Keywords:
Brassinosteroids
28-Homocastasterone
Cancer cell line A549
Anticancer
Reactive oxygen species

ABSTRACT

To explore the underlying mechanism of cancer cell growth inhibition by brassinosteroids (BS), reactive oxygen species (ROS) generation under treatment with 28-homocastasterone and its synthetic derivatives (22*S*,23*S*)-28-homocastasterone was measured in A549 human lung adenocarcinoma cells. BS induced ROS generation in A549 cells and their growth in a time and dose-dependent manner. The maximal effect was observed for (22*S*,23*S*)-28-homocastasterone which at 30 μM concentration showed a 6-fold increase of ROS generation in comparison with the control.

1. Introduction

Reactive oxygen species (ROS) are well established to play an important role in a wide variety of physiological and pathological processes both in plant and animal cells [1]. At the early stages of studies, ROS were mainly characterized in terms of their harmful effects [2–4]. Gradually, an understanding was reached that the ROS adverse effects were overestimated and the concept of "oxidative signaling" was developed [5]. In plants, the ROS signaling cascade is involved in cell differentiation [6], cell cycle, programmed cell death, hormone signaling, growth, and development [7]. In addition, ROS generation was found to be a common signal triggering downstream response to biotic and abiotic stresses [8,9]. The corresponding plant defence system is governed by a crosstalk between phytohormones which produce ROS as second messengers. The latter convey the hormonal information to mediate a wide range of adoptive responses [10].

Brassinosteroids (BS) have been recognized to be an integral part of the plant hormonal system [11–13]. Among various biological activities of BS, the most significant one is the ability to increase plant resistance to unfavourable biotic and abiotic environmental factors. Therefore, it was quite natural and expectable that interplay between this relatively new group of phytohormones and ROS was studied also. The first experiments studied the effect of epibrassinolide (EBl) on the accumulation of H_2O_2 in cucumber [14]. The EBl treatment was shown to increase H_2O_2 level in the apoplast of mesophyll cells, and this was accompanied by an enhanced tolerance to oxidative stress. It is worth mentioning that the foliar treatment resulted in accumulation of H_2O_2 not only in locally treated but also in the non-treated upper and lower leaves [15]. A transient increase of the superoxide anion radical generation was observed in wheat coleoptiles treated with BS [16,17].

Although BS have so far been found in plant sources only, they were shown to exhibit also various biological effects outside the plant kingdom [18]. In animal systems BS revealed antiviral, antifungal, antibacterial, neuroprotective, immunomodulatory, and other activities that makes them promising candidates for a variety of medicinal applications [19]. Of particular interest are numerous reports describing the anticancer properties of BS [20–31]. However, very little is known about the mechanism by which BS exert their cytotoxic effects. Knowledge of the influence of these

Abbreviations: BS, brassinosteroids; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; EBl, 24-epibrassinolide; Et-Br, ethidium bromide; FBS, fetal bovine serum; 28-homocastasterone, 28-homoCS; (22*S*,23*S*)-28-homocastasterone, (22*S*,23*S*)-28-homoCS; MTT, 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RFU, relative fluorescence units; ROS, reactive oxygen species.

* Corresponding author.
E-mail address: vz@iboch.bas-net.by (V.N. Zhabinskii).

http://dx.doi.org/10.1016/j.steroids.2016.06.010
0039-128X/

compounds on the redox status of animal cells is likely to contribute to the explanation of the observed effects.

In this paper, we provide the first experimental characterization of BS impact on the ROS level in cancer cell line A549 (lung adenocarcinoma). The experiments were carried out using the natural phytohormone 28-homocastasterone and its synthetic derivative (22*S*,23*S*)-28-homocastasterone (Fig. 1).

2. Experimental

2.1. Materials

28-Homocastasterone (28-homoCS), (22*S*,23*S*)-28-homocastasterone ((22*S*,23*S*)-28-homoCS), and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), respectively, were synthesized as previously described [32–34]. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin were purchased from Sigma and 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Serva Electrophoresis (Heidelberg, Germany).

