The Antitumor Effect of Lowered Doses of Cytostatics Combined with New Analogs of Vitamin D in Mice

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Abstract. *Active and less toxic vitamin D analogs could be useful for clinical applications. In the present study, the antitumor effects of two new synthetic analogs of vitamin D, namely PRI-2202 (24R calcipotriol) and PRI-2205 (5, 6-trans calcipotriol), were evaluated. Since the analogs PRI-2202 and PRI-2205 administered alone inhibited tumor growth only slightly, they were applied in a combined therapy with cytostatics. The in vitro results showed that the synergistic effect between vitamin D analogs and cytostatics was more pronounced when low concentrations of the latter were used. Due to this fact low doses of cytostatics were applied in the in vivo combined treatment schedules. The studies were performed in mouse mammary cancer 4T1 and Lewis lung cancer (LLC) models. Mice bearing subcutaneous tumors were treated with vitamin D analogs and cytostatics in different combinations. Statistically significant inhibition of tumor growth by the combined treatment was observed in 4T1 mammary cancer treated with cyclophosphamide and in LLC lung cancer-bearing animals treated with cisplatin. In contrast, no improved therapeutic effect of the combined treatment with low doses of doxorubicin and cyclophosphamide was observed in mice bearing LLC tumors. Moreover, the combined treatment with cisplatin led to increased toxicity, which did not depend on the calcemic activity of the vitamin D analogs. The general conclusion of this work is that combination of vitamin D analogs with cytostatics applied in low doses is not effective in vivo, despite the encouraging in vitro results. Nevertheless, combined treatment with vitamin D analogs was more effective than the treatment with cytostatics applied alone, when higher doses of cytostatics were used.*

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Calcitriol (a hormonally active form of vitamin D_3 (1, 25-dihydroxyvitamin D_3, 1, 25-$(OH)_2D_3$)), regulates calcium and phosphorus homeostasis, but also exerts antitumor activity both *in vitro* and *in vivo* (1-6). In addition to its antiproliferative and differentiation-inducing effects, calcitriol induces apoptosis in a number of cancer cell lines *in vitro* (7-10). Studies performed in tumor-bearing animals treated with calcitriol, have shown regression of tumors, inhibition of metastasis development, prolongation of survival time and an antiangiogenic effect (2, 11-14). Vitamin D used as chemopreventive agent can inhibit colon (15), breast (16, 17), lung (18) and prostate (19) carcinogenesis. This is supported by evidence obtained from a variety of preclinical experimental studies, epidemiological data and a few human clinical trials (15-17, 19-25). In human mammary breast cancer cell lines, calcitriol induces cell cycle arrest in the G0/G1 stage and/or apoptosis through vitamin D receptor (VDR) dependent mechanisms (13, 26, 27). In mouse lung carcinoma cells, the inhibition of metastasis formation and angiogenesis seemed to be a major mechanism responsible for the antitumor effects of vitamin D analogs (14).

In our previous studies, 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 were examined for their antiproliferative activity *in vitro* against various human normal and cancer cell lines (1, 7, 28, 29). 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 (1, 7, 29-32). The antitumor effect could be attributed to the induction of cancer cell differentiation (1, 7-9, 33).

Table I. *Experimental protocol of the treatment of mammary gland cancer 4T1-bearing mice with CY and vitamin D analogs (experiment was terminated at day 22).*

Group of mice	N[1]	Cytostatic[2]	Vitamin D analog[3]
Control	10	-	-
PRI-2202	6	-	PRI-2202
PRI-2205	6	-	PRI-2205
CY	8	CY	-
CY + PRI-2202	10	CY	PRI-2202
CY + PRI-2205	10	CY	PRI-2205

[1]Number of animals; [2]CY was administered *i.p.*, dose 20 mg/kg on days 2, 5, 7, 9, 12, 14, 16, 19 and 21; [3]vitamin D analogs were administered *s.c.*, dose 10 μg/kg on the same days as CY.

