Antiproliferative Activity of Vitamin D Compounds in Combination with Cytostatics

MARZENA PELCZYNSKA[1], MARTA SWITALSKA[1], MAGDALENA MACIEJEWSKA[1], IWONA JAROSZEWICZ[1,2], ANDRZEJ KUTNER[3] and ADAM OPOLSKI[1,4]

[1]*Department of Experimental Oncology, Institute of Immunology and Experimental Therapy, 12 R. Weigla St., 53-114 Wroclaw;*
[2]*Faculty of Chemistry, Wroclaw University, 14 F. Joliot-Curie St., 50-383 Wroclaw;*
[3]*Pharmaceutical Research Institute, 8 Rydygiera St., 01-793 Warsaw;*
[4]*Jan Dlugosz Academy, 13 Armii Krajowej Al., 42-201 Czestochowa, Poland*

Abstract. *Calcitriol is a potent antiproliferative agent against various tumour cells in vitro. Here, the results of a study on vitamin D compounds (calcitriol's analogues PRI-1906 and PRI-2191) as potential agents in combined antitumour therapy in vitro are presented. Applying antiproliferative SRB and MTT assays, the growth inhibitory effects of the vitamin D compounds, applied alone or in combination with either cisplatin or doxorubicin, were measured. The following cancer cell lines were employed: A549 (human non-small cell lung carcinoma), B16 (murine melanoma), CCRF, HL-60 (human leukaemia), SW707 (human colon cancer), MCF-7, T47D (human breast cancer), WEHI-3 (mouse leukaemia) and normal cells: BALB 3T3 (normal murine fibroblast cell line). It was shown that the treatment of tumour cells, which are sensitive to vitamin D compounds, with the combination of vitamin D compounds and cytostatics decreased the inhibitory concentration 50% (IC_{50}) values compared with the effects of the cytostatics applied alone.*

Calcitriol, the seco-steroid hormone, and its numerous analogues are proven, potent antiproliferative agents against various normal and neoplastic cells *in vitro* (1-3). They also have been shown to have the ability to induce the differentiation of human promyelocytic leukaemia, breast cancer, colon adenocarcinoma and prostate cancer cells, as well as of normal or psoriatic keratinocytes (4-7). In addition to its antiproliferative and differentiation-inducing effects, calcitriol induced apoptosis in a number of different cancer cell lines *in vitro*. Moreover, the inhibition of angiogenesis and regression of primary tumours were observed in tumour-bearing animals treated with calcitriol (8, 9). Such biological properties suggest potential therapeutic applications for calcitriol and its analogues, including antitumour therapy.

Unfortunately, the strong calcemic activity of calcitriol excludes its clinical use. These undesirable side-effects motivated the synthesis of new analogues, aiming at dissociating the calcemic and antiproliferative activities. One of the promising, low hypercalcemia-inducing analogues, PRI-2191 ((24R)-1,24-dihydroxyvitamin D_3), has been the object of our intensive studies (10). Its antiproliferative and antitumour effects against numerous human and mouse cancer cell lines were shown (11-13). Vitamin D_2 analogues are thought to be generally less toxic than the respective vitamin D_3 compounds. One of them, PRI-1906 ((24*E*)-(1*S*)-24-dehydro-24a-homo-1,25-dihydroxyergocalciferol), was also examined (14).

The PRI-1906 and PRI-2191 analogues revealed higher antiproliferative activity *in vitro* and similar *in vitro* toxicity against normal murine fibroblast (BALB 3T3) as calcitriol. However, an *in vivo* assay confirmed that the new analogues are less toxic than calcitriol (10 and Wietrzyk J., unpublished data). This suggests the possible application of these compounds in antitumour therapy, both as a single agents and in combined treatment protocols.

Materials and Methods

Compounds. Side-chain-modified calcitriol analogues (PRI-1906 and PRI- 2191) were used. The compounds were obtained from the

Abbreviations: VDR, vitamin D receptor; PRI-1906, new analogue of vitamin D_2; PRI-2191, new analogue of vitamin D_3; IC_{50}, inhibitory concentration 50%; SD, standard deviation.

Correspondence to: Marzena Pelczynska, MD, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla St. 12, 53-114 Wroclaw, Poland. Tel: (4871) 370 99 92, Fax: (4871) 370 99 42, e-mail: mapelcz@iitd.pan.wroc.pl

Key Words: Calcitriol, vitamin D analogues, combined treatment, cisplatin, doxorubicin, *in vitro* assay, cancer.

