Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 399–402

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Antitumor properties of (5*E*,7*E*) analogs of vitamin D_3 ☆

B. Filip [a], M. Milczarek [a], J. Wietrzyk [a,*], M. Chodyński [b], A. Kutner [b]

[a] *Department of Experimental Oncology, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 12 Rudolf Weigl Street, 53-114 Wroclaw, Poland*
[b] *Pharmaceutical Research Institute, 8 L. Rydygier Street, 01-793 Warsaw, Poland*

ARTICLE INFO

Article history:
Received 26 October 2009
Received in revised form 1 March 2010
Accepted 5 March 2010

Keywords:
Steroid
Calcitriol
(1*S*,24*R*)-1,24-dihydroxyvitamin D_3
(5*E*,7*E*) analogs antitumor activity
Calcemic effect

ABSTRACT

Geometric isomers (5*E*,7*E*) of major active metabolites of vitamin D_3 [1α,25$(OH)_2D_3$ and (24*R*)-1,24$(OH)_2D_3$] were synthesized by a new convenient procedure. Vitamin D triene system of the metabolites was first derivatized as a Diels–Alder adduct. Removal of the triene protecting group, in a key synthetic step, yielded the title compounds PRI-2208 and PRI-2209, respectively. The analogs were examined for their antiproliferative activity *in vitro* against human breast cancer cells (MCF-7) and promyelocytic leukemia (HL-60) cells. The activity was compared with one of the parent compounds. Both analogs examined revealed similar or higher antiproliferative activity compared to 1α,25$(OH)_2D_3$ or to (24*R*)-1,24$(OH)_2D_3$. The studies of calcemic activity *in vivo* showed that analogs PRI-2208 and PRI-2209 did not influence the serum calcium level in doses, in which 1α,25$(OH)_2D_3$ or (24*R*)-1,24$(OH)_2D_3$ significantly increased this level. The antitumor activity of these analogs in the LLC mice tumor model was studied. Analog PRI-2208 was found to be more active in inhibiting LLC tumor growth than 1α,25$(OH)_2D_3$, as well as than PRI-2191 and PRI-2209.

1. Introduction

The physiological level of the steroid hormone 1,25-$(OH)_2D_3$ (1,25-dihydroxyvitamin D_3 (calcitriol) – the most potent metabolite of vitamin D_3) is important mainly not only in regulation of calcium homeostasis and bone metabolism [1], but also in prevention of cancer. Epidemiological data suggest an inverse correlation between the serum level of 1,25-$(OH)_2D_3$ or sunlight exposure and incidence of some cancers such as prostate, colon, skin and breast [2–6]. In our previous studies, analogs of 1,25-dihydroxyvitamin D_3 with reversed configuration at C-1 or C-24 and *E* or *Z* geometry of the double bond at C-22 in the side-chain or at C-5 in the triene system were examined for their biological properties. On the basis of the *in vitro* antiproliferative activity, the effect of cell cycle *in vitro*, toxicity and antitumor activity *in vivo* we selected new vitamin D analogs with favorable biological profile. Selected analogs PRI-2191 and PRI-2205 are potent inhibitors of cancer cell proliferation both *in vitro* and *in vivo* with lowered toxicity [7–12].

Literature data [13,14] and our previous finding [12] of the enhanced biological activity of vitamin D (5*E*,7*E*) analog (PRI-2205) have further stimulated our interest in the other vitamin D compounds with the reversed geometry of the triene system. In this paper we communicate our evaluation of the anticancer activity of further 5,6-trans analogs of natural active metabolites of vitamin D_3, i.e. (5*E*,7*E*)-1,25-$(OH)_2D_3$ (PRI-2208) and (5*E*,7*E*,24*R*)-1,24-$(OH)_2D_3$ (PRI-2209), as well as their new straightforward syntheses [Schemes 1 and 2].

2. Materials and methods

2.1. Chemistry

Both analogs were prepared through the intermediate SO_2 Diels–Alder adducts, 2 and 3, respectively [Schemes 1 and 2]. Removal of the triene protecting group in alkaline ethanol gave the 5,6-trans analogs of interest, PRI-2208 and PRI-2209 [Fig. 1].

