

ANTIPROLIFERATIVE AND CYTOTOXIC EFFECT OF SELECTED VITAMIN D ANALOGS ON NASAL POLYPS FIBROBLASTS AND OTHER CELLS WITH HIGHER PROLIFERATIVE POTENTIAL*

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Abstract: Besides well-known effect on bone and mineral metabolism vitamin D is involved in essential non-calcemic regulatory mechanisms, such as cellular proliferation, differentiation and apoptosis in various cell types. Major limitation for therapeutic use of calcitriol, a hormonally active form of vitamin D, is its calcemic and phosphatemic action. Recently, more selective vitamin D analogs which retain clinically useful activities with reduced toxicity have been designed. The aim of the present study was to evaluate the *in vitro* effect of vitamin D analogs on proliferation rate and survivability of cells with increased proliferative activity. The effect of calcitriol, PRI-2191, PRI-1890, PRI-1906 and PRI-2205 was examined. The experiments were performed on cultures derived from nasal polyps and cancer cells lines (SNB-19, C32 and SH-4). Cultures were incubated 72 h with tested compounds, each at the concentration of 0.025, 0.25, 2.5 and 25 µg/mL. The cytotoxic effect of vitamin D analogs and their influence on growth rate were determined using WST-1 assay. RT-QPCR technique was used to evaluate the expression of anti-apoptotic *BCL-2* and pro-apoptotic *BAX* gene.

Each of the tested compounds presented significant effect at the concentrations above 0.25 µg/mL. The strongest inhibition of the growth rate and decrease in cell survivability was observed after treatment with PRI-1890 and PRI-2191. Stimulation with calcitriol and other vitamin D analogs led to decrease *BCL-2/BAX* mRNA ratio in each cell lines. The apparent pro-apoptotic action revealed PRI-2191 followed by PRI-1890. It might be hypothesized that vitamin D analogs supplementation may provide therapeutic benefits not only in oncological patients but also in chronic rhinosinuitis.

Keywords: vitamin D analogs, cytotoxicity, growth rate, apoptosis, nasal polyps, cancer, *BAX*, *BCL-2*

Besides well-known effect on bone and mineral metabolism vitamin D (VD) is involved in essential noncalcemic regulatory mechanisms, such as cellular proliferation, differentiation and apoptosis in various cell types (1). VD plays also an integral role in angiogenesis and number of immunologic effects towards lymphocytes, dendritic cells and macrophages (2). Calcitriol (1,25-dihydroxyvitamin D₃ or 1,25-dihydroxycholecalciferol) is a hormonally active form of VD made from calcidiol in kidneys and other tissues or delivered with food (Fig. 1). The most important downstream signal transduction pathways activated by calcitriol in target cells are realized through so called “genomic pathway” involving specific nuclear VD receptor (VDR). Activation of VDR leads to the relaxation of chromatin structure making DNA accessible for RNA

polymerase and subsequent initiation of transcription of target genes (3).

In vitro and *in vivo* observations have demonstrated that calcitriol is a potent inhibitor of cells growth (4). Anti-proliferative and pro-differentiating properties of 1,25-dihydroxyvitamin D₃ would make it a promising element of anticancer therapies (5). Major limitation for therapeutic use of calcitriol is its calcemic and phosphatemic action. The adverse effects motivated the search for new semi-selective analogs (VDA) which retain clinically useful activities with subsequent reduced calcium mobilizing potential.