Pharmaceutical Research Institute, Warsaw, Poland. Samples of the compounds were stored in amber ampullae, under argon, at –20˚C. The amount of each compound was determined by UV spectrometry (Carl Zeiss spectrophotometer, Jena, Germany) at 265 nm. Prior to usage, the compounds had been dissolved in absolute ethanol to a concentration of 10^{-4} M, and subsequently diluted in culture medium to reach the required concentrations.

Cell lines. A549 (human non-small cell lung carcinoma), B16 (murine melanoma), BALB 3T3 (normal murine fibroblast cell line), CCRF (human lymphoid leukaemia) MCF-7, T47D (human breast carcinoma), SW707 (human colon adenocarcinoma) and WEHI-3 (murine leukaemia) were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained in culture or frozen in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The human promyelocytic leukaemia HL-60 cell line was obtained from the European Type Culture Collection by courtesy of Professor Spik and Dr Mazurier (Laboratory of Biological Chemistry USTL, Lille, France). Twenty-four hours before addition of the test compounds, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of 10^4 cells per well and cultured in RPMI-1640 medium (CCRF, HL-60, WEHI-3) or in a mixture of RPMI 1640 and Opti-MEM (1:1) medium (A549, B16, MCF-7, T47D, SW707). RPMI 1640 was supplemented with 2 mM glutamine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 100 mg/ml streptomycin (Polfa, Tarchomin, Poland), 100 U/ml penicillin (Polfa), 1 mM sodium pyruvate (Sigma-Aldrich Chemie GmbH) (CCRF, HL-60, WEHI-3), 0.01 mg/ml insulin (Sigma-Aldrich Chemie GmbH) (MCF-7, T47D) and 5% (A549, B16, MCF-7, T47D, SW707) or 10% (HL-60, WEHI-3) fetal bovine serum (Sigma-Aldrich Chemie GmbH). The cells were cultured at 37˚C in a humid atmosphere saturated with 5% CO_2.

Antiproliferative assays in vitro. The *in vitro* cytotoxic effects of all the agents were examined after 72-h exposure of the cultured cells to varying concentrations of the test compounds, using the SRB assay for adherent cells (A549, B16, MCF-7, T47D SW707), as described by Skehan *et al.* (15) or the MTT assay for leukaemia cells (CCRF, HL-60, WEHI-3), as described by Marcinkowska *et al.* (16).

The SRB assay: The cells attached to the plastic were fixed by gently layering cold 50% trichloroacetic acid (TCA, Sigma-Aldrich Chemie GmbH) on the top of the culture medium in each well. The plates were incubated at 4˚C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (Sigma-Aldrich Chemie GmbH) and dissolved in 1% acetic acid (POCH, Gliwice, Poland) for 30 min. Unbound dye was removed by rinsing (4x) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered TRIS base (POCH) for determination of the optical density (at 540 nm wavelength) in a computer-interfaced, 96-well microtitre plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland).

The MTT assay: For the last 3-4 h of incubation, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT: Sigma, St. Louis, MO, USA) stock solution (5 mg/ml) were added to each well. The viable cells reduce the pale yellow MTT to a navy blue formazan, so the more viable cells present in a well, the more MTT will be reduced to formazan. On completion of the incubation, 80 μl of the lysing mixture were added to each well (lysing mixture: 225 ml dimethylformamide, 67.5 g sodium dodecyl sulphate (both from Sigma, USA) and 275 ml of distilled water). After 24 h, when the formazan crystals had been dissolved, the optical densities of the samples were read on an Multiskan RC photometer (Labsystems) at a 570 nm wavelength.

The results were presented as an IC_{50} (inhibitory concentration 50%) – the concentration of the test agent which inhibits the proliferation of 50% of the cancer cell population. The IC_{50} values were calculated separately for each experiment. Each compound at every concentration was tested in triplicate in a single experiment, which was repeated three to five times. The percent growth inhibition for all the experiments was counted and presented on graphs with standard deviation. Ethanol was used as a solvent (in dilution corresponding to the highest concentration applied to the test compounds) and it did not exert any inhibitory effect on cell proliferation ($p<0.05$).

Graphical data evaluation. The graphs presented in this work show the inhibition of cell proliferation (%) caused by the cytostatics used alone (first line) or in combination with calcitriol or its analogues (second line), according to Peters *et al.* (17). The third line on the graph expresses the hypothetical inhibition of proliferation by the two agents, calculated according to the following formula:

$$\%H = 100 - \frac{(100-\%cyt)*(100-\%vit)}{100}$$

where: *%H* - hypothetical inhibition of cell proliferation by both agents applied together; *%cyt*=inhibition of proliferation by cytostatic alone; *%vit*=inhibition of proliferation by vitamin D compound alone.