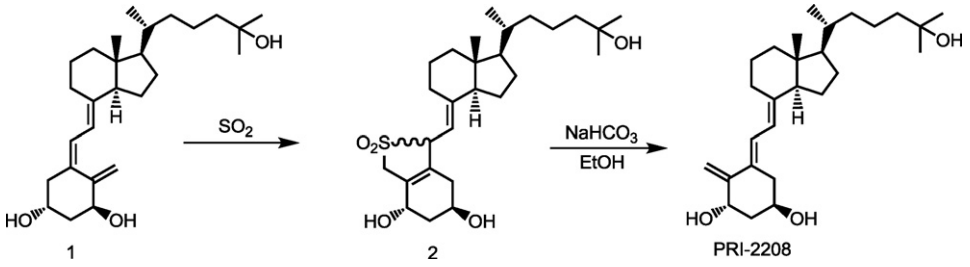
The starting (24*R*)-1,24-$(OH)_2D_3$ (PRI-2191) was obtained by our convergent strategy (5,6) from the vitamin D synthon (C-22 sulfon 4). Deprotonated sulfon 4 was alkylated with tosylate 5 and the resulting β-hydroxysulfone 6 was desulfonylated with sodium amalgam in buffered methanol to give alcohol 7. Desysilation of 7 yielded the analog PRI-2191 [Scheme 3], used as a starting material in our preparation of target analog PRI-2209 [Scheme 2].

Samples of the compounds were stored in amber ampoules, under argon at −20 °C. Prior to usage, the compounds were dissolved in 99.8% ethanol then diluted in 80% propylene glycol to reach the required concentrations, and administered to mice in a

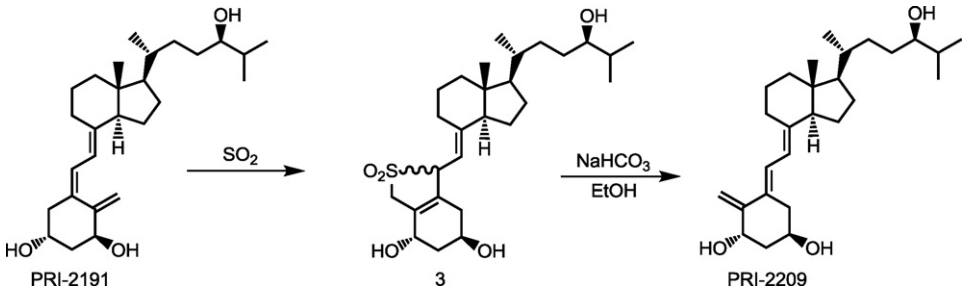
☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

* Corresponding author. Tel.: +48 71 3371172; fax: +48 71 3371382.
E-mail address: wietrzyk@iitd.pan.wroc.pl (J. Wietrzyk).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.03.017



Scheme 1. Synthesis path of analog PRI-2208.



Scheme 2. Synthesis path of analog PRI-2209.

volume 5 μL/1 g of body weight. For *in vitro* studies the compounds were diluted in culture media.

2.2. Cell lines

Human HL-60 (leukemia), MCF-7 (breast) cancer cell lines were obtained from American Type Culture Collection (Rockville, Maryland, USA). Both cell lines are being maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Leukemia cell line HL-60 was cultured in RPMI 1640 medium (Gibco, Scotland, UK) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 1.0 mM sodium pyruvate, 10% fetal bovine serum. MCF-7 cells were cultured in Eagle medium (IIET, Wroclaw, Poland) supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, 10% fetal bovine serum and 0.8 mg/L of insulin (all from Sigma–Aldrich Chemie GmbH, Steinheim, Germany). All culture media were supplemented with 100 units/mL penicillin, and 100 μg/mL streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland). Cell lines were grown at 37 °C with 5% CO_2 humidified atmosphere.

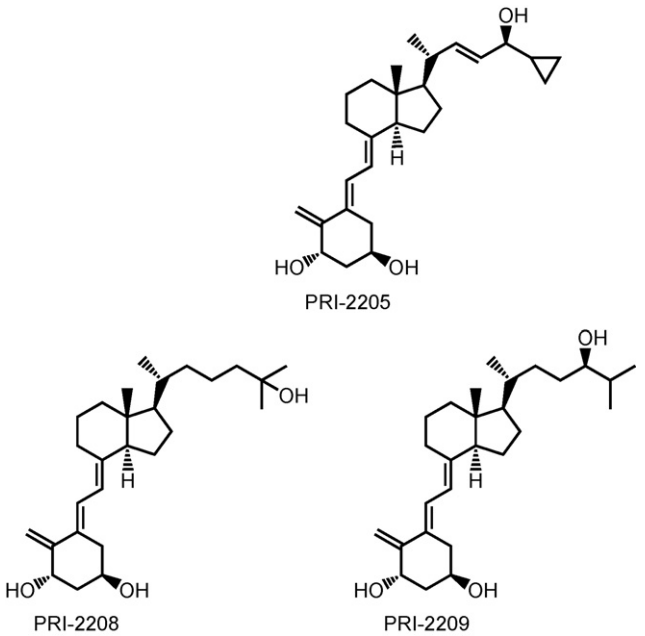


Fig. 1. (5*E*,7*E*) analogs of vitamin D_3-PRI-2208 and PRI-2209.