2.2. Cell culture

Human lung adenocarcinoma cell line A549 was purchased from the Russian Cell Culture Collection. A549 cells were cultivated in DMEM medium. The medium was supplemented with 10% FBS, L-glutamine (250 mg/L), penicillin (100 U/mL), streptomycin (100 mg/L). A549 cells were kept under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Cells were subcultured twice per week following standard trypsinization protocols.

2.3. MTT cell viability assay

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 [35]. In these assays 1.0×10^4 A549 cells in medium were added per well to 96-well plates, and grown for 24 h. The cells (70–80% confluent) were treated with BS in concentrations of 1–200 μM for 24 h in cell culture medium. Cells used as control were incubated solely with the maximum used amount of the diluent DMSO. After incubation for 24 h, MTT solution (5 mg/mL) was added and the cells were incubated for 4 h. The concentration leading to 50% inhibition of viability (IC_{50}) after 24 h was determined by measuring absorbance at 570 nm, using a microplate reader, as an indicator used to measure 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.

2.4. Measurement of intracellular ROS generation

ROS generation was analyzed by flow cytometry using DCFH-DA [34]. For this assay A549 cells were grown in 6-well plates, and grown for 24 h. At 70–80% confluence cells were treated with BS in concentrations of 1–100 μM and 10 μM DCFH-DA and incubated for 2 h in 5 mM PBS buffer. Cells used as control were incubated solely with 10 μM DCFH-DA and the maximum used amount of the diluent DMSO. Fluorescence generation due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein (DCFH) by non-specific cellular esterases and the subsequent oxidation of DCFH by peroxides was measured by means of flow cytometry (Beckman Coulter Cytomics FC 500 (λ_{ex} = 495 nm and λ_{em} = 520 nm)).

2.5. Cell cycle determination

For this purpose the cells were seeded at densities of 4.0×10^4 - cells/cm^2 in DMEM culture media in T-25 cm^2 culture flasks. After 24 h incubation, the cells were treated with effective concentrations as determined by the intracellular ROS formation assay described above. Cells treated with DMSO alone were used as controls. After 24 h incubation, the cells were washed twice with PBS (pH = 7.4), trypsinized and washed from trypsin by centrifugation at 360*g* for 5 min. Then supernatant was removed and OptiLyse C (Beckman Coulter) was added for 10 min. Afer adding lysis buffer cells were washed twice with centrifugation and fixed in chilled ethanol (70%; v/v). To determine their DNA contents, the cells were stained with Et-Br and analyzed using a Beckman Coulter Cytomics FC 500 flow cytometer. The obtained results were processed using Multicycle AV Software (Phoenix Flow Systems, USA).

2.6. Determination of dead cells

The percentage of dead cells was determined using ethidium bromide (Et-Br) staining protocol [36]. Et-Br only stains cells that have lost their membrane integrity. Briefly, after incubation for 2 h with DCF-DA and BS in appropriate concentrations, cells were detached using 0.25% trypsin-EDTA solution, counted and treated with 10 μl of Et-Br for 10 min in the dark. The amount of dead cells was determined with the help of flow cytometry. The obtained results were processed using CXP Software (Beckman Coulter, USA).

2.7. Statistical analysis

The data were expressed as mean ± S.D. All statistics were calculated using the STATISTICA program (StatSoft, USA). A p-value of <0.05 was considered as significant.