If the hypothetical line fell below the experimental one, obtained for the combination of the two agents, this indicated a synergistic effect of the compounds applied.

Statistical evaluation. The Student's *t*-test for independent samples was applied.

Results and Discussion

Treatment with either calcitriol or PRI-1906 or PRI-2191 applied alone. The results of the studies on the antiproliferative activities of either calcitriol, PRI-1906 or PRI-2191 applied alone are summarized in Table I. The HL-60 and WEHI-3 leukaemia cells were the most sensitive to the inhibitory effects of calcitriol and both of its analogues.

Combined treatment with cytostatics and either PRI-1906 or PRI-2191 analogues. The results of the combined treatment with vitamin D compounds and cisplatin or doxorubicin are presented in Tables II – V.

From a 55-fold to a few-fold decrease of cisplatin's IC_{50} values were observed after incubation of the leukaemia cells

Table I. *Antiproliferative activity in vitro of 10 nM calcitriol, PRI-1906 and PRI-2191.*

Cell line	% of inhibition (10 nM)		
	Calcitriol Mean±SD	PRI-1906 Mean±SD	PRI-2191 Mean±SD
HL-60	35.8±10.6	44.7±15.2	41.8±10.3
WEHI-3	53.5±9.2	43.0±24.6	60.5±2.1
CCRF	12.2±7.6	6.8±8.9	22.1±16.9
T47D	8.2±7.0	21.3±15.2	17.0±5.0
MCF-7	4.7±1.5	0.0±0.0	19.2±7.0
SW707	1.0±0.5	0.4±0.9	0.3±1
B16	0.3±0.6	0.0±0.0	0.3±0.6
A549	1.5±0.7	0.0±0.0	1.0±0.6
BALB 3T3	27.0±8.2	35.7±8.7	39.3±2.1

Table II. *IC_{50} values for cisplatin alone or applied in combined treatment with 10 nM of PRI-1906.*

Cell line	IC_{50} for cisplatin [μg/ml] applied		IC_{50} alone/ IC_{50} with PRI-1906
	alone Mean±SD	with PRI-1906 Mean±SD	
WEHI-3	2.650±0.274	0.789±0.706*	**3.4**
HL-60	0.303±0.065	0.139±0.037*	**2.2**
CCRF	1.341±0.102	0.537±0.275*	**2.5**
T47D	3.554±1.859	1.801±0.708	2.0
MCF-7	6.886±1.828	4.947±0.527	1.4
SW707	2.590±0.941	2.788±0.850	0.9

* – $p<0.05$ combined treatment *versus* cisplatin alone.

(WEHI-3, HL-60 and CCRF) or the breast cancer cells (T47D, MCF-7) with the calcitriol analogues (Tables II and III, Figures 1, 2). The most significant decrease in IC_{50} value was observed for the combination of cisplatin and PRI-2191 against WEHI-3 (Table III). There were no changes in the IC_{50} values for combined therapy against other cell lines (Tables II and III). These data show that significant synergistic effects between cisplatin and vitamin D analogues occured in the leukaemia and breast cancer cells.

A decrease of doxorubicin's IC_{50} value was observed after combined treatment with PRI-1906 or PRI-2191 against leukaemia cells (Tables IV and V, Figures 3, 4). The most significant, 66-fold, decrease of IC_{50} value was observed for the combination of doxorubicin and PRI-1906 against WEHI-3 (Table IV, Figure 3). In the case of interaction between doxorubicin and PRI-2191, this decrease of IC_{50} value led only to a sub-additive effect (Figure 4). The cells of the CCRF leukemia cell line were sensitive to the PRI-2191 analogue but resistant to PRI-1906. In combined treatment, it was noticed that against these cells only

Table III. *IC_{50} values for cisplatin alone or applied in combined treatment with 10 nM of PRI-2191.*

Cell line	IC_{50} for cisplatin (μg/ml±SD)		IC_{50} alone/ IC_{50} with PRI-2191
	alone	with PRI-2191	
HL-60	0.230±0.060	0.011±0.050*	**6.2**
WEHI-3	0.055±0.005	0.001±0.000*	**55.0**
CCRF	1.837±0.994	1.493±0.484	1.2
T47D	6.255±2.700	0.952±0.705*	**6.6**
MCF-7	4.808±1.128	2.454±0.826*	**2.0**
SW707	1.840±1.138	1.768±1.098	1.0
B16	3.107±0.598	2.395±0.318	1.1
A549	1.725±0.392	1.855±0.643	1.0

* – $p<0.05$ combined treatment *versus* cisplatin alone.