Due to the multidirectional activities and lower toxicity, supplementation of newer VDA is hypothesized to have a positive therapeutic effect also in chronic inflammatory diseases (6). Previously, we

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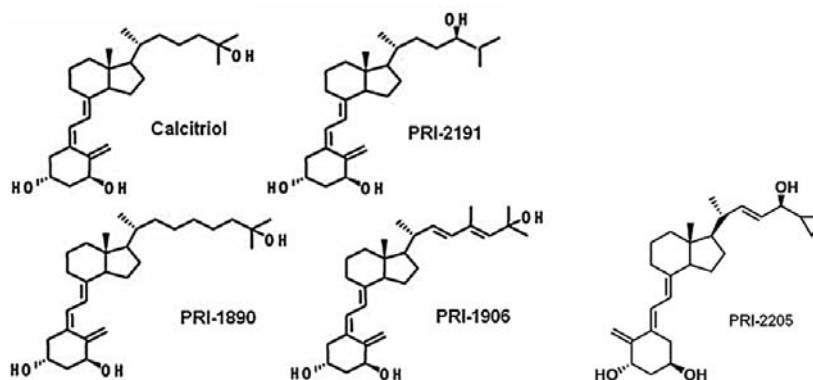


Figure 1. Structures of calcitriol and its analogs: PRI-2191, PRI-1890, PRI-1906 and PRI-2205.

have presented potential applicability of tacalcitol (PRI-2191) in the prevention and treatment of chronic rhinosinusitis with nasal polyps (NP) (7).

Chronic rhinosinusitis (CRS) is a common disease with a substantial health care impact. Due to the complex and unclear etiology, CRS remains unresolved and presents difficult therapeutical challenge. Due to well-known side effects related to steroids, there is a need for the investigation of new agents suitable for CRS management. Mulligan et al. (8) showed that patients with CRS demonstrate insufficient level of VD when compared to the control. Supplementary treatment of CRS involving VDA would be similar to broad spectrum of dermatological conditions where a moderate to strong recommendation was given for the use of topical VD in combination with steroids (9). Additionally, there is constant need to study the effectiveness of particular, newly designed analog especially in terms of unknown structure-function relationship.

The objective of this study was to determine the effect of VDA on growth rate and survivability of selected cells with increased proliferative activity (i.e., nasal polyps' fibroblasts, SNB-19, C32 and SH-4 cell line). VDA included calcitriol, tacalcitol [(24R)-1,24-dihydroxyvitamin D₃ or PRI-2191] and three newer compounds marked as PRI-1890, PRI-1906 and PRI-2205 (Fig. 1).

EXPERIMENTAL

Cells culture and treatment

The experiments were performed on the following cells: fibroblasts derived from NP, astrocytoma cell line SNB-19 (DSMZ no. ACC 325), ame-

lanotic melanoma cell line C32 (ATCC® CRL-1585™) and melanoma cell line SH-4 (ATCC® CRL-7724™). NP samples were obtained from 3 patients with CRS during routine surgical procedure performed in the Dept. of Otolaryngology, Wrocław Medical University in Poland. All the subjects met the diagnostic criterion for CRS as established by the European position paper on rhinosinusitis and nasal polyps (EPOS 2012). Patients had been free of any medications for at least 4 weeks before surgery and had bilateral NPs on endoscopic examination and computed tomography (CT). The presence of the comorbidities was excluded. The study was approved by Local Ethical Committee of Wrocław Medical University.

NP specimens were immediately rinsed in phosphate buffered saline (PBS), cut into small fragments and placed into a sample tubes containing 1 mL PBS. The tubes were directly transported on ice to the laboratory for further investigations. A part of each sample was fixed in 10% buffered neutral formalin (Chempur, Piekary Śl., Poland), processed routinely, and embedded in paraffin wax (Bio-Plast, Wrocław, Poland) for subsequent immunohistochemical examination to establish diagnosis and to exclude other pathologies.