2.3. An anti-proliferative assay in vitro

24 h before addition of the tested compounds, the cells were plated in 96-well plates (Sarstedt, Germany) at density of 1×10^4 for cells per well. An SRB assay was performed after 72 h exposure to varying concentrations of the tested agents as described previously [12]. The optical densities of the samples were read on a Multiskan RC photometer (Labsystems, Helsinki, Finland).

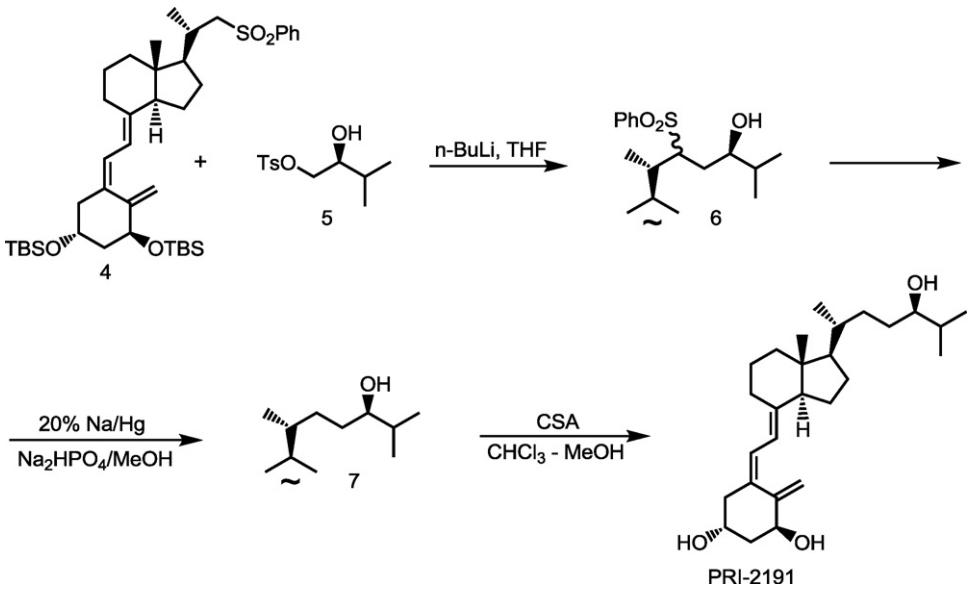
The results were calculated as an IC_{50} (inhibitory concentration 50) – the dose of tested agent which inhibits proliferation of 50% of the cancer cell population. Each compound in each concentration was tested in triplicate in a single experiment, which was repeated four times.

2.4. Mice

C57Bl/6 female, supplied from the Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, Poland, were maintained in standard laboratory conditions. All experiments were performed according to Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education issued by the New York Academy of Sciences' Ad Hoc Committee on Animal Research and were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

2.5. Calcemic activity

Mice were s.c. injected with calcitriol, PRI-2191, PRI-2205, PRI-2208 or PRI-2209 for two consecutive days at the dose of 10 μg/kg/day. The control group was treated with 80% propylene glycol. Animals were sacrificed three days after the first injection of the compound, and blood sera were collected. The calcium level



Scheme 3. Synthesis path of PRI-2191 (no. 3 in Scheme 2).

was measured in each individual serum sample using the photometric Arsezano 3 method (Olympus AU400; Olympus America Inc., Melville, NY, USA).

2.6. Antineoplastic effect

Mouse Lewis Lung Carcinoma LLC was obtained as a gift from Dr. I. Wodinsky from the Southern Research Institute in Birmingham, Alabama, USA. The cell line was cultured *in vitro* in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Mice were subcutaneously inoculated in the right flank of the abdomen with a suspension of 3×10^5cells per mouse. Mice were injected s.c. with one of the agents: calcitriol, PRI-2191, PRI-2208 or PRI-2209, according to following schedule: 1 μg/kg/day for calcitriol and PRI-2191 and 1 or 10 μg/kg/day for PRI-2208 and PRI-2209 from day 4 to 15 (three times a week, at days: 4, 6, 8, 11, 13, 15).