3. Results and discussion

The cytotoxicity of BS was evaluated by the MTT assay [34]. As shown in Fig. 2, both compounds reduced the proliferation of A549

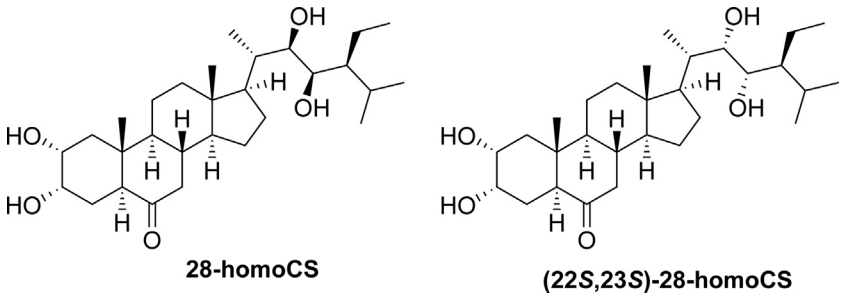

Fig. 1. Chemical structure of 28-homocastasterone and (22*S*,23*S*)-28-homocastasterone.

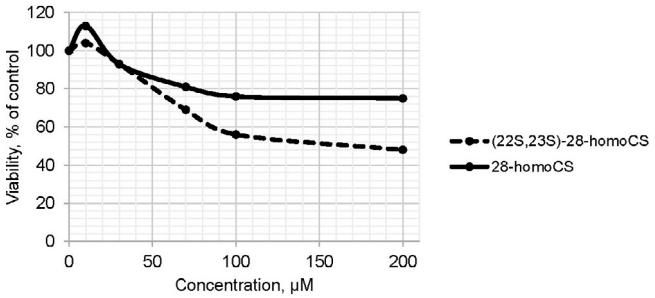

Fig. 2. Effect of BS towards A549 cells as determined by the MTT assay.

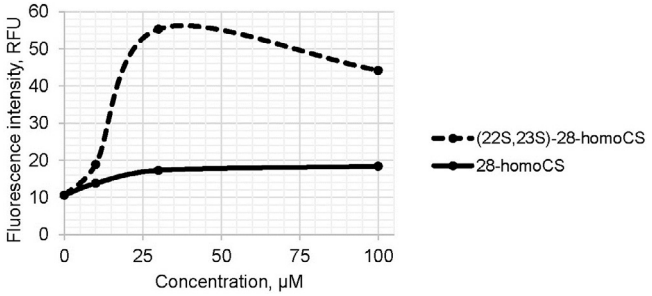

Fig. 4. Effect of BS on intracellular ROS formation in A549 cells as determined by measuring the average fluorescence intensity.

cells in a dose-dependent manner. The more pronounced effect was observed for (22*S*,23*S*)-28-homocastasterone.

To measure intracellular levels of ROS, A549 human lung adenocarcinoma cells were stained with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). This widely used indicator is converted inside the cells after cleavage of the acetate groups by intracellular esterases and oxidation by ROS into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [37]. In combination with flow cytometry, it is a very easy to use and extremely sensitive method to evaluate the redox state of cells [38].

As an initial step, we measured the distribution of the fluorescence intensity in the presence or absence of the studied BS. It was found that the treatment of A549 cells with (22*S*,23*S*)-28-homoCS led to a marked shift to greater fluorescence peak intensities compared to the untreated control (Fig. 3) thus reflecting the increase in ROS intracellular level.

This increase was concentration dependent over a range of 10–100 μM, as was determined by measuring the average fluorescence (Fig. 4). The fluorescence intensity of BS-treated A549 cells was much higher than those of untreated controls ($p < 0.05$). The maximum effect was observed for the compound (**2**) which at 30 μM concentration showed a 6-fold increase of the fluorescence intensity in comparison with the control.

For a more detailed analysis of BS action on cancer cells, two parameter flow cytometry protocol was employed for the differentiation of the cell population using ethidium bromide (Et-Br) in addition to DCFH-DA. Et-Br, belonging to the fluorescent DNA dyes, is known as an intercalating agent to stain nucleic acids. It is not able to permeate the undamaged plasma membrane of normal cells. The compromised membrane of non-viable cells allows the penetration of Et-Br into the cell, and after binding to DNA the stain then emits orange fluorescent light [39]. Representative histograms depicting Et-Br and DCF fluorescence in A549 cells treated with BS are given in Fig. 5. All cells can be divided into certain subpopulations in the coordinates Et-Br vs. DCF. Dead cells are distinguished based on a high Et-Br fluorescence intensity whereas living cells display a low fluorescent intensity of this DNA-bound fluorochrome. The latter cells could be divided further into two groups: those with low and those with high fluorescence of DCF. Splitting the histogram into quadrants B1-B4 allows the differentiation of the cells' response to oxidative stress not only for the entire population but also for specific groups of cells. The percentage of cells in the two lower quadrants reflect the content of living cells, and the two top ones that of dead cells characterized by a high value of fluorescence Et-Br.

