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ORIGINAL ARTICLE

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

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Abstract
Purpose Active and less toxic vitamin D analogs could be useful for clinical applications. In the present study, we evaluated the toxicity and antitumor effect of two new synthetic analogs of vitamin D, namely PRI-1906 [(24*E*)-24a-Homo-(1*S*)-1,25-dihydroxyergocalciferol] and its side-chain unsaturated homo analog PRI-1907.
Methods The toxicity and calcemic activity, as well as antitumor effect of calcitriol analogs was investigated in vivo. The studies were performed in a mouse mammary 16/C cancer model. Since calcitriol and its analogs inhibited 16/C tumor growth only slightly, we applied them in the combined therapy with cyclophosphamide (CY). Moreover, cell cycle analysis and VDR and p27 expression were investigated.
Results The LD_{50} values after five daily subcutaneous (s.c.) injections were 7.8, 10.0 and 2.4 μg/kg per day for calcitriol, PRI-1906 and PRI-1907, respectively. The serum calcium level increased to 40, 23 and 63% over the control for these compounds. We also compare the antitumor activity of the PRI-1906 with the calcitriol and previously studied PRI-2191 (1,24-dihydroxyvitamin D_3, tacalcitol). Statistically significant inhibition of tumor growth by calcitriol up to the eighth day was observed in all schedules applied. PRI-1906 inhibited the tumor growth at doses 1 and 5 μg/kg per day, and PRI-2191 only at the dose 5 μg/kg per day.
Conclusion Addition of vitamin D analogs increased the antitumor effect of CY. PRI-1906 exhibited toxicity higher than PRI-2191 but lower than calcitriol and antitumor activity similar to both PRI-2191 and calcitriol. This new analog seems to be a good candidate for the combined treatment of mammary cancer.

Keywords Steroid · Calcitriol · (1*S*,24*R*)-1,24-dihydroxyvitamin D_3 · Chemotherapy · Antitumor · Analogs

Introduction

Calcitriol (1,25-dihydroxyvitamin D_3, 1,25-$(OH)_2D_3$), a hormonally active form of vitamin D_3, generally regulates calcium and phosphorus homeostasis, but also exerts antitumor activity both in vitro and in vivo [1–7]. In addition to its antiproliferative and differentiation-inducing effects, calcitriol induces apoptosis in a number of different cancer cells in vitro [8, 12]. In vivo studies in tumor-bearing animals treated with calcitriol, showed regression of tumors, inhibition of the metastases, prolongation of survival time, and, in addition, the antiangiogenic effect [4, 13–15]. Recent findings have indicated that vitamin D used as an chemopreventive agent can modulate and inhibit colon [16], breast [17, 18], and prostate [19] carcinogenesis. Supporting evidence has been obtained from a variety of preclinical experimental studies, epidemiological data,

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and a few human clinical trials [16–25]. In mammary epithelial cells, including cells derived from breast cancers, calcitriol induces cell cycle arrest in G0/G1 stage and/or apoptosis through VDR dependent mechanisms [26].

A number of studies on the combined treatment with calcitriol or its analogs and different chemotherapeutic agents have been reported in the last decade both in vitro [27–30] and in vivo [31, 32]. The application of potentially effective, hyper-physiological doses of calcitriol in anticancer therapy is limited by its calcemic activity and subsequent risk of hypercalcemia [20, 33, 34]. These undesired side effects motivated the synthesis of new analogs, aiming to dissociate calcemic and antiproliferative effects [35].

In our previous studies, we have shown that the vitamin D_3 metabolite, (24R)-1,24-dihydroxyvitamin D_3 (tacalcitol, 1,24-$(OH)_2D_3$, PRI-2191) revealed higher antitumor and lower calcemic activity as well as lower toxicity than calcitriol [36]. We also examined a series of vitamin D_2 analogs with a highly unsaturated side-chain and a series of vitamin D_3 analogs with an additional one or two hydroxyl groups in the side-chain for their antiproliferative activity in vitro against various human normal and cancer cell lines [1, 37, 38]. In these studies we revealed the higher antiproliferative activity of synthetic analogs of vitamin D_2 with the extended and rigid side chain, coded PRI-1906 [(24E)-24a-Homo-(1S)-1,25-dihydroxyergocalciferol] and its side-chain unsaturated homo analog PRI-1907 [1, 37]. We have also shown that this effect could be attributed to the induction of cancer cell-differentiation [1, 8–11].

In this article, we describe the effect of calcitriol and its analogs PRI-1906, PRI-1907 on the mouse serum calcium levels, their lethal toxicity (LD_{50}), and antitumor activity in vivo in combination with CY in mice with mammary cancer 16/C. Tacalcitol (PRI-2191), the previously tested analog of calcitriol [36], was used as an additional reference compound.

Materials and methods

Compounds

Calcitriol and its side-chain-modified analogs PRI-1906, PRI-1907 and PRI-2191 (Fig. 1) were certified synthetic materials obtained from Pharmaceutical Research Institute, Warsaw, Poland. 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 volume 50 µl/10 g of body weight.