Table IV. *IC_{50} values for doxorubicin alone or applied in combined treatment with 10 nM of PRI-1906.*

Cell line	IC_{50} for doxorubicin [μg/ml] applied		IC_{50} alone/ IC_{50} with PRI-1906
	alone Mean±SD	with PRI-1906 Mean±SD	
WEHI-3	0.0066±0.0049	0.0001±0.0001*	**66**
HL-60	0.9562±0.1304	0.2336±0.0636*	**4.1**
CCRF	0.2740±0.0160	0.2313±0.1592	1.2
T47D	0.2830±0.1171	0.2735±0.1257	1.0
MCF-7	3.0843±0.6580	2.9563±0.7387	1.0
SW707	1.3888±0.1775	1.4305±0.1768	1.0

* – $p<0.05$ combined treatment *versus* doxorubicin alone.

Table V. *IC_{50} values for doxorubicin alone or applied in combined treatment with 10 nM of PRI-2191.*

Cell line	IC_{50} for doxorubicin (μg/ml±SD)		IC_{50} alone/ IC_{50} with PRI-2191
	alone	with PRI-2191	
HL-60	0.084±0.010	0.022±0.019*	**3.8**
WEHI-3	0.058±0.070	0.020±0.068	2.9
CCRF	0.356±0.090	0.078±0.090*	**4.6**
T47D	1.010±0.485	0.871±0.460	1.2
MCF-7	3.296±1.081	3.127±1.304	1.1
SW707	0.941±0.422	1.023±0.551	0.9
B16	0.022±0.011	0.017±0.000	1.3
A549	0.941±0.422	1.023±0.551	0.9

doxorubicin with PRI-2191 acted synergistically (Table V). Furthermore, there were no changes in the IC_{50} values for combined therapy against the other cell lines, which means that there is no synergistic effect between vitamin D compounds and doxorubicin on vitamin D weakly-sensitive or resistant cells.

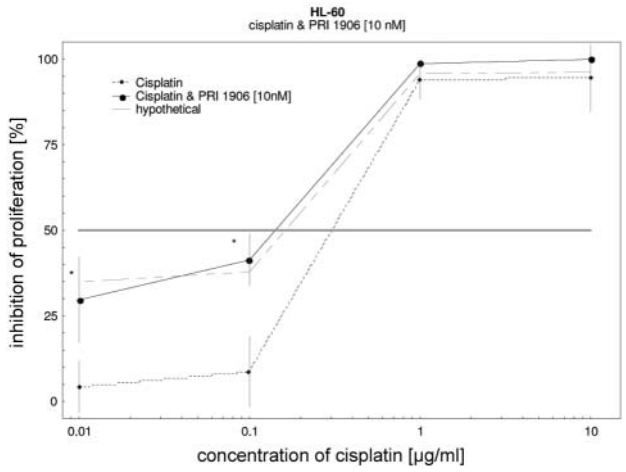

Figure 1. *Synergism between cisplatin and PRI-1906 in the HL-60 cell in vitro model. *- statistically significant, p<0.05, combined treatment versus cisplatin alone.*

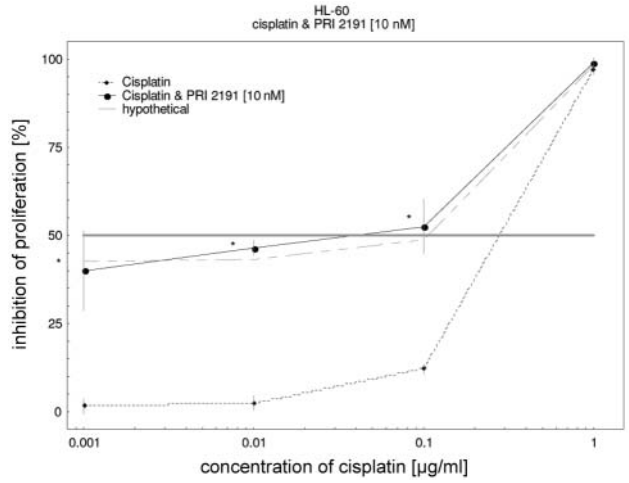

Figure 2. *Synergism between cisplatin and PRI-2191 in the HL-60 cell in vitro model. *- statistically significant, p<0.05, combined treatment versus cisplatin alone.*