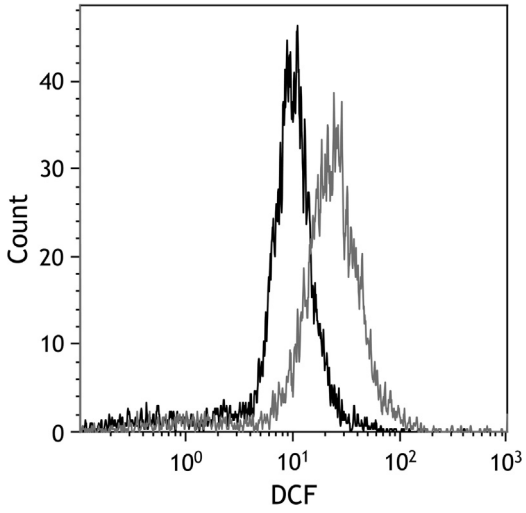

Fig. 3. Fluorescence histograms of A549 human lung adenocarcinoma cells in the absence (left panel) or in the presence (right panel) of (22*S*,23*S*)-28-homoCS. The vertical axis gives the number of events in linear scale, and the horizontal axis shows the fluorescence intensity in a logarithmic scale.

As follows from Fig. 5, under the action of (22*S*,23*S*)-28-homoCS the cell population shifts from the bottom left quadrant (B3) to the lower right quadrant (B4), which is the area with a greater intensity of DCF fluorescence and hence an increased level of ROS. Simultaneously, a shift of the cell population along the ordinate axis to a higher level of Et-Br can be noticed. This may indicate the appearance of membrane damages through which the fluorochrome can enter a cell. This is especially noticeable at the high concentration of (22*S*,23*S*)-28-homoCS (100 μM). Two more effects are observed in this case: the appearance of a considerable amount of cells (13.4%) in the left upper quadrant (B1) and an almost two-fold (from 35% to 19%) reduction in the proportion of cells in quadrant B4, compared to the 10 μM concentration, i.e. characterized by a high fluorescence of DCF. The latter is consistent with the dome-like dependence of the mean fluorescence intensity of DCF on the concentration of (22*S*,23*S*)-28-homoCS. The observed effect may be due to the fact that high concentrations of BS lead to the appearance of a large number of non-viable cells, possibly by a mechanism unrelated to the intracellular levels of reactive oxygen species. In general, especially in the lower concentration range, increased ROS level and the number of dead cells with damaged membrane increases proportionally to the concentration of (22*S*,23*S*)-28-homoCS.

Furthermore, a 2-fold increase in the level of apoptosis under the treatment of natural 28-homoCS as compared to the control was observed in the experiments on apoptosis and cell cycle determination of A549 cell line, while no such effect was found for (22*S*,23*S*)-28-homoCS (Table 1). These data suggest that mechanism of cell death under the treatment of synthetic BS could be associated with necrosis. Such effects of the studied compounds are not surprising, since it is known that an increase of the ROS level in cancer cells often leads to the switching mechanism of their death from apoptotic to necrotic [40].