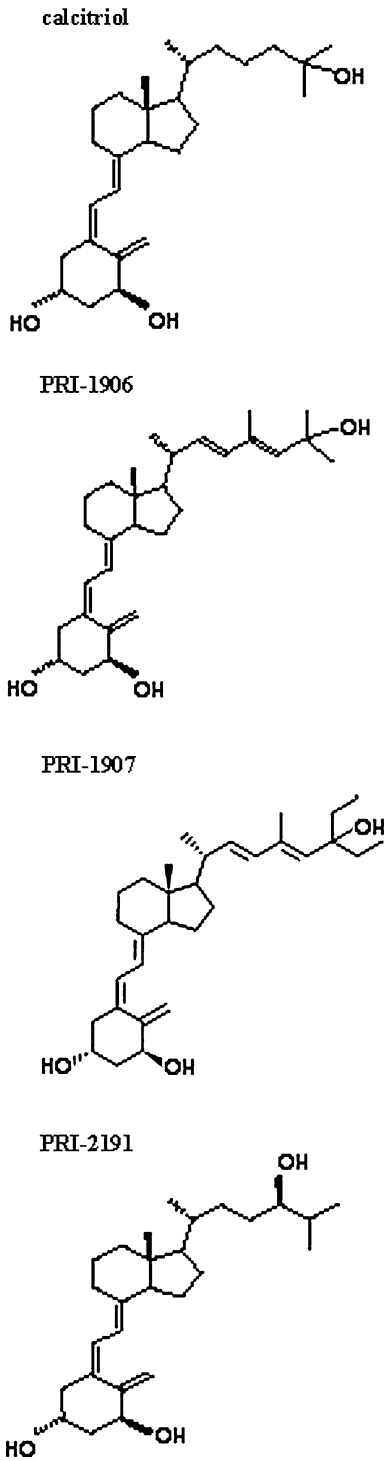

Fig. 1 The structural formulas of calcitriol and its analogs (PRI-1906, PRI-1907 and PRI-2191). Calcitriol, 1,25- dihydroxycholecalciferol; PRI-1906, (24E)-(1-S)-24-dehydro-24a-homo-1,25-dihydroxyergocalciferol; PRI-1907, (24E)-(1-S)-24-dehydro-24a,26,27-trihomo-1,25-dihydroxyergocalciferol; PRI-2191, (24R)-1,24-dihydroxycholecalciferol

Cyclophosphamide (CY)—Endoxan, ASTA Medica AG, Frankfurt, Germany was diluted in *aqua pro injection* to reach the required concentrations, and administered to mice in a volume 100 µl/10 g of body weight.

Mice

C3H female or [C57Bl/6 × DBA/2]F_1 (BDF_1) male, 12–16 weeks old mice, weighing 20–25 g, supplied from the Animal Breeding Centre of the Institute of Immunology and Experimental Therapy and from the Animal Facility of the Medical University, Wroclaw, 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.

Lethal toxicity

Subacute toxicity (3-week observation) after five subcutaneous (s.c.) administrations was determined in BDF_1 male mice. Dose levels were established according to preliminary toxicity evaluation, as follows: from 100 down to 0.1 μg/kg per day. The dose lethal for 50% of mice (LD_{50}) was computed using the STATISTICA software.

Calcemic activity

Male BDF_1 mice were s.c. injected with calcitriol, PRI-1906 or PRI-1907 for 2 consecutive days at the dose of 5 μg/kg per day. The control group was treated with propylene glycol. Animals were sacrificed 3 days after the first injection of the compound, and blood sera were collected. The calcium level was measured in each individual serum sample using the photometric Arsezano 3 method (Olympus AU400; Olympus America Inc., Melville, NY, USA).

Antineoplastic effect

Mouse mammary adenocarcinoma 16/C 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 and/or in vivo in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

C3H/W female mice were orthotopically inoculated into the right mammary fat pad with a 20% (w/v) suspension of tumor cells (approximately 1×10^5 viable cells per mouse) in 0.05 ml of saline. Alternatively, some mice were s.c. inoculated in the right flank of the abdomen with a 5% (w/v) suspension of tumor cells (approximately 1×10^5 viable cells per mouse) in 0.2 ml of saline.

Details of the treatment schedules used in this study as following. All schedule days were counted starting from the transplantation of tumor cells (day 0).

1. In single agent therapy mice bearing 16/C cancer transplanted orthotopically were injected s.c. with one of the agents: calcitriol, PRI-1906 or PRI-2191, according to following schedules: (1) 5 μg/kg per day from day 1 to 5; (2) 2.5 μg/kg per day from day 1 to 8; (3) 1 μg/kg per day from day 1 to 13. The first treatment protocol was also used in mice receiving the tested agents by oral (p.o.) route.
2. In combined treatment with CY the mice bearing 16/C cancer transplanted s.c. were injected with: (A) calcitriol or PRI-1906 s.c. at a dose of 0.1 μg/kg starting from the day 1 for 11 consecutive days, or starting from the day 6 after inoculation of tumor cells for 6 consecutive days, and then from day 13 every second day until the end of the experiment. (B) PRI-2191 or PRI-1906 s.c. at a dose of 1.0 μg/kg for 9 consecutive days, starting from the day 1 or day 5 after inoculation of tumor cells. CY was injected i.p. at a single dose of 50 mg/kg at day 5 after tumor cell inoculation in schedule A or at the single dose of 100 mg/kg at day 4 after tumor cell inoculation in schedule B.