Table II. *Experimental protocol of the treatment of Lewis lung carcinoma-bearing mice with CIS and vitamin D analogs (experiment was terminated at day 21).*

Group of mice	N[1]	Cytostatic[2]	Vitamin D analog[3]
Control	9	-	-
PRI-2202	8	-	PRI-2202
PRI-2205	8	-	PRI-2205
CIS	8	CIS	-
CIS + PRI-2202	8	CIS	PRI-2202
CIS + PRI-2205	8	CIS	PRI-2205

[1]Number of animals; [2]CIS was administered *i.p.*, dose 1 mg/kg on days 1, 3, 6, 8, 10, 13, 15, 17 and 20; [3]vitamin D analogs were administered *s.c.*, dose 10 μg/kg on the same days as CIS.

Table III. *Experimental protocol of the treatment of Lewis lung carcinoma-bearing mice with DX, CY and vitamin D analogs (experiment was terminated at day 21).*

Group of mice	N[1]	Cytostatic[2]	Vitamin D analog[3]
Control	13	-	-
PRI-2202	11	-	PRI-2202
PRI-2205	10	-	PRI-2205
CY	11	CY	-
CY + PRI-2202	11	CY	PRI-2202
CY + PRI-2205	11	CY	PRI-2205
DX	11	DX	-
DX + PRI-2202	11	DX	PRI-2202
DX + PRI-2205	11	DX	PRI-2205

[1]Number of animals; [2]DX was administered *i.p.*, dose 1 mg/kg on days 1, 3, 6, 8, 10, 13, 15 and 18; CY was administered *i.p.*, dose 10 mg/kg on days 1, 3, 6, 8, dose 20 mg/kg on days 10, 13 and dose 50 mg/kg on days 15 and 18; [3]vitamin D analogs were administered *s.c.*, dose 10 μg/kg on the same days as cytostatics.

Table IV. *Experimental protocol of the treatment of Lewis lung carcinoma-bearing mice with CIS and vitamin D analogs. Clodronate was used in this experiment to prevent calcemic effects in the therapy by vitamin D analogs (experiment was terminated at day 22).*

Group of mice[1]	N[2]	Cytostatic[3]	Vitamin D analog[4]
Clodronate alone (Control)	6	-	-
Clodronate + PRI-2191	7	-	PRI-2191
Clodronate + PRI-2205	7	-	PRI-2205
Clodronate + CIS	6	CIS	-
Clodronate + CIS + PRI-2191	7	CIS	PRI-2191
Clodronate + CIS + PRI-2205	7	CIS	PRI-2205

[1]Mice in all six groups received clodronate at dose 1.5 mg/mouse on days 2, 7 and 11; [2]number of animals; [3]CIS was administered *i.p.*, dose 1.5 mg/kg on days 2, 4 and 7; [4]PRI-2191 was administered *s.c.*, dose 1 μg/kg and PRI-2205 was administered *s.c.*, dose 10 μg/kg on days 2, 4, 7, 9, 11, 14, 16, 18 and 21.

A number of studies on the effect of combined treatment with calcitriol or its analogs and different chemotherapeutic agents have been reported in the last decade both *in vitro* (7, 30, 31, 34-36) and *in vivo* (37, 38). In our own studies, the combination of cyclophosphamide (CY) or cisplatin (CIS) with other vitamin D analogs (tacalcitol or (24*E*)-24-dehydro-24a-homo-1, 25-dihydroxyergocalciferol) resulted in an alteration of its antitumor effect [Wietrzyk, unpublished data and (32)].

The application of potentially effective, hyper-physiological doses of calcitriol in anticancer treatment is limited by its calcemic activity and subsequent risk of hypercalcemia (20, 39, 40). These undesired side effects motivated the synthesis of new analogs, aiming to dissociate calcemic and antiproliferative effects (41). The new vitamin D analogs 24R calcipotriol (PRI-2202) and 5, 6-trans calcipotriol (PRI-2205) were selected because of their observed effect on the cell cycle stage and antiproliferative activity *in vitro*, toxicity and antitumor activity *in vivo* (42). These derivatives with diastereomeric and geometric modifications were obtained in the course of synthesis of calcipotriol (43). In this article, the effect of these two vitamin D analogs, PRI-2202 and PRI-2205 on mouse serum calcium levels, blood leukocyte number and antitumor activity *in vivo* in combined treatment with CY, CIS or doxorubicin (DX) in mice bearing transplanted mammary (4T1) or Lewis lung cancer (LLC) is described.