Survivability assay

Cultures were set and carried out in Nunc 75 mL non-treated cell culture flasks. Cells were seeded at 10,000/well in a 96-well plate and incubated for next 24 h. Cells grown in DMEM (Lonza, Basel, Switzerland) medium supplemented with 10% fetal bovine serum (FBS) (Biological Industries Ltd., Kibbutz Beit-Haemek, Israel), penicillin (10000

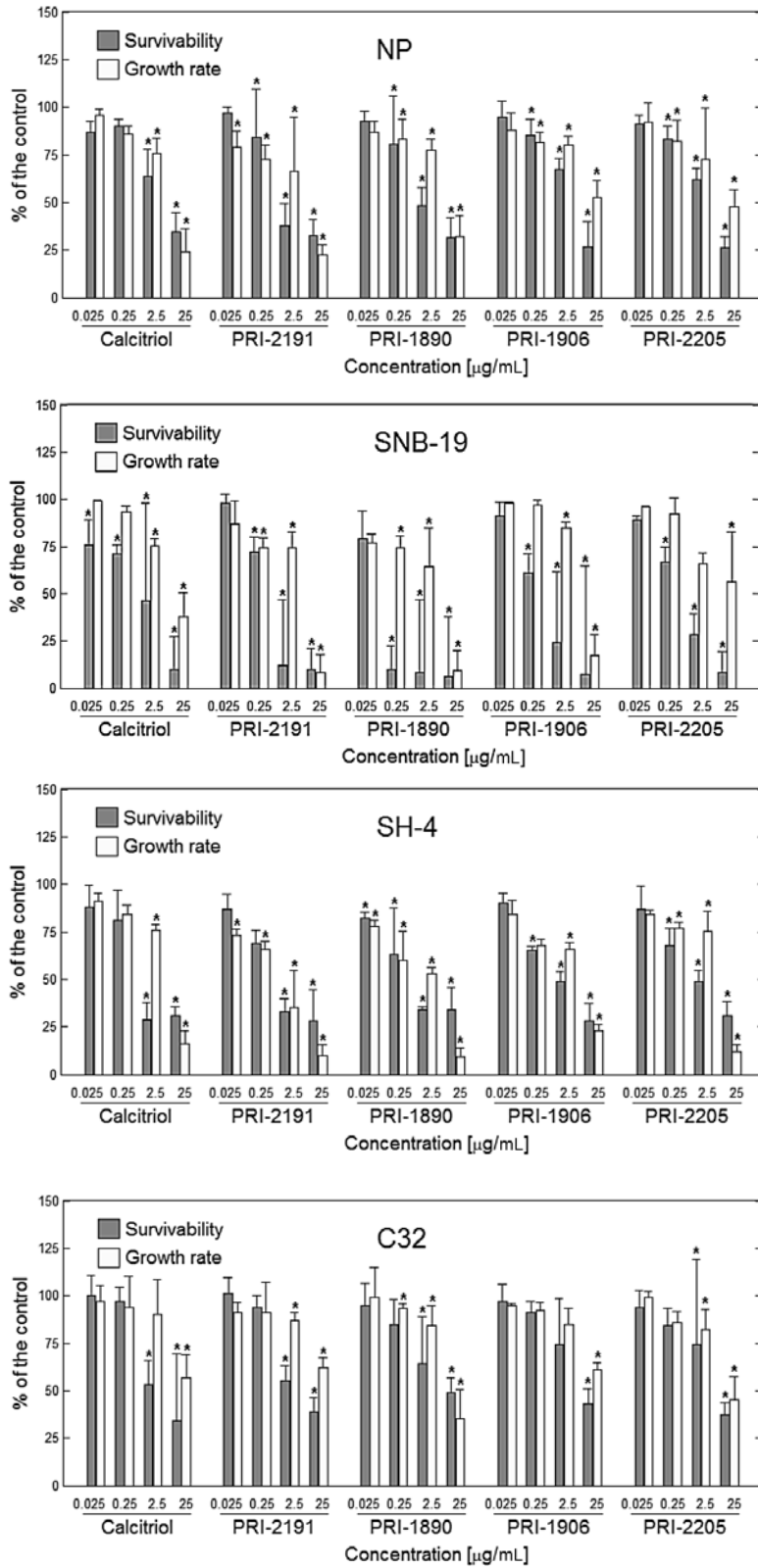


Figure 2. The results of WST-1 assay to determine cells survivability and proliferation rates of NP fibroblasts, SNB-19, SH-4 and C32 cells treated with calcitriol and vitamin D analogs (PRI-2191, PRI-1906, PRI-2205 and PRI-1890). Data are shown as the mean percentages (\pm SD) of the control culture. *Significant difference from the respective control culture ($p < 0.05$)

U/mL) and streptomycin (10 mg/mL) (Biological Industries Ltd., Kibbutz Beit-Haemek, Israel).