Evaluation of the therapeutic effects

Tumor volume was calculated using the formula $(a^2 \times b)/2$, where a = shorter tumor diameter in mm and b = longer tumor diameter in mm. Inhibition of tumor growth was calculated from the following formula: tumor growth inhibition (TGI) [%] = $(W_T/W_C) \times 100 - 100\%$, W_T the median tumor weight of treated mice and W_C that of untreated control animals. The expected inhibition used to estimate the effect of combination of two compounds was evaluated using the formula $\%H = 100 - [(100 - \text{TGI CY}) \times (100 - \text{TGI calcitriol or its analog})/100]$ [39].

Evaluation of the molecular mechanism

Mice bearing 16/C adenocarcinoma transplanted orthotopically were injected s.c. with one of the agents: PRI-1906 or PRI-2191, according to the following schedule: 1 μg/kg per day from day 1 to 13 after the transplantation of tumor cells. Mice were sacrificed on the day 14 and primary tumors and blood were collected.

Cell cycle analysis

16/C mammary adenocarcinoma cells from primary tumors were obtained by mincing fresh tumor tissue with a scalpel, passing them through plasma filter, and suspending in phosphate-buffered saline (PBS). Cell suspension was washed once with PBS supplemented with 2% of fetal bovine serum. Then the cells were washed in PBS and counted in a

hemacytometer. 1×10^6 cells were fixed for 24 h in 70% ethanol at −20°C. Then the cells were washed twice in PBS and incubated with RNAse (50 μg/ml, Fermentas, Germany) at 37°C for 1 h. The cells were stained for 30 min with propidium iodide (50 μg/ml, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at 4°C and the cellular DNA content was determined using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Determination of the VDR and p27 expression

To determine VDR or p27 expression by Western blot, the collected tumors frozen at -80°C were homogenized (Medimachine, Dako, Denmark), rinsed once in PBS and lysed on ice for 45 min in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 1% triton × 100, supplemented with the complete-TM mixture of protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA). Lysates were sonificated and cleared by micro centrifugation at 11,000 rpm for 10 min. Protein concentrations were determined using the protein assay (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (50 μg) were separated in a 10% (VDR, anti-actin) or 14.5% (p27) SDS polyacrylamide gel and transferred to a nitrocellulose membrane (0.45 Micron; Osmonics, GE Water & Technologies, Trevose, PA, USA). Protein loading and efficiency of transfer were monitored by 0.1% Ponceau S-Red staining. Membranes were blocked for 1 h at room temperature in 1% blocking reagent (Roche Diagnostics, Mannheim, Germany) in PBS, then washed three times (3 × 10 min) with 0.05% PBST (PBS/Tween-20) and incubated overnight with the primary antibody: rabbit anti-VDR polyclonal antibody or anti-p27 polyclonal antibody (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or rabbit anti-actin antibody (Sigma-Aldrich, Poznan, Poland). After incubation the blot was washed three times with 0.1% PBST and incubated for 1 h with the secondary antibody: biotinylated polyclonal goat anti-rabbit immunoglobulins (Dako Cytomation, Glostrup, Denmark). Afterwards the blot was washed three times with 0.1% PBST and incubated for 45 min with avidin conjugated to horseradish peroxidase (Dako Cytomation, Glostrup, Denmark). After incubation the blot was washed briefly with distilled water and three times with 0.1% PBST. Blotted proteins were detected with ECL Western blotting detection reagents (Roche Diagnostics, Mannheim, Germany) and then exposed to Kodak X-OMAT X-ray film (Sigma, Steinheim, Germany).

Statistical analysis

One–way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test or a Mann–Whitney *U* Test was applied; *p* values lower than 0.05 were considered as a significant.

Results

First, we evaluated the toxicity and in vivo antitumor effect of PRI-1906 and PRI-1907—calcitriol analogs synthesized with the aim of obtaining a compound with antitumor activity comparable or higher than calcitriol but with a lower calcemic effect.

In vivo toxicity of PRI-1906 and PRI-1907 versus calcitriol

The results of the s.c. toxicity evaluations, expressed as LD_{50} values estimated 3 weeks after the first drug injection, are presented in Table 1. After five consecutive s.c. injections of calcitriol, its median LD_{50} values varied from 4.6 to 8.0 μg/kg per day in three independent determinations, whereas those for PRI-1906 were significantly higher (lower toxicity) and ranged from 8.5 to 11.0 μg/kg per day. Subcutaneous administration of PRI-1907 appeared to be more toxic at this point than either calcitriol or PRI-1906, and ranged from 2.4 to 2.7 μg/kg per day (Table 1).

Body weight changes (BWC), showed similar time-course profiles for both calcitriol and PRI-1906. A maximal decrease in body weight was observed from the third to eighth day following s.c. administration, after which, in surviving mice, recovery started. However, the decrease in body weight in animals treated with calcitriol was higher and lasted longer than that in mice treated with the same dose of PRI-1906. The kinetics of body weight variations at a dose of 5 μg/kg per day (s.c.) is illustrated in Fig. 2. All mice treated with PRI-1907 in this dose died after the eighth day of the experiment.