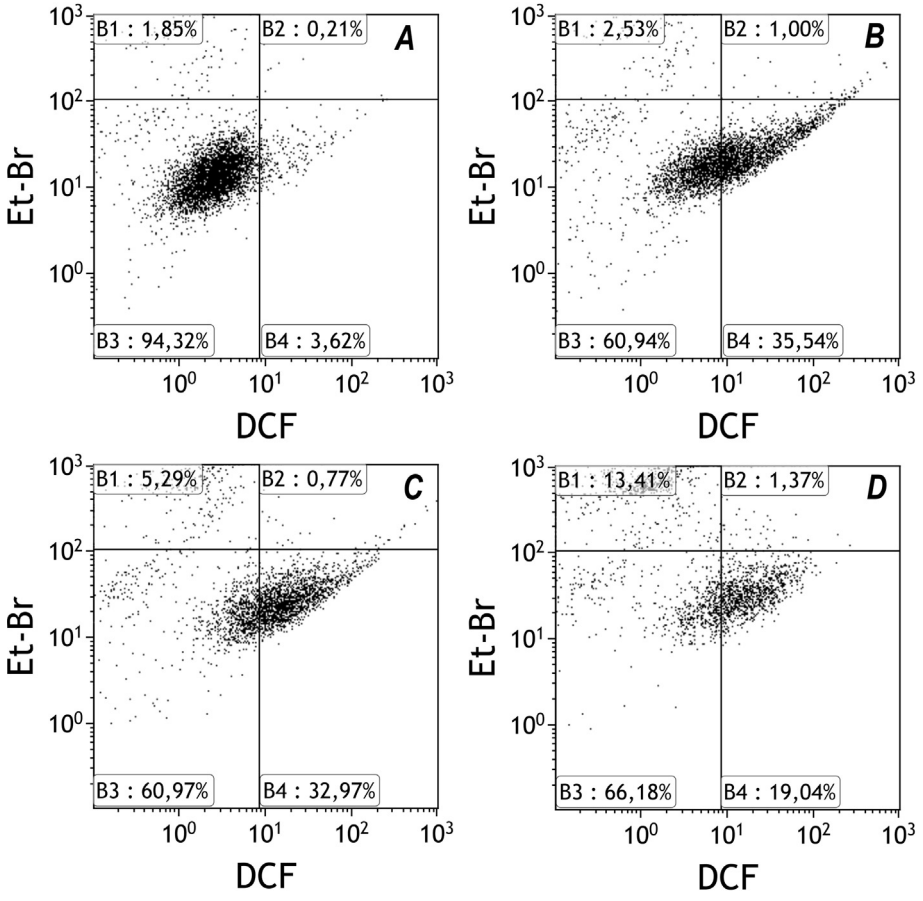

Fig. 5. Scatter histogram of fluorescence Et-Br vs. DCF in A549 cells at different concentrations of (22*S*,23*S*)-28-homoCS. A) Control. B) 10 μM. C) 30 μM. D) 100 μM.

Table 1
Apoptosis level and cell cycle distribution of A549 cells after treatment with 28-homoCS and (22*S*,23*S*)-28-homoCS

Control/BS (24 h)	[BS], μM	Apoptosis subG$_1$ (%)	Cell cycle distribution (%)			
			G$_1$	G$_2$	S	G$_2$/G$_1$
Control	1% DMSO	12	56	0	44	2
28-homoCS	100	24	63	0	37	2
(22*S*,23*S*)-28-homoCS	50	14	64	0	37	2

4. Conclusion

Our studies revealed that BS induce ROS generation in A549 cells in a time and dose-dependent manner. The maximal effect was observed for (22*S*,23*S*)-28-homocastasterone which at 30 μM concentration exhibited a 6-fold increase of ROS generation in comparison with the control. To the best of our knowledge, this is the first report demonstrating the effect of steroid plant hormones on ROS generation in cancer cells. It may be responsible, at least partly, for the cytotoxic properties of BS toward tumor cells. A common feature of nearly all cancer cells is the elevated level of ROS resulting from a disruption of redox homeostasis due to various reasons (increased metabolic activity, mitochondrial disfunction, oncogene activity, etc.) [41]. Excessive levels of ROS are known to be toxic to all living cells causing progressive oxidative damage to cellular proteins, lipids and DNA and ultimately cell death. It is reasonable to expect that a further increase of ROS levels will be more harmful for cancer cells than for normal ones. In this way, compounds with prooxidant properties, capable of increasing ROS generation and raising oxidative stress over the toxicity threshold, may be considered as potential pharmacological agents for anticancer therapy [42,43]. A number of ROS-generating agents are currently in use or in clinical trials as chemotherapeutic drugs, such as cisplatin [44], doxorubicin [45], bleomycin [46], and arsenic trioxide [47]. The results of the present investigation showed that, based on their ROS-generating properties, BS may serve as new lead compounds for the development of novel cancer therapeutics.