Restriction of cells viability (resulting from the cytotoxicity of VDA) was assessed in cultures carried out in medium without growth stimulating factors necessary for cell divisions. For that reason, the WST-1 assay (Roche Molecular Biochemicals, Mannheim, Germany) was performed after 72 h cells incubation with calcitriol and VDA (PRI-2191, PRI-1890, PRI-1906 or PRI-2205) (Fig. 1) at 0.025, 0.25, 2.5 or 25 µg/mL in FBS-free medium, according to the manufacturer's protocol. Calcitriol and VDA were manufactured and certified by the Pharmaceutical Research Institute (Warszawa, Poland). Control wells were filled with a medium without calcitriol and VDA. WST-1 assay is based on reduction of a substrate (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan. Reduction in enzyme activity leads to a decrease in the amount of formazan dye, which directly correlates to the number of metabolically active cells in the culture. The intensity of the colorimetric reaction was measured by microplate reader UVM340 (Biogenet, Piaseczno, Poland) at 440 nm.

Growth rate assay

To assess the influence of calcitriol, PRI-2191, PRI-1890, PRI-1906 and PRI-2205 on growth rate tested cells were incubated for 72 h in medium containing FBS and further supplemented with each agent at various concentrations (0.025, 0.25, 2.5 and 25 µg/mL). Total number of living cells in the cultures was estimated by cytotoxicity-proliferation WST-1 assay (Roche Molecular Biochemicals, Mannheim, Germany). Control wells were filled with a medium without calcitriol and VDA under the same conditions.

Determination of transcriptional activity of *BAX* and *BCL-2* genes

In order to evaluate the expression of anti-apoptotic *BCL-2* gene and pro-apoptotic *BAX* gene QRT-PCR technique was used. The cells were first exposed for 24 h to calcitriol, PRI-2191, PRI-1890, PRI-1906 and PRI-2205 at 0.25 µg/mL. Extraction of RNA from the cells was performed using QuickRNA™ MiniPrep Kit (Zymo Research, Irvine, CA, USA). To determine the transcriptional activity of *BCL-2* and *BAX* gene the fluorescence detector DNA Engine OPTICON™ (MJ Research, San Francisco, USA) and set of reagents from QuantiTect™ SYBR® Green RT-PCR Kit (Qiagen, Hilden, Germany) were used.

Statistical analysis

Statistical analysis was performed using Statistica PL 7.0 (Statsoft, Kraków, Poland). The data were presented as the mean ± SD. Each experimental sample was at least in triplicate and each experiment was performed at least three times. The differences between groups were explored with ANOVA and *post-hoc* Duncan test was applied. A level of $p < 0.05$ was considered to be significant.

RESULTS

Influence of calcitriol and other VDA on survivability of the cells

Calcitriol and VDA presented cytotoxic effect towards cells in each of the tested colony carried out in FBS-free medium (Fig. 2). A lack of growing stimuli (FBS) limited cells divisions within the cultures. Calcitriol and VDA action was dose-dependent. Dose dependency was most clearly visible in SNB-19 and SH-4 cell lines. Calcitriol and VDA significantly decreased cells viability even in lower concentration i.e., starting at 0.025 µg/mL in SNB-19, SH-4 and NP cell lines. In C32 cell line calcitriol and VDA had to be applied in higher concentrations ≥ 0.25 µg/mL to obtain the same results. Susceptibility to the cytotoxic action of calcitriol and VDA in the cell lines showed origin-specific manner. SNB-19 cells were the most susceptible, whereas C32 were the least sensitive to VD compounds. PRI-1890 and PRI-2191 restricted cells viability the most efficiently independently from cells origin.