Materials and Methods

Compounds. Analogs of calcitriol - PRI-2191, PRI-2201, PRI-2202 and PRI-2205 were certified synthetic materials obtained from the 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 5 μl/1 g of body weight.

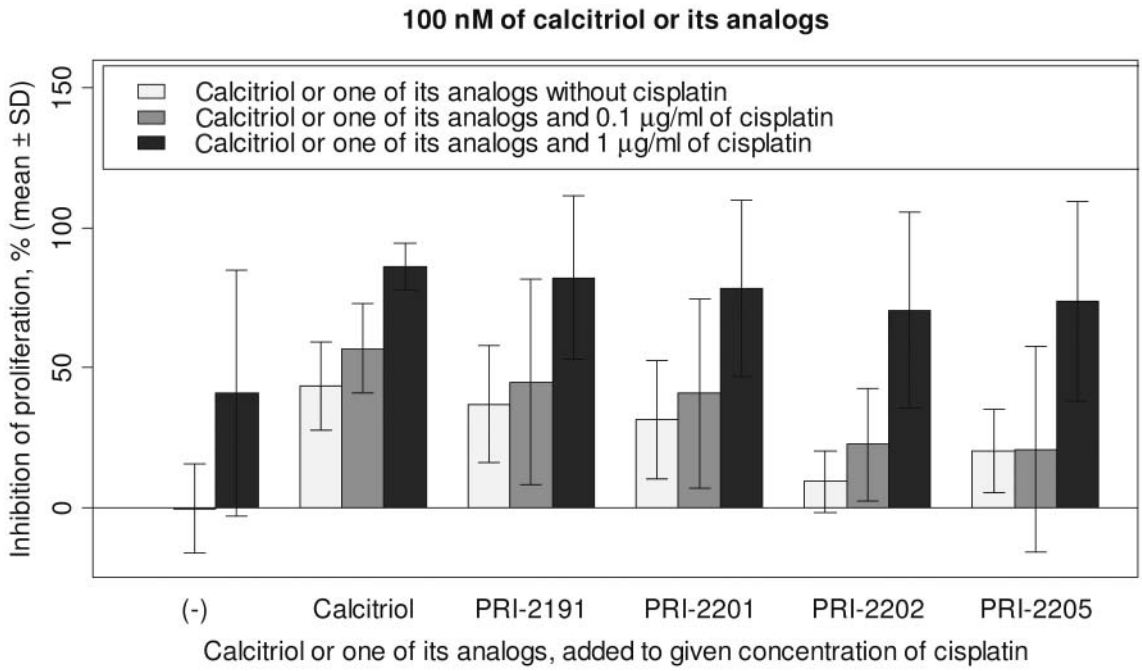


Figure 1. *Inhibition of proliferation of Lewis lung carcinoma cells in vitro exposed to CIS and calcitrol or its analogs. The data are represented as mean values with SD of proliferation inhibition (%). Calcitriol or its analogs were used in 100 nM concentration.*

Cyclophosphamide (Endoxan, ASTA Medica AG, Frankfurt, Germany), cisplatin (cisplatinum, Ebewe, Unterach, Austria), doxorubicin (Institute of Biotechnology and Antibiotics, Warsaw, Poland) and clodronate (Bonefos, Leiras Oy, Finland) were diluted in *aqua pro injectione* to reach the required concentrations and administered to mice in a volume 10 μl/1 g of body weight.

Cell lines. The mouse mammary adenocarcinoma 4T1 cells were obtained from American Type Culture Collection (ATCC) and the Lewis lung carcinoma (LLC) cells were received as a gift from Dr. I. Wodinsky, National Cancer Institute, Bethesda, USA.