Acknowledgments

The financial support of the Belarusian Foundation for Fundamental Research (project X16MC-005) is greatly appreciated. We thank Prof. Ae de Groot (Wageningen, the Netherlands) for helpful discussion and comments.

References

[1] B.C. Dickinson, C.J. Chang, Chemistry and biology of reactive oxygen species in signaling or stress responses, Nat. Chem. Biol. 7 (8) (2011) 504–511.
[2] K.M. Holmstrom, T. Finkel, Cellular mechanisms and physiological consequences of redox-dependent signalling, Nat. Rev. Mol. Cell Biol. 15 (6) (2014) 411–421.
[3] U. Theopold, Developmental biology: a bad boy comes good, Nature 461 (7263) (2009) 486–487.
[4] P. Sharma, A.B. Jha, R.S. Dubey, M. Pessarakli, Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions, J. Bot. 2012 (2012) 26.
[5] C.H. Foyer, G. Noctor, Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context, Plant, Cell Environ. 28 (8) (2005) 1056–1071.

[6] R. Schmidt, J.H.M. Schippers, ROS-mediated redox signaling during cell differentiation in plants, Biochim. Biophys. Acta, Gen. Subj. 1850 (8) (2015) 1497–1508.
[7] N. Garg, G. Manchanda, ROS generation in plants: Boon or bane?, Plant Biosyst 143 (1) (2009) 81–96.
[8] M. Fujita, Y. Fujita, Y. Noutoshi, F. Takahashi, Y. Narusaka, K. Yamaguchi-Shinozaki, K. Shinozaki, Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks, Curr. Opin. Plant Biol. 9 (4) (2006) 436–442.
[9] H.K. Kang, K.H. Nam, Reverse function of ROS-induced CBL10 during salt and drought stress responses, Plant Sci. 243 (2016) 49–55.
[10] C.G. Bartoli, C.A. Casalongué, M. Simontacchi, B. Marquez-Garcia, C.H. Foyer, Interactions between hormone and redox signalling pathways in the control of growth and cross tolerance to stress, Environ. Exp. Bot. 94 (2013) 73–88.
[11] V.A. Khripach, V.N. Zhabinskii, A. de Groot, Brassinosteroids. A New Class of Plant Hormones, Academic Press, San Diego, 1999.
[12] S. Hayat, A. Ahmad, Brassinosteroids: Bioactivity and Crop Production, Kluwer Academic Publisher, 2003. p. 246.
[13] S. Hayat, A. Ahmad, Brassinosteroids: A Class of Plant Hormone, Springer, Dordrecht, 2011, p. 462.
[14] X.J. Xia, Y.J. Wang, Y.H. Zhou, Y. Tao, W.H. Mao, K. Shi, T. Asami, Z. Chen, J.Q. Yu, Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber, Plant Physiol. 150 (2) (2009) 801–814.
[15] X.J. Xia, Y.H. Zhou, J. Ding, K. Shi, T. Asami, Z. Chen, J.Q. Yu, Induction of systemic stress tolerance by brassinosteroid in *Cucumis sativus*, New Phytol. 191 (3) (2011) 706–720.
[16] A.A. Vayner, Y.E. Kolupaev, T.O. Yastreb, V.A. Khripach, The participation of reactive oxygen species in the induction of thermotolerance of wheat coleoptiles caused by exogenous brassinosteroids, Visn. Kharkiv. Nats. Agrarn. Univ., Ser. Biol. 30 (3) (2013) 39–45.