Table 1 Lethal toxicity, expressed as LD_{50} for the new vitamin D analogs (PRI-1906, PRI-1907) and calcitriol in healthy mice

	LD_{50}[a], μg/kg per day		N[b]
	Median	Min.–max.	
Calcitriol	**7.8**	4.6–8.0	50
PRI-1906	**10.0**[c]	8.5–11.0	93
PRI-1907	**2.4**[d]	2.4–2.7	87

Subacute toxicity (3-week observation) after 5 consecutive daily s.c. administrations was determined in BDF_1 male mice

[a] LD_{50} the dose lethal for 50% of mice

[b] *N* number of mice used for evaluation

[c] $p < 0.05$ as compared to calcitriol (Mann–Whitney *U* Test)

[d] $p < 0.05$ as compared to calcitriol and PRI-1906 (Mann–Whitney *U* Test)

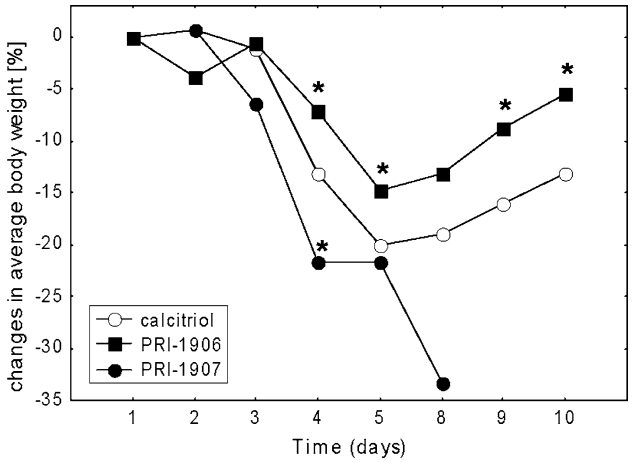

Fig. 2 Kinetics of body weight changes in healthy mice treated with PRI-1906, PRI-1907 and calcitriol. Male BDF_1 mice were treated s.c. during five consecutive days, at the dose 5 μg/kg per day. Asterisks denotes $p < 0.05$ as compared to calcitriol (ANOVA, Tukey-Kramer multiple comparisons test). There were six mice in each group

Calcemic activity of PRI-1906 and PRI-1907 compounds in comparison with calcitriol

The results of the serum calcium level evaluation after two daily s.c. injections of calcitriol or its analogs (5 μg/kg per day) are presented in Table 2. The calcium level in the serum of mice treated with PRI-1906 was significantly lower than that in animals treated with calcitriol ($p < 0.05$). In mice treated with PRI-1907, the serum calcium level was significantly higher than that in animals treated with either calcitriol or PRI-1906 (Table 2). The serum calcium level increased to 40, 23 and 63% compared to the control in mice treated with calcitriol, PRI-1906 and PRI-1907, respectively. Healthy, animals treated with dilution solution propylene glycol were used as the control group for comparison.

Antitumor activity of PRI-1906 in comparison with calcitriol and PRI-2191

Due to the high toxicity of PRI-1907, we selected PRI-1906 for further studies of its antitumor effect. The studies were performed in a mouse estrogen receptor-positive mammary adenocarcinoma model (16/C) of C3H mice. Statistically significant inhibition of tumor growth by calcitriol until day 8 was observed in all schedules of administration. After day 8 the difference with control was not significant. PRI-1906 inhibited the tumor growth in the doses 1 and 5 μg/kg per day, and PRI-2191 only in the dose 5 μg/kg per day (Fig. 3). The first schedule described above was also applied in experiments with oral administration. In this case, we observed statistically significant inhibition of tumor growth only for PRI-2191 until day 18 (Fig. 4).

We also compared the changes in body weight after s.c. or p.o. administrations of the dose of 5 μg/kg per day according to schedule 1 (Fig. 5). In the case of s.c. injection, the highest weight loss was observed in mice receiving calcitriol (30%) and the lowest in animals injected with PRI-2191 (16%). However, after oral administration, the weight loss was similar for all compounds and did not exceed 15% (Fig. 5). It confirmed our previous observations that oral dministration of calcitriol was significantly less toxic as compared to the s.c. injection, but the toxicity of PRI-2191 did not differ according to the mode of administration. Moreover, no significant differences in toxicity of PRI-2191 in comparison with calcitriol were observed when the drugs were administered p.o. [36]. Notwithstanding, in the case of PRI-1906, observation of body weight changes suggests a profile of toxicity similar to calcitriol. For this analog we have observed more profound maximal body weight loss after subcutaneous (25.6%) than after p.o. (14.5%) route of administration (Fig. 5).

It is believed that antiproliferative activity of calcitriol and its analogs is mediated through the binding to a ligand-bound vitamin D receptor (VDR) and also by regulation of cell cycle inhibitor p27 expression. We have shown that 16/C mammary adenocarcinoma expresses VDR in whole cell lysates of tumors (Fig. 6a). Moreover, the elevation of p27 expression was investigated after treating mice with PRI-1906 or PRI-2191 analogs (Fig. 6a). The level of p27 protein was significantly increased in the tumors deriving from mice treated with PRI-2191 analog. However, we have not observed the increased expression of p27 in the tumors deriving from mice treated with PRI-1906.