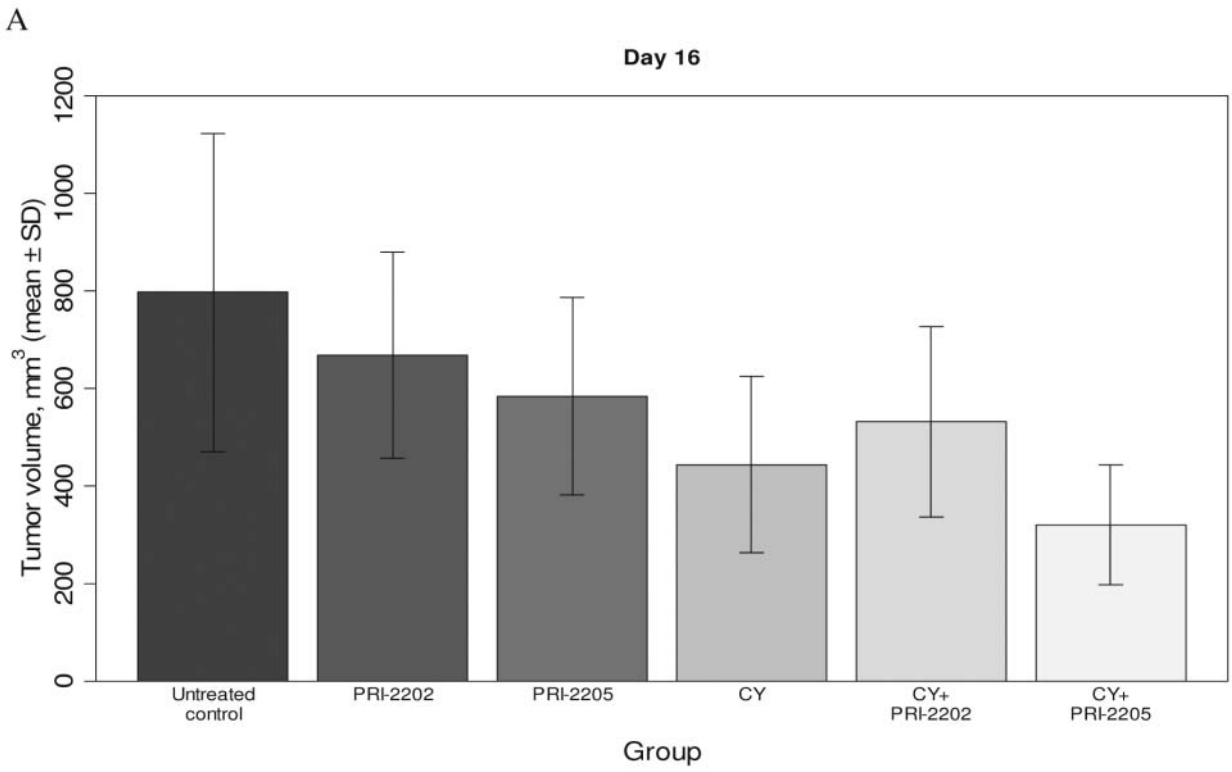
The 4T1 cells were maintained in RPMI-1460 GlutaMAX adjusted to contain 4.5 g/L glucose, 2 mM glutamine and 1.0 mM sodium pyruvate with fetal bovine serum (10%). The LLC cells were maintained in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, 4 mM L-glutamine and 1 mM sodium pyruvate and supplemented with 10% fetal bovine serum.

An anti-proliferative assay in vitro. The cells were placed in 96-well flat bottom plates (Sarstedt, Inc., Newton, NC, USA) at a density of $5x10^3$ for 4T1 or $2x10^3$ for the LLC cells per well, 24 hours before addition of the tested compounds. In the combined treatment the cells were exposed for 120 hours to various concentrations (1, 10, 100 and 1000 nM) of calcitriol or its analogs and simultaneously treated with the following doses of CIS 10, 100, 1000 and 10, 000 ng/ml. The sulforhodamine B (SRB) assay for evaluation of the cytostatic effect was performed as described previously (42). The results were calculated as a percent of proliferation inhibition by the tested compounds. The average values were counted using the data from 3-7 repetitions.

Mice. BALB/c female or C57BL/6 male, 12-16 week old mice, weighing 20-25 g, supplied from the Nofer Institute of Occupational Medicine (Lodz, Poland) and the Polish Academy of Sciences Medical Research Center (Warszawa, 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.

Design of the in vivo experiments. BALB/c female mice were orthotopically inoculated into the right mammary fat pad with $5x10^5$ viable 4T1 tumor cells per mouse in 0.05 ml saline and then randomly divided into six groups receiving different combinations of treatment agents (Table I). In three subsequent experiments, C57BL/6 mice were subcutaneously inoculated in the right flank of the abdomen with $3x10^5$ of LLC tumor cells suspended in 0.2 ml saline and then randomly divided into different groups (day 0). One of three experimental protocols was applied in respective experiments (Table II-IV). All the experiments described above were planned in full factorial design with two factors controlled simultaneously, the type of cytostatic and the type of vitamin D analog respectively. At the end of some experiments blood was harvested before subsequent sacrifice of the mice and metastases were counted in the lungs of the animals during autopsy.