[17] Y.E. Kolupaev, A.A. Vayner, T.O. Yastreb, A.I. Oboznyi, V.A. Khripach, The role of reactive oxygen species and calcium ions in the implementation of the stress-protective effect of brassinosteroids on plant cells, Appl. Biochem. Microbiol. 50 (6) (2014) 658–663.
[18] V.N. Zhabinskii, N.B. Khripach, V.A. Khripach, Steroid plant hormones: effects outside plant kingdom, Steroids 97 (2015) 87–97.
[19] A.B. Pereira-Netto, Brassinosteroids: Practical Applications in Agriculture and Human Health, Bentham Science Publishers, 2012.
[20] Y.D. Wu, Y.J. Lou, Brassinolide, a plant sterol from pollen of *Brassica napus* L., induces apoptosis in human prostate cancer PC-3 cells, Pharmazie 62 (5) (2007) 392–395.
[21] F.V. Drozdov, A.P. Mekhtiev, G.E. Morozevich, V.P. Timofeev, A.Y. Misharin, Cytotoxic derivatives of (22*R*,23*R*)-dihydroxystigmastane, Russ. J. Bioorg. Chem. 33 (3) (2007) 326–333.
[22] J. Malíková, J. Swaczynová, Z. Kolář, M. Strnad, Anticancer and antiproliferative activity of natural brassinosteroids, Phytochemistry 69 (2) (2008) 418–426.
[23] A.Y. Misharin, A.R. Mehtiev, G.E. Morozevich, Y.V. Tkachev, V.P. Timofeev, Synthesis and cytotoxicity evaluation of 22,23-oxygenated stigmastane derivatives, Bioorg. Med. Chem. 16 (2008) 1460–1473.
[24] J. Steigerová, J. Oklestkova, M. Levková, L. Rárová, Z. Kolář, M. Strnad, Brassinosteroids cause cell cycle arrest and apoptosis of human breast cancer cells, Chem. Biol. Interact. 188 (3) (2010) 487–496.
[25] A.Y. Misharin, A.R. Mehtiev, V.N. Zhabinskii, V.A. Khripach, V.P. Timofeev, Y.V. Tkachev, Toxicity of (22*R*,23*R*)-22,23-dihydroxystigmastane derivatives to cultured cancer cells, Steroids 75 (3) (2010) 287–294.
[26] V.A. Khripach, V.N. Zhabinskii, O.V. Gulyakevich, O.V. Konstantinova, A.Y. Misharin, A.R. Mekhtiev, V.P. Timofeev, Y.V. Tkachev, Synthesis of secasterol and 24-episecasterol and their toxicity for MCF-7 cells, Russ. J. Bioorg. Chem. 36 (6) (2010) 746–754.
[27] J. Steigerová, L. Rárová, J. Oklešťková, K. Křížová, M. Levková, M. Šváchová, Z. Kolar, M. Strnad, Mechanisms of natural brassinosteroid-induced apoptosis of prostate cancer cells, Food Chem. Toxicol. 50 (2012) 4068–4076.
[28] V.A. Khripach, V.N. Zhabinskii, Y.V. Ermolovich, O.V. Gulyakevich, A.R. Mekhtiev, P.A. Karalkin, Synthesis and biological activity of the probable biosynthetic precursors of 24^1 norbrassinolide, Russ. J. Bioorg. Chem. 38 (4) (2012) 438–446.
[29] M.K. Kanwar, R. Bhardwaj, S.P. Chowdhary, P. Arora, P. Sharma, S. Kumar, Isolation and characterization of 24-Epibrassinolide from *Brassica juncea* L. and its effects on growth, Ni ion uptake, antioxidant defense of Brassica plants and in vitro cytotoxicity, Acta Physiol. Plant 35 (4) (2013) 1351–1362.
[30] P. Obakan, E.D. Arisan, A. Calcabrini, E. Agostinelli, Ş. Bolkent, N. Palavan-Unsal, Activation of polyamine catabolic enzymes involved in diverse responses against epibrassinolide-induced apoptosis in LNCaP and DU145 prostate cancer cell lines, Amino Acids 46 (3) (2014) 553–564.