Table 2 Serum calcium and phosphorus levels after s.c. administration of calcitriol, PRI-1906 and PRI-1907, expressed as mean ± SD

Parameter measured	Group			
	Control (propylene glycol)	Calcitriol	PRI-1906	PRI-1907
Calcium (mEq/l)	4.7 ± 0.1	6.6 ± 0.3[a]	5.8 ± 0.3[ab]	7.7 ± 0.1[ab]
Inorganic phosphorus (mg/dl)	9.0 ± 0.4	8.8 ± 0.8	8.7 ± 0.6	8.0 ± 0.9
Number of mice in group	4	6	6	6

Male BDF_1 mice were treated s.c. for 2 consecutive days, at dose of 5 μg/kg per day

[a] $p < 0.05$ as compared to propylene glycol

[b] $p < 0.05$ as compared to calcitriol (ANOVA, Tukey-Kramer multiple comparisons test)

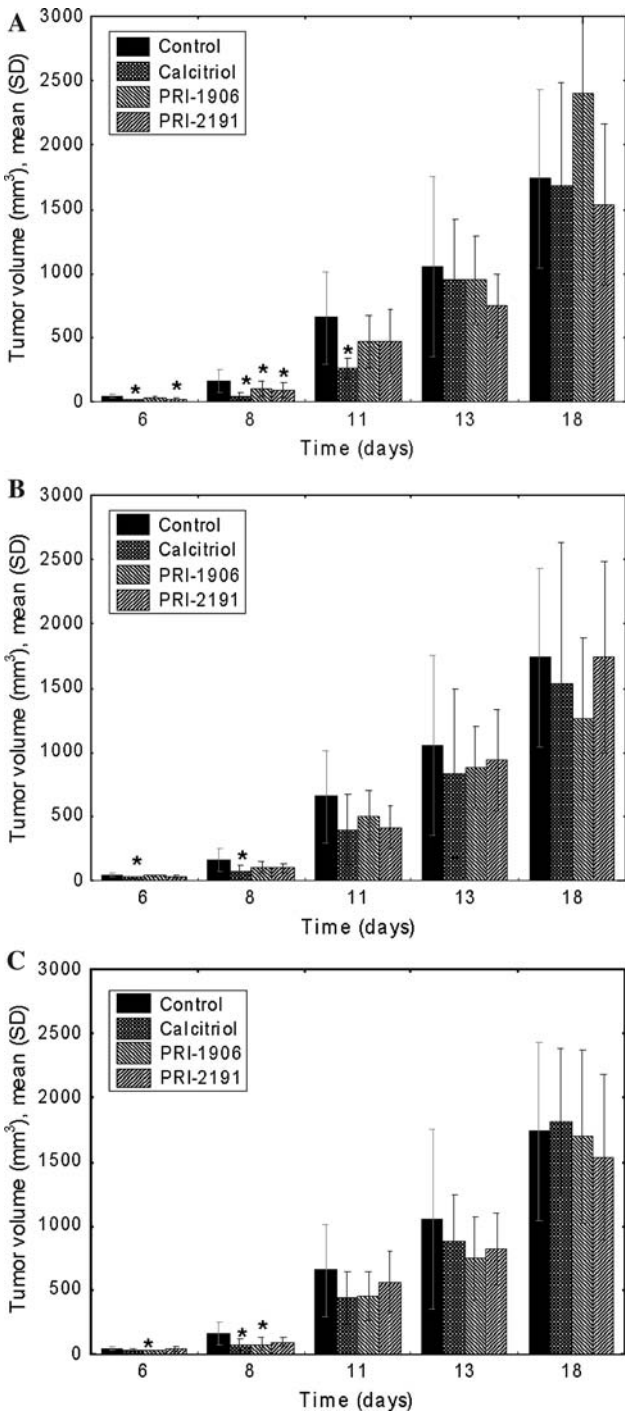

Fig. 3 The effect of subcutaneous administration of calcitriol and PRI-2191 or PRI-1906 on the growth of 16/C mouse mammary tumor inoculated orthotopically. Mice bearing 16/C cancer transplanted orthotopically were injected s.c. with one of the agents: calcitriol, PRI-1906 or PRI-2191, according to following schedules: **a** 5 μg/kg per day from day 1 to 5; **b** 2.5 μg/kg per day from day 1 to 8; **c** 1 μg/kg per day from day 1 to 13. Number of mice used for evaluation: control group 12; treated groups 6. *Asterisks* denotes $p < 0.05$ as compared to control (Mann–Whitney *U* test)

Cell cycle analysis of tumor cells obtained from mice treated with PRI-2191 revealed that there was a statistically significant increase in the percentage of cells in the G0/G1

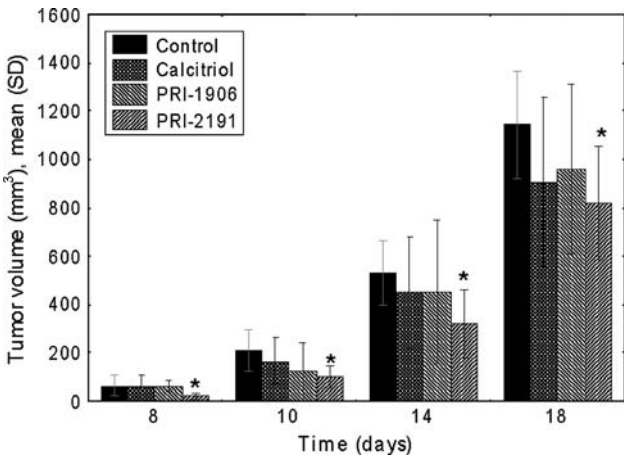

Fig. 4 The effect of oral administration of calcitriol and its analogs PRI-2191, PRI-1906 on the growth of 16/C mouse mammary tumor inoculated orthotopically. Mice bearing 16/C cancer transplanted orthotopically were administered p.o. with one of the following agents—calcitriol, PRI-1906 or PRI-2191, 5 μg/kg per day from day 1 to day 5 of the experiment. Number of mice used for evaluation: control group 12, treated groups 6 mice. *Asterisks* denotes $p < 0.05$ as compared to control (Mann–Whitney *U* test)

and decrease in the percentage of cells in the S stages, as compared to control. A similar tendency was also true for tumor cells obtained from mice treated with PRI-1906, but these changes were not statistically significant (Fig. 6b).