Evaluation of the therapeutic effect. The tumor volume was calculated using the formula (a^2 x b)/2, where a = shorter tumor diameter in mm and b=longer tumor diameter in mm. The inhibition of tumor growth was calculated from the following formula: TGI [%] (tumor growth



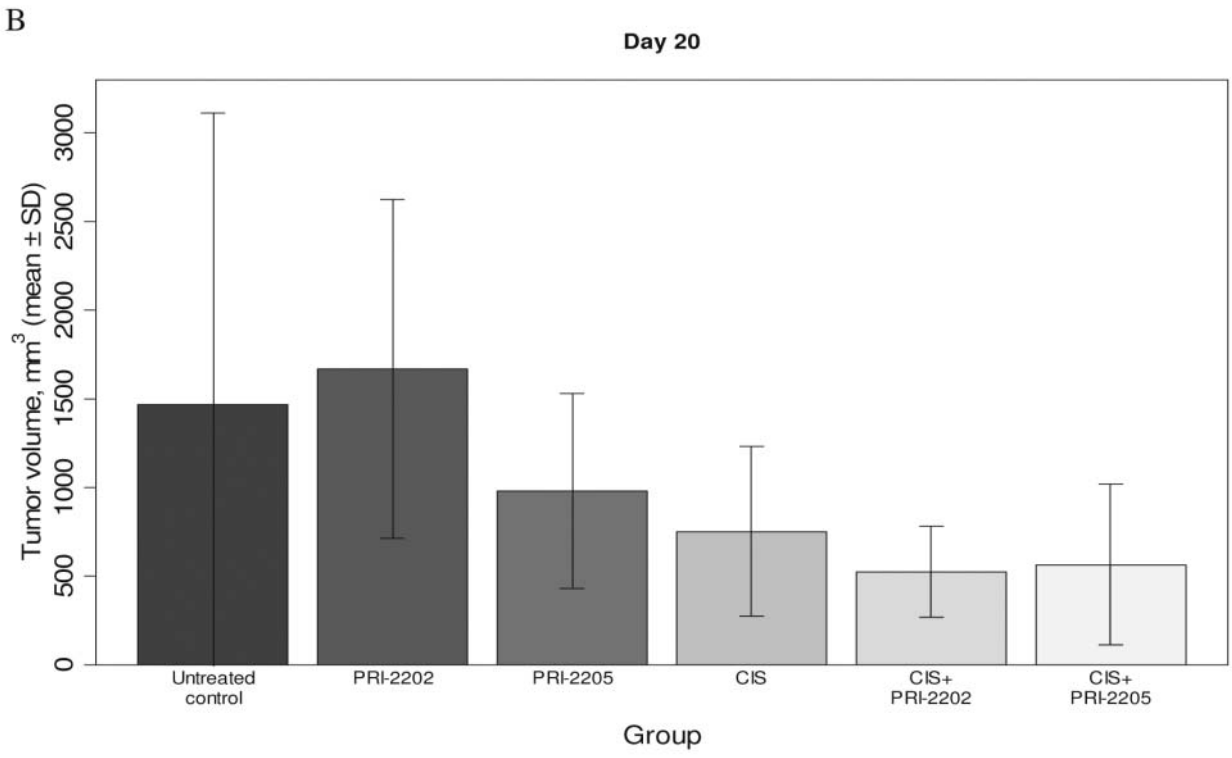


Figure 2. *continued*

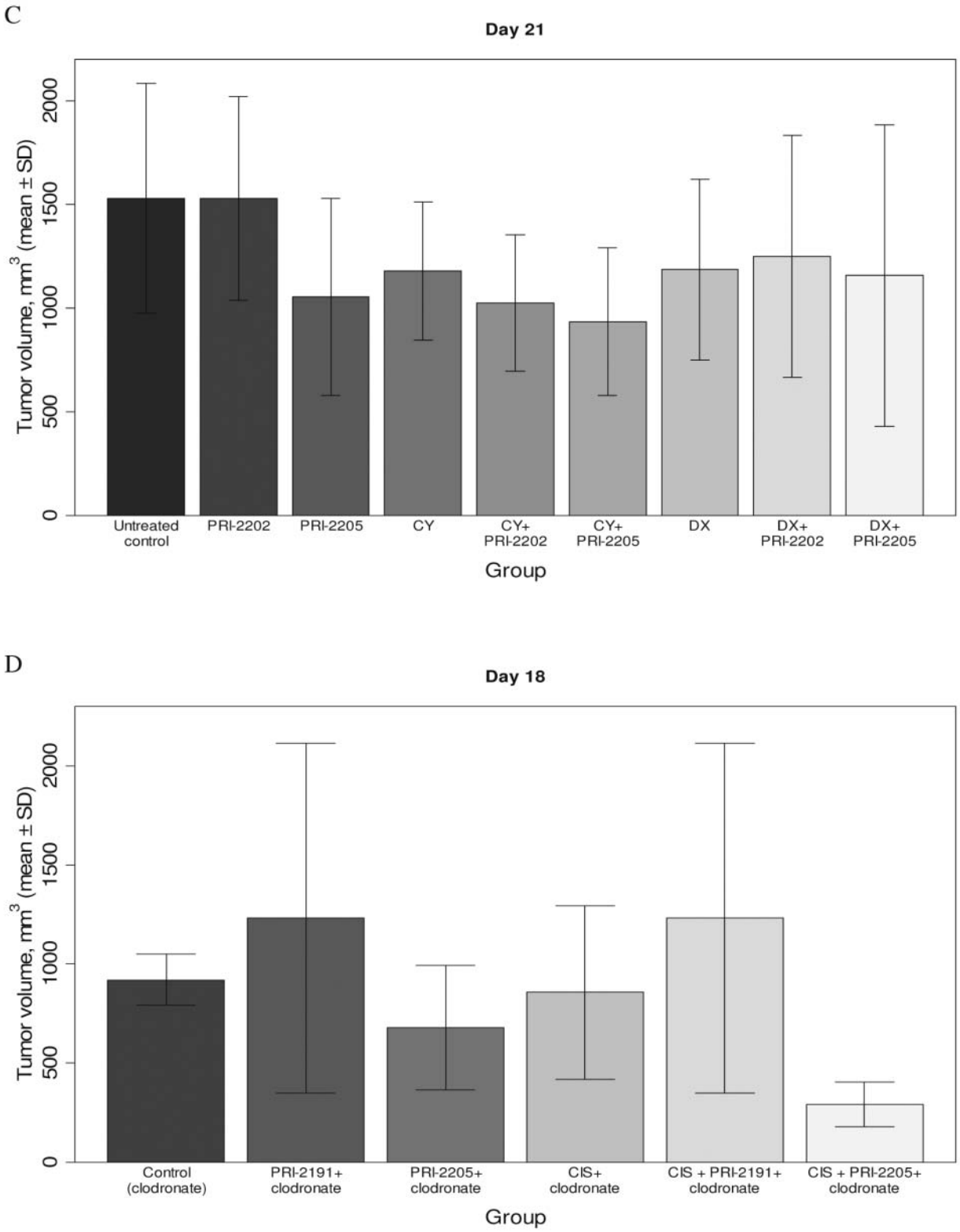


Figure 2. *Tumor volume in mice in different groups from four sequential experiments. Mice were treated with different combinations of calcitriol analogs and cytostatics: A) mammary gland cancer 4T1, treatment with CY (only data for day 16 is shown for clarity). B) Lewis lung carcinoma, treatment with CIS (only data for day 20 is shown for clarity). C) Lewis lung carcinoma, treatment with CY and DX (only data for day 21 is shown for the sake of clarity). D) Lewis lung carcinoma, treatment with CIS with parallel administration of clodronate (only data for day 18 is shown for the sake of clarity).*