[31] P. Obakan, C. Barrero, A. Coker-Gurkan, E.D. Arisan, S. Merali, N. Palavan-Unsal, SILAC-based mass spectrometry analysis reveals that epibrassinolide induces apoptosis via activating endoplasmic reticulum stress in prostate cancer cells, PLoS One 10 (9) (2015) e0135788.
[32] V.A. Khripach, V.N. Zhabinskii, G.I. Ivanova, V.K. Olkhovick, A new synthesis of homobrassinolide, Vesti AN Belarusi, Ser. Khim. Nav. 1 (1992) 70–72.
[33] A.A. Akhrem, F.A. Lakhvich, V.A. Khripach, N.V. Kovganko, V.N. Zhabinskii, A new synthesis of (22*S*,23*S*)-homocastasterone, Dokl. Akad. Nauk 275 (1984) 1089–1091.
[34] R. Brandt, A.S. Keston, Synthesis of diacetyldichlorofluorescin: a stable reagent for fluorometric analysis, Anal. Biochem. 11 (1) (1965) 6–9.
[35] J. van Meerloo, G.J. Kaspers, J. Cloos, Cell sensitivity assays: the MTT assay, Methods Mol. Biol. 731 (2011) 237–245.
[36] S.E. Petersen, Accuracy and reliability of flow cytometric DNA analysis using a simple, one-step ethidium bromide staining protocol, Cytometry 7 (4) (1986) 301–306.
[37] X. Chen, Z. Zhong, Z. Xu, L. Chen, Y. Wang, 2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: forty years of application and controversy, Free Radical Res. 44 (6) (2010) 587–604.
[38] E. Eruslanov, S. Kusmartsev, Identification of ROS using oxidized DCFDA and flow-cytometry, Methods Mol. Biol. 594 (2010) 57–72.
[39] L.R. Castilho, A.M. Moraes, E.F.P. Augusto, M. Butler, Animal Cell Technology: From Biopharmaceuticals to Gene Therapy, Taylor & Francis, New York, 2008, pp. 157–158.
[40] J. Chandra, A. Samali, S. Orrenius, Triggering and modulation of apoptosis by oxidative stress, Free Radical Biol. Med. 29 (3–4) (2000) 323–333.
[41] G.-Y. Liou, P. Storz, Reactive oxygen species in cancer, Free Radical Res. 44 (5) (2010) 479–496.
[42] T.W. Stief, The physiology and pharmacology of singlet oxygen, Med. Hypotheses 60 (4) (2003) 567–572.
[43] D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?, Nat Rev. Drug Discov. 8 (7) (2009) 579–591.
[44] C. Casares, R. Ramirez-Camacho, A. Trinidad, A. Roldan, E. Jorge, J.R. Garcia-Berrocal, Reactive oxygen species in apoptosis induced by cisplatin: review of physiopathological mechanisms in animal models, Eur. Arch. Otorhinolaryngol. 269 (12) (2012) 2455–2459.
[45] S. Zhang, X. Liu, T. Bawa-Khalfe, L.S. Lu, Y.L. Lyu, L.F. Liu, E.T. Yeh, Identification of the molecular basis of doxorubicin-induced cardiotoxicity, Nat. Med. 18 (11) (2012) 1639–1642.
[46] S.B. Wallach-Dayan, G. Izbicki, P.Y. Cohen, R. Gerstl-Golan, A. Fine, R. Breuer, Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway, Am. J. Physiol. Lung Cell. Mol. Physiol. 290 (4) (2006) L790–L796.
[47] Y.H. Han, H.J. Moon, B.R. You, S.Z. Kim, S.H. Kim, W.H. Park, Effects of arsenic trioxide on cell death, reactive oxygen species and glutathione levels in different cell types, Int. J. Mol. Med. 25 (1) (2010) 121–128.