Antitumor activity of PRI-1906 in comparison with calcitriol and PRI-2191 in a combined treatment with CY

As shown above, since calcitriol and its analogs revealed a slight effect on 16/C tumor growth, they were examined in combined antitumor therapy in the experimental protocols described below. Bearing in mind the characteristics of murine transplantable mammary adenocarcinoma 16/C, and the sensitivity of the primary tumor to treatment with drugs used in breast cancer therapy, we chose CY as the reference agent in combined therapy [40].

Schedule A with the 0.1 μg/ml dose of calcitriol and its analogs combined with 50 mg/kg of CY

CY was injected i.p. at the 50 mg/kg dose at fifth day after tumor cells inoculation. Calcitriol or PRI-1906 were administered s.c. at the 0.1 μg/kg dose for 11 consecutive days, starting from the first day or for 6 consecutive days, starting from the sixth day after inoculation of tumor cells and then from day 13 every second day (Table 3). Single therapy with calcitriol did not affect tumor growth. PRI-1906 inhibited tumor growth by 49% ($p < 0.05$) only when administered from the first day after 16/C cells inoculation. The tumor weights in both groups with combined

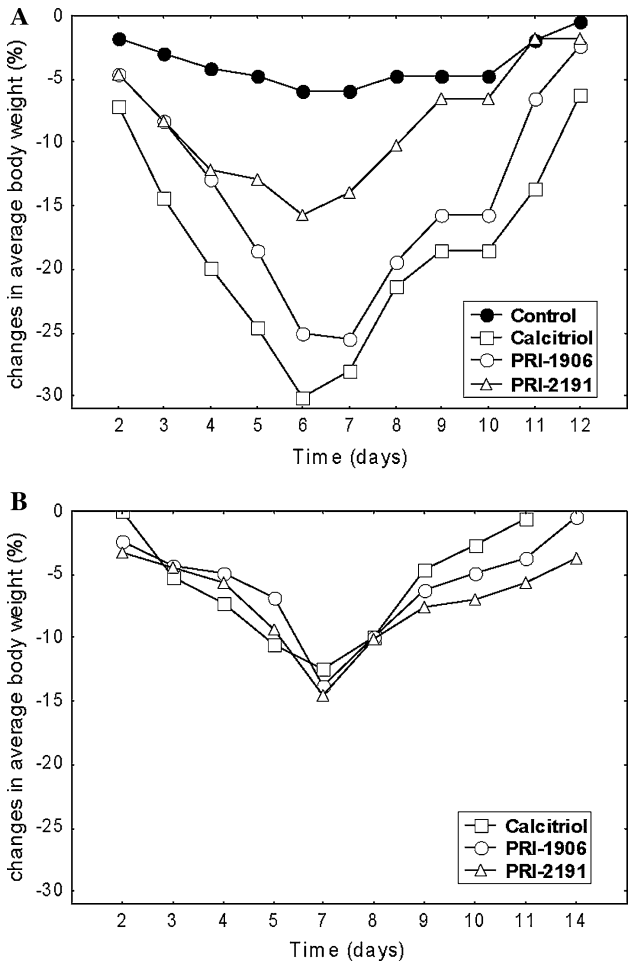

Fig. 5 Kinetics of average body weight changes in mice bearing 16/C tumors, treated 5 μg/kg per day of calcitriol, PRI-1906 and PRI-2191. **a** Subcutaneous administration; **b** oral administration. Number of mice used for evaluation: control group 12, treated groups 6 mice

CY + PRI-1906 therapy and group with combined CY + calcitriol therapy started from the first day, were lower than in group treated with single CY monotherapy. However, these differences were not statistically significant in the model used, therefore we can only presume an additive effect of both compounds in groups treated by combined therapy (Table 3).

Schedule B with the dose of 1.0 μg/ml of calcitriol analogs combined with 100 mg/kg of CY

CY was injected i.p. at the 100 mg/kg dose at the fourth day after tumor cells inoculation. PRI-2191 and PRI-1906 were administered s.c. at a dose of 1.0 μg/kg for 9 consecutive days, starting from the first or fifth day after inoculation of tumor cells (Table 4). The monotherapy by the either of analogs was not effective regardless of the starting day of the treatment. A slight potentiation effect of both analogs on the CY efficacy was noted in the groups with combined therapy started from the first day. However, although the tumor weights in these groups were lower than those in the group treated with CY monotherapy, the differences were not statistically significant in the model used (Table 4). Combined therapy started from the fifth day did not show any influence of the analogs on the efficacy of the CY in this schedule.

Discussion

Since calcitriol is effective as an antitumor agent only at doses, which are highly toxic, it seemed appropriate to search for new vitamin D analogs with low calcemic and high antitumor activity that could be potentially applied in anticancer therapy, both in single drug protocols and in combination with other antitumor agents [19, 24, 27, 29, 31, 32, 34, 36, 38, 41–48]. Mice treated with synthetic analog of vitamin D_2 with the extended and rigid side chain, coded PRI-1906 (Fig. 1) had shown lower mortality and toxicity in comparison with calcitriol-treated mice. Another analog, PRI-1907 (Fig. 1), which revealed slightly lower antiproliferative activity than PRI-1906 [8, 37], was significantly more toxic than both calcitriol and PRI-1906.