Modulation of cells' growth by calcitriol and other VDA

In the second part of the experiment, the influence of calcitriol and VDA on the cells growing under the conditions enabling cell divisions were assessed. Such conditions made it possible to evaluate not only cytotoxic properties of VDA but also the impact of calcitriol and VDA on cell cycles progression and cells divisions. The action of calcitriol and VDA in each cell line was dose-dependent. Under those conditions, calcitriol and VDA efficacy to decrease cells number was weaker in most of the concentrations compared to that obtained in FBS-free medium. The exception included SH-4 cell line where calcitriol and other VDA at the highest concentration used (25 µg/mL) suppressed cells number more significantly than in FBS-free medium. The same results were obtained with PRI-1890 and PRI-2191 at 25 µg/mL in C32 and SNB cell lines, respectively. In NP fibroblasts, PRI-1890 (at 0.025,

0.25 and 25 $\mu\text{g}/\text{mL}$), PRI-2191 (at 25 $\mu\text{g}/\text{mL}$) and PRI-1906 (at 0.025 and 0.25 $\mu\text{g}/\text{mL}$) impaired growth rate more efficiently.

Modulation of *BCL-2* and *BAX* gene expression by calcitriol and VDA

Calcitriol and its analogs were used at 0.25 $\mu\text{g}/\text{mL}$ each to assess the influence on the expression of pro-apoptotic *BAX* and anti-apoptotic *BCL-2* gene. The results were shown in a form of *BCL-2/BAX* ratio which better reflects the apoptosis process than isolated *BCL-2* and *BAX* expression levels (Fig. 3). Stimulation with calcitriol and other VDA led to a decrease of *BCL-2/BAX* mRNA ratio in each tested cell lines. The strongest pro-apoptotic action had PRI-2191 followed by PRI-1890.

DISCUSSION AND CONCLUSION

In the present paper we investigated the cytotoxic and anti-proliferative effects of calcitriol and VD analogs in selected cancer cell lines and fibroblast derived from nasal polyps. Although the tested compounds were differentially active in different cell lines, PRI-1890 and PRI-2191 restricted cells viability most efficiently, independently from cells origin. It might be suggested that PRI-1890, PRI-2191 and PRI-1906 at the higher concentrations may additionally impair cells number not only due to their cytotoxic activity but also through the modulation of the cell cycle progression and divisions.

According to the published data, the therapeutic efficacy of calcitriol and many VDA, as single

agents, for the treatment of cancers has not yet fulfilled its promise. However, newer VDA including PRI-2191, PRI-1906, PRI-2205 and PRI-2202 were seen to be more potent inhibitors of cancer cells proliferation than calcitriol in monotherapy or in combination with cyclophosphamide or cisplatin (10, 11). PRI-2205, a geometric analog of calcitriol, which exerted antiproliferative activity *in vitro* and antitumor activity *in vivo*, revealed less calcemic at the doses which inhibit tumor growth (12). Similarly, PRI-1906, VDA with the extended and rigid side chain has higher antiproliferating activities with lower risk of hypercalcemia and toxicity than calcitriol. In particular, Bauska et al. (13) observed approximately 30% inhibition of proliferation in prostate cancer cells in response to PRI-1906. From the experiments carried on AML derived cells it is known that PRI-1906 has stronger than 1,25-dihydroxyvitamin D3 pro-differentiating activities. Antiproliferative potency of VDA has not been clearly explained, although the Cdk inhibitor p27^{Kip1} seems to be important in that process (14). It was showed also that calcitriol induced cell cycle arrest is accompanied by decreased Cdk inhibitor p21^{Kip1} expression and Rb dephosphorylation what further inactivates members of E2F family of transcription factors (15, 16). The outcomes of those studies showed distinct difference between the action of the compounds and the origin of cells what agrees with our results. While in cancer therapy pro-differentiating properties of VD seems to be essential, in the case of treatment of inflammatory diseases antiproliferative, pro-apoptotic and immunomodulating