Tumor volume was calculated using the formula $(a^2 \times b)/2$, where a is the shorter tumor diameter in mm and b the longer tumor diameter in mm. Inhibition of tumor growth was calculated from the following formula: TGI [%] (tumor growth inhibition) = $(W_T/W_C) \times 100 - 100\%$, where W_T is the median tumor weight of treated mice and W_C – that of untreated control animals.

2.7. Statistical analysis

Kruskal–Wallis ANOVA and median test followed by a Mann–Whitney *U*-test was applied. p values lower than 0.05 were considered as a significant.

Table 1
Antiproliferative activity *in vitro* of calcitriol and its analogs against human promyelocytic leukemia cell line HL-60 and human breast cancer cell line MCF-7.

Compound	ID50 nM [mean ± SD]	
	HL-60	MCF-7
Calcitriol	8.59 ± 3.82	210 ± 53.52
PRI-2208	26.276 ± 11.83	425.09 ± 90.08
PRI-2191	6.379 ± 1.484	483.24 ± 147.7
PRI-2209	22.906 ± 0.323	354.30 ± 65.42

3. Results and discussion

In this paper we present new straightforward syntheses and preliminary evaluation of the anticancer activity of 5,6-trans analogs of natural active metabolites of vitamin D_3, i.e. (5*E*,7*E*)-1,25-$(OH)_2D_3$ (PRI-2208) and (5*E*,7*E*,24*R*)-1,24-$(OH)_2D_3$ (PRI-2209).

3.1. In vitro antiproliferative activity

Both analogs showed a significant proliferation inhibition of HL-60 and MCF-7 cells. In the case of human MCF-7 breast carcinoma cells the antiproliferative effect of PRI-2208 was two times lower than this of calcitriol. However, the antiproliferative effect of PRI-2209 was about 1.5 times higher than this of PRI-2191 [Table 1]. Focusing on human leukemia HL-60 cells proliferation, the IC_{50} for

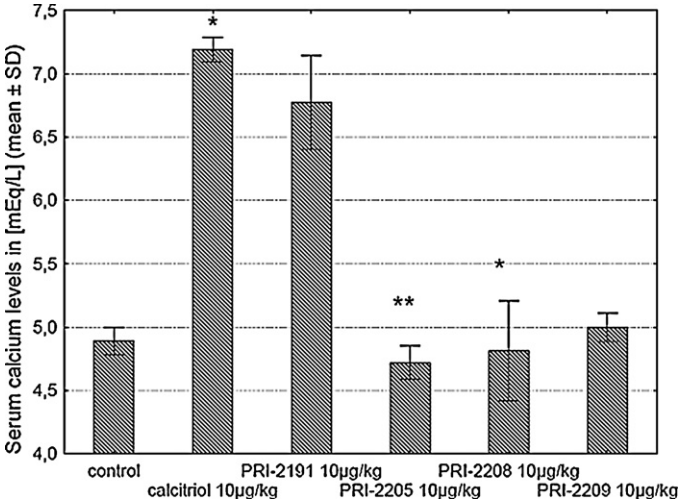


Fig. 2. Serum calcium level of healthy mice after vitamin D analogs treatment. Kruskal–Wallis multiple comparison test, nonparametric, $p < 0.05$. *: Calcitriol in comparison to control group (propylene glycol), PRI-2208 in comparison to calcitriol, **: PRI-2205 in comparison to calcitriol and PRI-2191. Calcium levels were measured in serum of healthy mice after two days treatment with 10 μg/kg/day of calcitriol, PRI-2191 or their analogs: PRI-2205, PRI-2208, PRI-2209. Control group was treated with 80% propylene glycol.

Table 2
The effect of PRI-2208 and PRI-2209 on the growth of mouse Lewis lung cancer LLC inoculated s.c.