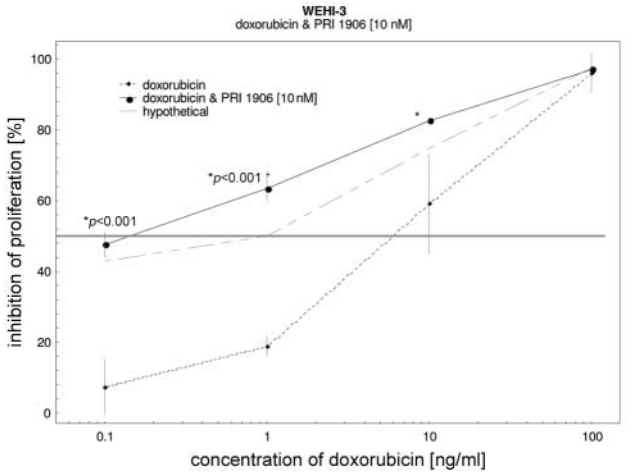

Figure 3. *Synergism between doxorubicin and PRI-1906 in the WEHI-3 cell in vitro model. *- statistically significant, p<0.05, combined treatment versus doxorubicin alone. +- statistically significant, p<0.01, combined treatment versus hypothetical.*

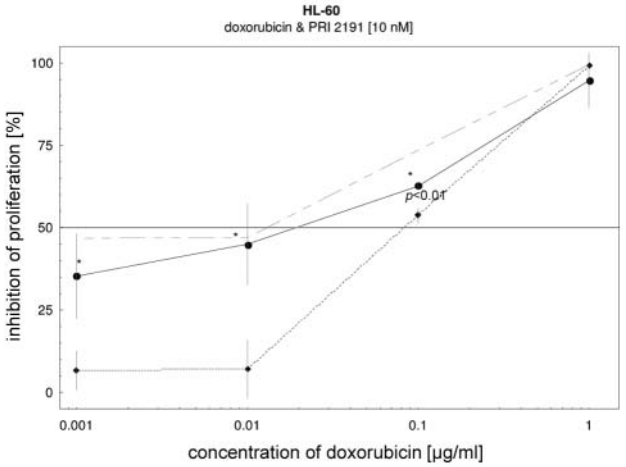

Figure 4. *Synergism between doxorubicin and PRI-2191 in the HL-60 cell in vitro model. *- statistically significant, p<0.05, combined treatment versus doxorubicin alone.*

In our previous study, we found that PRI-2191 was more effective in combined treatment with cytostatics than calcitriol (10, 13), and both of the presented analogues were less toxic then the referential compound (10 and Wietrzyk J., unpublished data). Previously, we have shown that pre-treatment of HL-60 cells with PRI-1906 and PRI-2191 decreased the IC_{50} values of the cytostatics (18). In this study, it was confirmed that either cisplatin or doxorubicin simultaneously applied with the vitamin D analogues led to the same effect. Moreover, in this study, we tried to find an interaction between these compounds and the cytostatics in another model of cancer cells weakly-sensitive to vitamin D compounds. In these models, the applied compounds did not act synergistically. The only exception was a model of CCRF leukaemia cells, where cisplatin and PRI-1906 acted synergistically. This observation suggests that synergistic growth inhibition by the analogue of calcitriol and the cytostatics mainly depended on the sensitivity of cells to the antiproliferative activity of vitamin D compounds, confirming the results from our previous assay (13). It is worth noting that doxorubicin acted synergistically with the vitamin D compounds only in leukaemia cells, and cisplatin in leukaemia and breast cancer cells. In the assay with doxorubicin, better interaction was obtained using the PRI-1906 analogue rather than PRI-2191.

In conclusion, both new vitamin D analogues revealed strong antiproliferative activity against the WEHI-3 and

HL-60 cell lines, but both were also active against normal fibroblasts. In combined simultaneous treatment, a statistically significant synergistic activity was noted mainly in the leukaemia and mammary gland cancer cell lines.

Thus, because of the good biological properties of the new vitamin D analogues (high antitumour and low calcemic activity), PRI-1906 and PRI-2191 seem to be good candidates for further preclinical and/or clinical studies, especially as partner compounds in combined treatment.

Acknowledgements

This work was partly supported by grants 3P05A08725 and PBZ-KBN-091/P05/2003 from MiIN (Ministry of Informatics and Science), Warsaw, Poland.