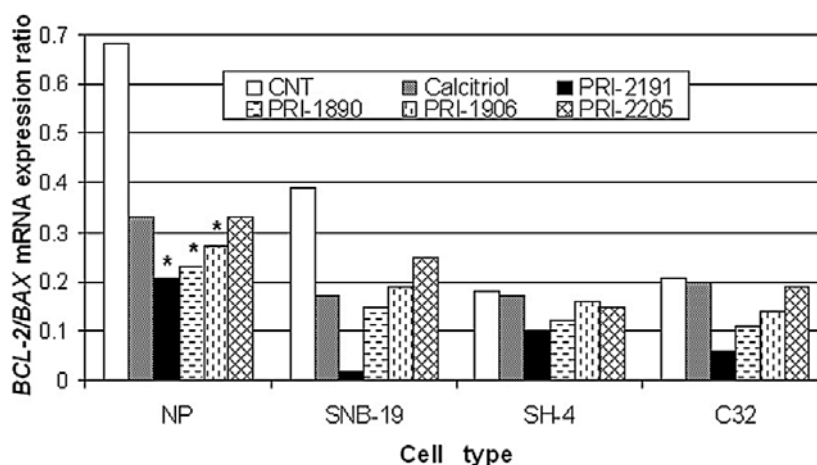


Figure 3. Effect of calcitriol and other vitamin D analogs (PRI-2191, PRI-1906, PRI-2205 and PRI-1890) on the *BAX/BCL-2* mRNA ratio in NP fibroblasts, SNB-19, SH-4 and C32 cells lines. Values are the means \pm SD. *Significant difference from the respective control culture ($p < 0.05$)

activity would be much more important. Our previous study showed that tacalcitol appeared to be more active in inhibition of NP fibroblasts proliferation than calcitriol (7).

The way in which VDA modulate apoptosis is dependent on the type of cells to be treated and is not apparent. McGuire et al. (17) reported that the pro-apoptotic effect of 1,25-dihydroxyvitamin D3 in murine squamous cell cancer is associated with up-regulation of MEKK-1. Although downstream pathway of MEKK-1 that leads to apoptosis remains undefined, there is evidence that MEKK-1 activation results in conformational changes in the pro-apoptotic BAK protein (18). Other publication indicates the role for 1,25-dihydroxyvitamin D3 in alteration of BAX subcellular distribution in the pro-apoptotic effect (19). Previously, we have revealed slightly better efficiency of tacalcitol over calcitriol in reducing *BCL-2/BAX* mRNA ratio in fibroblasts derived from NP (20). Herein, calcitriol and other tested VDA decreased *BCL-2/BAX* mRNA ratio in each cell line compared to the respective control. Tacalcitol (PRI-2191) showed the strongest pro-apoptotic activity among the all tested compounds. However, PRI-1890 revealed very similar pro-apoptotic efficacy in NP fibroblasts, SH-4 and C32 cells. Stronger pro-apoptotic activity of PRI-2191 and PRI-1890 may in part explain the pronounced effect of both compounds limiting cells survival observed in our study. PRI-2205 has been shown before to cause apoptosis of HL-60 cells at a dose of 10 nM, but at a higher dose (100 nM) it caused cells differentiation (10). Contrary, according to Dehghani et al. (21), 1,25-dihydroxyvitamin D3 may suppress cells apoptosis as it was showed in multiple sclerosis patients.

Our and other studies performed on the anti-inflammatory effects of VDA suggest the possibility of their application in CRS to develop treatment strategies that include the use of steroids in combination with either VDA or phototherapeutics. Few of the same pathways affected by dexamethasone overlapped with the VD responses (22). Additionally, it was observed that VDA stimulate innate immunity and enhance antimicrobial activity by upregulating synthesis of the anti-infective molecules like cathelicidin (23). Xystrakis et al. (24) revealed that administration of VDA enhance subsequent responsiveness to dexamethasone for induction of IL-10 what indicates that VDA could potentially increase the therapeutic response to steroids in CRS.