In our previous studies, the presence of estrogen (ER) or progesterone (PR) receptors was detected in 66.7% (10 out of 15 mice) of 16/C tumors analyzed. There were high individual variations in ER and PR receptor levels in 16/C tumors. In ER positive tumors, the level varied from 44.20 fmol/mg of cytosol protein to 213.60 fmol/mg of cytosol protein, whereas the level of PR varied from 31.96 to 202.68 fmol/mg of cytosol protein [49]. Love-Schimenti et al. [50] suggested that estradiol in the ER positive cells blocks the antiproliferative effects of the analogs. They also suggested the potential effectiveness of combined therapy applying both by antiestrogen and calcitriol analogs in ER positive human breast tumors. They further speculated that calcitriol analogs might be effective alone in ER negative breast tumors. In our mouse mammary cancer model, we observed that the antitumor effect of calcitriol and its analogs is low and is revealed only in the initial stage of tumor growth. Moreover, the effectiveness of treatment is related to the protocol and route of administration. However, combination of one of these agents with a cytotoxic drug, for example CY or cisplatin [36], may increase the antitumor effect. We observed the potentiation of anticancer activity of CY when the analogs of calcitriol were administered from first day after tumor cells inoculation, and this effect was more pronounced for higher doses of the analogs. Assessment of combined therapeutic effect was done multiplying the effects of each drug alone and comparing the estimated effect with the observed one. When the observed effect exceeded the estimated one, potentiation was

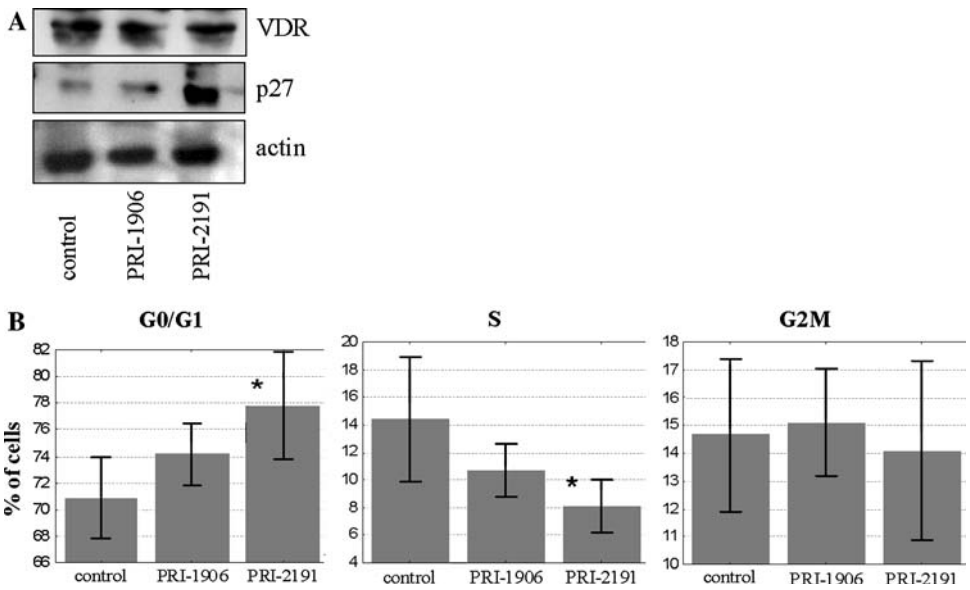

Fig. 6 The influence of tumor growth and some physiological and molecular parameters in serum and tumors from mice bearing 16/C mammary gland carcinoma treated with PRI-1906 or PRI-2191 calcitriol analogs. All parameters from day 13, PRI-1906 or PRI-2191 were administered s.c. in the dose 1 μg/kg per day during 11 consecutive days, starting from day 3. Tumors and blood were collected. **a** VDR and p27 expression—representative immunoblot. **b** Cell cycle distribution—FACS analysis: the results are presented as a mean (±SD) percentage of the cell population attributed to one of the cell cycle phases: G0/G1, S, G2/M, analysis was performed using the ModFit software. *Asterisks* denotes $p < 0.05$ (Kruskall Wallis test) as compared to a control. Number of mice used for evaluation: control = 9, PRI-1906 = 6, PRI-2191 = 7

Table 3 The effect of calcitriol and PRI-1906 on the growth of 16/C mouse mammary tumor inoculated s.c. Schedule A: single therapy with either calcitriol or PRI-1906, started from day 1 or day 6, and combined therapy with the CY

Treatment	Tumor weight (mg) median mean ± SD		TGI (%)	
	Day 1[a]	Day 6[a]	Day 1[a]	Day 6[a]
CY	641 ± 332	565[c]	44	
PRI-1906	515[c] 482 ± 258	717 876 ± 358	49	29
PRI-1906 + CY	454[c] 549 ± 267	405[c] 453 ± 165	55 (71[b]) additive effect	60 (60[b]) additive effect
Calcitriol	762 729 ± 230	1138 1058 ± 290	25	−12
Calcitriol + CY	417[c] 465 ± 220	588[c] 576 ± 273	59 (58[b]) additive effect	42 (37[b]) no interaction
Control	1016 983 ± 537		–	