References

1 van Leeuven J and Pols H: Vitamin D: cancer and differentiation. *In*: Vitamin D. Feldman D, Pike JW, Glorieux FH (eds.). San Diego, Elsevier Academic Press, pp. 1571-1598 2005.
2 Jones G, Strugnell SA and DeLuca HF: Current understanding of the molecular actions of vitamin D. Physiol Rev *78*: 1193-1231, 1998.
3 Pelczynska M, Jaroszewicz I, Switalska M and Opolski A: Biological activity of calcitriol and its new analogues – potential therapeutical applications. Postepy Hig Med Dosw *59*: 129-139, 2005.
4 Zimber A, Chedeville A, Abita JP, Barbu V and Gespach C: Functional interactions between bile acids, all-trans retinoic acid, and 1,25-dihydroxy-vitamin D_3 on monocytic differentiation and myeloblastin gene down-regulation in HL60 and THP-1 human leukemia cells. Cancer Res *60*: 672-678, 2000.
5 Segaert S, Degreef H and Bouillon R: Vitamin D receptor expression is linked to cell cycle control in normal human keratinocytes. Biochem Biophys Res Commun *279*: 89-94, 2000.
6 Lin R and White JH: The pleiotropic actions of vitamin D. Bioessays *26*: 21-28, 2004.
7 Friedrich M, Rafi L, Mitschele T, Tilgen W, Schmidt W and Reichrath J: Analysis of the vitamin D system in cervical carcinomas, breast cancer and ovarian cancer. Rec Res Cancer Res *164*: 239-246, 2003.
8 Blutt SE, Polek TC, Stewart LV, Kattan MW and Weigel NL: A calcitriol analogue, EB1089, inhibits the growth of LNCaP tumors in nude mice. Cancer Res *60*: 779-782, 2000.
9 Colston K and Welsh J: Vitamin D and breast cancer. *In*: Vitamin D. Feldman D, Pike JW, Glorieux FH (eds.). San Diego, Elsevier Academic Press, pp. 1663-1678, 2005.
10 Wietrzyk J, Pelczynska M, Madej J, Dzimira S, Kusnierczyk H, Kutner A, Szelejewski W and Opolski A: Toxicity and antineoplastic effect of (24R)-1,24-dihydroxyvitamin D_3 (PRI-2191). Steroids *69*: 629-635, 2004.
11 Opolski A, Wietrzyk J, Chrobak A, Marcinkowska E, Wojdat E, Kutner A and Radzikowski C: Antiproliferative activity *in vitro* of side-chain analogues of calcitriol against various human normal and cancer cell lines. Anticancer Res *19*: 5217-5222, 1999.
12 Opolski A, Wietrzyk J, Siwinska A, Marcinkowska E, Chrobak A, Radzikowski C and Kutner A: Biological activity *in vitro* of side-chain modified analogues of calcitriol. Curr Pharm Des *6*: 755-765, 2000.
13 Pelczynska M, Wietrzyk J, Jaroszewicz I, Nevozhay D, Switalska M, Kutner A, Zabel M and Opolski A: Correlation between VDR expression and antiproliferative activity of vitamin D_3 compounds in combination with cytostatics. Anticancer Res *25*: 2235-2240, 2005.
14 Chodynski M, Wietrzyk J, Marcinkowska E, Opolski A, Szelejewski W and Kutner A: Synthesis and antiproliferative activity of side-chain unsaturated and homologated analogs of 1,25-dihydroxyvitamin D(2). (24E)-(1S)-24-Dehydro-24a-homo-1,25-dihydroxyergocalciferol and congeners. Steroids *67*: 789-798, 2002.
15 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR: New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst *82*: 1107-1112, 1990.
16 Marcinkowska E, Kutner A and Radzikowski C: Cell differentiating and anti-proliferative activity of side-chain modified analogues of 1,25-dihydroxyvitamin D_3. J Steroid Biochem Mol Biol *67*: 71-78, 1998.
17 Peters GJ, van der Wilt CL, van Moorsel CJ, Kroep JR, Bergman AM and Ackland SP: Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol Ther *87*: 227-253, 2000.
18 Siwinska A, Opolski A, Chrobak A, Wietrzyk J, Wojdat E, Kutner A, Szelejewski W and Radzikowski C: Potentiation of the antiproliferative effect *in vitro* of doxorubicin, cisplatin and genistein by new analogues of vitamin D. Anticancer Res *21*: 1925-1929, 2001.

Received March 10, 2006
Accepted March 27, 2006