On the basis of the present study it might be hypothesized that vitamin D analogs have a thera-

peutic function not only in selected neoplastic disorders but also in chronic inflammatory diseases such chronic rhinosinuitis.

REFERENCES

1. Leyssens C., Verlinden L., Verstuyf A.: *Endocr. Relat. Cancer* 22, R31 (2013).
2. Mann E.H., Chambers E.S., Pfeffer P.E., Hawrylowicz C.M.: *Ann. N.Y. Acad. Sci.* 1317, 57 (2014).
3. Campbell M.J.: *Front. Physiol.* 14, 181 (2014).
4. Krishnan A.V., Trump D.L., Johnson C.S., Feldman D.: *Rheum. Dis. Clin. North Am.* 38, 161 (2012).
5. Bikle D.D.: *Endocrine* 46, 29 (2014).
6. Mann E.H., Chambers E.S., Pfeffer P.E., Hawrylowicz C.M.: *Ann. N. Y. Acad. Sci.* 4, 16 (2014).
7. Rostkowska-Nadolska B., Fraczek M., Gawron W., Latocha M.: *Acta Biochim. Pol.* 56, 235 (2009).
8. Mulligan J.K., Bleier B.S., O'Connell B.: *Clin. Exp. Immunol.* 164, 312 (2011).
9. Wat H., Dytoc M.: *J. Cutan. Med. Surg.* 1, 91 (2014).
10. Wietrzyk J., Nevozhay D., Filip B., Milczarek M., Kutner A.: *Anticancer Res.* 27, 3387 (2007).
11. Wietrzyk J., Nevozhay D., Milczarek M., Filip B., Kutner A.: *Cancer Chemother. Pharmacol.* 62, 787 (2008).
12. Milczarek M., Rosinska S., Psurski M., Maciejewska M., Kutner A., Wietrzyk J.: *Anticancer Res.* 33, 433 (2013).
13. Bauska H., Kłopot A., Kielbinski M., Chrobak A., Wijas E. et al.: *J. Steroid Biochem. Mol. Biol.* 126, 46 (2011).
14. Rosato R.R., Wang Z., Gopalkrishnan R.V., Fisher P.B., Grant S.: *Int. J. Oncol.* 19, 181 (2001).
15. Hershberger P.A., Modzelewski R.A., Shurin Z.R., Rueger R.M., Trump D.L.: *Cancer Res.* 59, 2644 (1999).
16. Alagbala A.A., Johnson C.S., Trump D.L., Posner G.H., Foster B.A.: *Oncology* 70, 483 (2006).
17. McGuire T.F., Trump D.L., Johnson C.S.: *J. Biol. Chem.* 276, 26365 (2001).
18. Mandic A., Viktorsson K., Molin M., Akusjarvi G., Eguchi H. et al.: *Mol. Cell Biol.* 21, 3684 (2001).
19. Hershberger P.A., McGuire T.F., Yu W.D.: *Mol. Cancer Ther.* 1, 821 (2002).

20. Fraczek M., Rostkowska-Nadolska B., Sliupkas-Dyrda E., Kusmierz D., Pniak J., Latocha M.: *Adv. Clin. Exp. Med.* 19, 679 (2010).
21. Dehghani L., Meamar R., Etemadifar M., Sheshde Z.D., Shaygannejad V. et al.: *Int. J. Prev. Med.* 4, S211 (2013).
22. Cheng X., Zhao X., Khurana S., Bruggeman L.A., Kao H.Y.: *PLoS One* 4, e60213 (2013).
23. Wolff A.E., Jones A.N., Hansen K.E.: *Nat. Clin. Pract. Rheumatol.* 4, 580 (2008).
24. Xystrakis E., Kusumakar S., Boswell S., Peek E., Urry Z., Richards D.F.: *J. Clin. Invest.* 116, 146 (2006).

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