Treatment		Tumor weight (mg)				TGI (%)		No. of mice with lung metastases/no. of all mice in group	Serum calcium level
		Day 13		Day 15		Day 13	Day 15		Mean ± SD
		Mean ± SD	Median	Mean ± SD	Median				
Calcitriol	1 μg/kg	433.8 ± 143	414	495.8 ± 136	458	3	30	3/5 (60%)	7.3 ± 0.31[a]
PRI-2191	1 μg/kg	409.7 ± 103	425	528.8 ± 187	592	−3	9	1/3 (33.3%)	5.02 ± 1.8
PRI-2208	1 μg/kg	392.2 ± 154	330	545.2 ± 172	497	20	23	1/6 (16.67%)	5.12 ± 0.26[b]
	10 μg/kg	503.8 ± 66	531	671.8 ± 154	615	−28	5	2/5 (40%)	5.43 ± 0.12
PRI-2209	1 μg/kg	480.6 ± 177	389	662.8 ± 98	644	6	1	2/5 (40%)	5.2 ± 0.15[b]
	10 μg/kg	434.2 ± 196	462	538.2 ± 99	573	−11	12	1/4 (25%)	4.74 ± 1.1
Control (80% propylene glycol)		457.8 ± 124	414	628.8 ± 168	650	–	–	4/5 (80%)	5.09 ± 0.2

(Schedule: single therapy with either PRI-2208 or PRI-2209, from day 4 to 15, three times per week. Calcitriol and PRI-2191 were used as control vitamins for PRI-2208 and PRI-2209 appropriately.) Calcemic activity: Kruskal–Wallis multiple comparison test, nonparametric. Calcium levels were measured in serum of tumor bearing mice after 20 days treatment with 1 μg/kg/day of calcitriol, PRI-2191 or their analogs: PRI-2208, PRI-2209 (1 or 10 μg/kg/day).

[a] Calcitriol in comparison to control group.

[b] PRI-2208 1 μg/kg and PRI-2209 1 μg/kg in comparison to calcitriol.

PRI-2208 and PRI-2209 was about four times lower than IC_{50} for calcitriol or PRI-2191.

Comparing these results and our previous findings obtained for PRI-2205 we can conclude, that the analogs with the reversed geometry of the triene system have similar, high antiproliferative potential, especially on HL-60 cells [12].

3.2. Calcemic activity of PRI-2208 and PRI-2209 in healthy mice

The results of the serum calcium level evaluation after two daily s.c. injections of calcitriol or its analogs (10 μg/kg/day) are presented in Fig. 2. The calcium level in the serum of healthy BDF_1 mice treated with PRI-2208, PRI-2205 and PRI-2209 was similar to the serum calcium level in control group. In mice treated with PRI-2208 or PRI-2205, the serum calcium level was significantly lower than that in animals treated with calcitriol ($p < 0.05$) or PRI-2191 [Fig. 2].

3.3. Antitumor and calcemic activity of PRI-2208 and PRI-2209 in LLC tumors bearing mice

The antitumor activity of these analogs in the LLC tumor model was tested. PRI-2208 in the dose of 1 μg/kg/day inhibited the tumor growth evidently when compared to the control group or to calcitriol [Table 2]. Moreover, this effect was observed even after we finished the administration of compounds. In all other treated groups an increase in tumor volume was observed after day 15 (data not shown). We did not observe a tumor growth inhibition in groups treated with PRI-2209. Both analogs exhibit a tendency to diminish the number of LLC lung metastases, but these results need to be confirmed.

The analog PRI-2208 reveals lower anticancer activity than PRI-2205 [10], but it shows no toxicity which usually reveals oneself in loss of body weight and high serum calcium level. Only calcitriol treated group exhibits a loss of body weight and statistically significant increase in calcium level (used even in the smaller dose of 1 μg/kg/day). PRI-2208 and PRI-2209 used in both concentrations (1 or 10 μg/kg/day) did not influence the serum calcium level in comparison to control group [Table 2].

A serious limitation to the clinical use of calcitriol is hypercalcemia as the result of application of higher than physiological doses. The undesired hypercalcemia after calcitriol application explains the motivation to develop analogs which could enable differentiation of the calcemic and bone metabolism effects from the antiproliferative activity [15,16]. Our data demonstrate that the analogs PRI-2208 and PRI-2209 are non-toxic and potent inhibitors of cancer cell proliferation *in vitro*. The antitumor activity of these analogs tested in the LLC mice tumor model, show that the analog PRI-2208 appears to be more effective than calcitriol. It revealed no calcemic activity in the dose which inhibits tumor growth or at higher dose. In particular, their effect in a combined treatment protocol with cytostatics should be considered for further study.