[a] Day after tumor cells inoculation of first calcitriol injection (or analog)

[b] Minimal expected inhibition

[c] $p < 0.05$, compared to control group (Mann–Whitney *U* Test)

Calcitriol or PRI-1906 were s.c. administered daily at the dose of 0.1 μg/kg for 11 or 6 consecutive days, starting from first or sixth day after inoculation of tumor cells. CY was injected i.p. in the dose of 50 mg/kg at fifth day after tumor cell inoculation

Day of evaluation, 20th; number of mice, 6 per group and 12 in control

The expected inhibition used to estimate the effect of combination of two compounds was evaluated using the formula %H = 100−[(100−TGI CY) × (100−TGI calcitriol or its analog) /100]. When the observed effect is above the expected one, potentiation was concluded. When the observed effect is under the expected one but above that observed for each drugs used alone, additive effect was concluded

Table 4 The effect of PRI-2191 and PRI-1906 on the growth of 16/C mouse mammary tumor inoculated s.c. Schedule B: single therapy with either PRI-2191 or PRI-1906, started from day 1 or day 5, and combined therapy with the CY

Treatment	Tumor weight (mg) median mean ± SD		TGI (%)	
	Day 1[a]	Day 5[a]	Day 1[a]	Day 5[a]
CY	267[c] 384 ± 220		72	
PRI-1906	936 908 ± 582	1098 1250 ± 477	3	−13,5
PRI-1906 + CY	107[c] 250 ± 327	230[c] 226 ± 130	89 (73[b])potentiation	76 (68[b]) no interaction
PRI-2191	1055 948 ± 275	726 860 ± 313	−9	25
PRI-2191 + CY	184[c] 195 ± 67	232[c] 282 ± 313	81 (69.5[b]) potentiation	76 (79[b]) no interaction
Control	968 925 ± 385		–	

[a] Day after tumor cells inoculation of first calcitriol analogs injection

[b] Minimal expected inhibition

[c] $p < 0.05$, compared to control group (Mann–Whitney U Test)

PRI-2191 and PRI-1906 were administered s.c. at the dose of 1.0 μg/kg for nine consecutive days, starting from the day 1 or 5 after inoculation of tumor cells. CY was injected i.p. in the dose of 100 mg/kg on the fourth day after tumor cell inoculation

Day of evaluation, 20th; number of mice, 6 per group and 12 in control

The expected inhibition used to estimate the effect of combination of two compounds was evaluated using the formula %H = 100 − [(100 − TGI CY) × (100 − TGI calcitriol or its analog)/100]. When the observed effect is above the expected one, potentiation was concluded. When the observed effect is under the expected one but above that observed for each drugs used alone, additive effect was concluded

concluded as described elsewhere [39]. We also performed a formal statistical analysis and we have not observed statistically significant differences in inhibition of the tumor growth between groups treated with CY alone and CY combined with calcitriol or its analogs. Therefore we are rather cautious in the interpretation of our results (Tables 3 and 4). It seems that supportive therapy with the analogs needs to be started early and has to be conducted for longer to reveal substantial improvement in the CY therapy.

It is generally accepted that calcitriol and its analogs primarily exert their pleiotropic effects after binding to a specific receptor, which is a member of the steroid hormone receptor superfamily [51]. The ligand-bound vitamin D receptor (VDR) then interacts with its cognate binding site, termed vitamin D-response element (VDRE), to affect the transcription of target genes like cell cycle inhibitor p21 [51–53]. However, other vitamin D targeted genes, like other cell cycle inhibitor p27, which lack VDRE could be regulated by calcitriol due to interaction with the transcription factor Sp1 [54, 55]. Several studies suggest that growth inhibition by calcitriol may be attributed to inhibition of the G1 to S transition in the cell cycle, which probably is due, at least in part, to stimulation of expression of the cyclin-dependent kinase inhibitors (CDKIs), p21 and p27 as well as programmed cell death [12, 41, 45, 53–55]. Our present observations are in accordance with previous results on the pro-differentiating activity of vitamin D analogs. We have shown that HL-60 leukemia cells began to resemble phenotypically mature macrophages after their exposure to PRI-1906 or PRI-2191 in vitro. The differentiating activities of vitamin D analogs were higher than that activity shown by calcitriol [1, 8]. Moreover, the potencies of the analogs to induce differentiation paralleled their activation of Erk, JNK and p38 mitogen-activated protein kinase (MAPK) pathways, and the anti-proliferative activity closely correlated with the extent of hypophosphorylation of retinoblastoma protein (pRb) [10]. These data suggest that the higher antitumor effect of the combined treatment with calcitriol analogs and CY may be the result of the cell cycle arrest in the G0/G1 cell cycle phase and increased p27 expression following the treatment with PRI-2191. However, lack of significant increase in the p27 expression following the treatment with PRI-1906 suggests that some differences exist between the mechanisms of action of these two analogs.

In conclusion, PRI-1906 exhibited higher toxicity than PRI-2191 but lower than calcitriol and antitumor activity similar to PRI-2191 or calcitriol. However, the ability of PRI-1906 to induce differentiation of mammary adenocarcinoma cells in vivo was limited and lower than PRI-2191.

The new vitamin D analog PRI-1906 seems to be a potential candidate for further preclinical studies in the anticancer treatment.

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