References

[1] H. Reichel, H.P. Koeffler, A.W. Norman, The role of the vitamin D endocrine system in health and disease, N. Engl. J. Med. 320 (15) (1989) 980–991.
[2] C.F. Garland, G.W. Comstock, F.C. Garland, K.J. Helsing, E.K. Shaw, E.D. Gorham, Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study, Lancet 2 (8673) (1989) 1176–1178.
[3] H.L. Newmark, M. Lipkin, Calcium, vitamin D, and colon cancer, Cancer Res. 52 (1992) 2067s–2070s.
[4] E.H. Corder, H.A. Guess, B.S. Hulka, G.D. Friedman, M. Sadler, R.T. Vollmer, B. Lobaugh, M.K. Drezner, J.H. Vogelman, N. Orentreich, Vitamin D and prostate cancer: a prediagnostic study with stored sera, Cancer Epidemiol. Biomarkers Prev. 2 (5) (1993) 467–472.
[5] H.S. Cross, P. Bareis, H. Hofer, M.G. Bischof, E. Bajna, S. Kriwanek, E. Bonner, M. Peterlik, 25-Hydroxyvitamin D (3)-1alpha-hydroxylase and vitamin D receptor gene expression in human colonic mucosa is elevated during early cancerogenesis, Steroids 66 (3–5) (2001) 287–292.
[6] E. Giovannucci, The epidemiology of vitamin D and colorectal cancer: recent findings, Curr. Opin. Gastroenterol. 22 (1) (2006) 24–29.
[7] A. Opolski, J. Wietrzyk, A. Chrobak, E. Marcinkowska, E. Wojdat, A. Kutner, C. Radzikowski, Antiproliferative activity in vitro of side-chain analogues of calcitriol against various human normal and cancer cell lines, Anticancer Res. 19 (6B) (1999) 5217–5222.
[8] A. Opolski, J. Wietrzyk, A. Siwinska, E. Marcinkowska, A. Chrobak, C. Radzikowski, A. Kutner, Biological activity in vitro of side-chain modified analogues of calcitriol, Curr. Pharm. Des. 6 (7) (2000) 755–765.
[9] J. Wietrzyk, M. Pelczynska, J. Madej, S. Dzimira, H. Kusnierczyk, A. Kutner, W. Szelejewski, A. Opolski, Toxicity and antineoplastic effect of (24R)-1,24-dihydroxyvitamin D_3 (PRI-2191), Steroids 69 (2004) 629–635.
[10] J. Wietrzyk, D. Nevozhay, B. Filip, M. Milczarek, A. Kutner, The antitumor effect of lowered doses of cytostatics combined with new analogs of vitamin D in mice, Anticancer Res. 27 (5A) (2007) 3387–3398.
[11] J. Wietrzyk, D. Nevozhay, M. Milczarek, B. Filip, A. Kutner, Toxicity and antitumor activity of the vitamin D analogs PRI-1906 and PRI-1907 in combined treatment with cyclophosphamide in a mouse mammary cancer model, Cancer Chemother. Pharmacol. 62 (5) (2008) 787–797.
[12] J. Wietrzyk, M. Chodynski, H. Fitak, E. Wojdat, A. Kutner, A. Opolski, Antitumor properties of diastereomeric and geometric analogs of vitamin D_3, Anticancer Drugs 18 (4) (2007) 447–457.
[13] W.R. Wecksler, A.W. Norman, Studies on the mode of action of calciferol XXV. 1 alpha,25-dihydroxy-5,6-trans-vitamin D_3, the 5E-isomer of 1 alpha,25-dihydroxyvitamin D_3, Steroids 35 (4) (1980) 419–425.
[14] J. Hisatake, T. Kubota, Y. Hisatake, M. Uskokovic, S. Tomoyasu, H.P. Koeffler, 5,6-trans-16-ene-vitamin D_3: a new class of potent inhibitors of proliferation of prostate, breast, and myeloid leukemic cells, Cancer Res. 59 (16) (1999) 4023–4029.
[15] H.A. Pols, J.C. Birkenhager, J.P. van, Leeuwen, Vitamin D analogues: from molecule to clinical application, Clin. Endocrinol. (Oxf.) 40 (3) (1994) 285–292.
[16] J.Y. Zhou, A.W. Norman, M. Akashi, D.L. Chen, M.R. Uskokovic, J.M. Aurrecoechea, W.G. Dauben, W.H. Okamura, H.P. Koeffler, Development of a novel 1,25$(OH)_2$-vitamin D_3 analog with potent ability to induce HL-60 cell differentiation without modulating calcium metabolism, Blood 78 (1) (1991) 75–82.