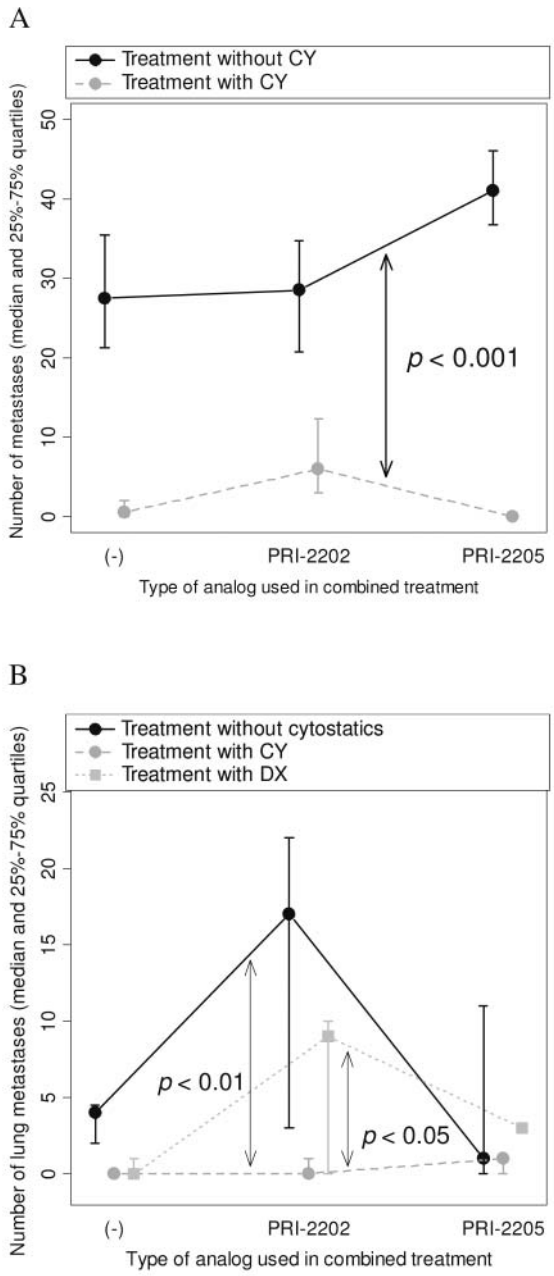


Figure 3. *Lung metastases in mice treated with the cytostatics alone or in combination with vitamin D analogs. A) mammary gland cancer 4T1, treatment with CY; B) Lewis lung carcinoma, treatment with CY or DX.*

inhibition) = (W_T/W_C) x 100-100%, where W_T was the median tumor volume of treated mice and W_C that of the untreated control animals.

Calcemic activity. Blood sera were collected and then the animals were sacrificed at the end of the experiment. The calcium level was measured in each individual serum sample using the photometric Arsezano 3 method (Olympus AU400, Olympus America Inc., Melville, NY, USA).

Statistical evaluation. Statistical analysis was performed using STATISTICA version 7.1 (StatSoft Inc., USA) and R version 2.3.1 (R Foundation for Statistical Computing, Austria). The data were analysed by two-way ANOVA for the type of chemotherapy agent (first factor) and the type of vitamin D analog (second factor). Tukey's pairwise comparisons were performed for further *post-hoc* analysis. The assumptions of ANOVA were checked using PP-plots, Shapiro-Wilk's test and Levene's test. In case of violations of ANOVA assumptions, a nonparametric stratified permutation Kruskal-Wallis overall test from the coin package in R was used with subsequent pairwise comparisons and Bonferroni correction. *P*-values less than 0.05 were considered significant.

Results

Combined treatment with vitamin D analogs and cytostatics in vitro. The combined application of vitamin D analogs and CIS caused inhibition of the proliferation of LLC cells *in vitro* in a additive manner. The best additive effect was obtained when CIS was used at the low concentration of 1 μg/ml and calcitriol or its analogs at a concentration of 100 nM (data for these concentrations are shown Figure 1).

The mouse mammary gland cancer 4T1 cells appeared to be not sensitive to the antiproliferative activity of vitamin D analogs *in vitro*. The maximum proliferation inhibition which was observed did not exceed 14%. In the combined application, antagonistic antiproliferative effects between vitamin D analogs and CIS were observed (data not shown).

PRI-2202 and PRI-2205 administration alone or in combination with the CY in the mouse mammary gland cancer 4T1 model. In all the experimental groups in which treatment included cyclophosphamide, the mice had statistically significant lower tumor volumes than in the remaining groups without cyclophosphamide for all days of measurement ($p<0.01$, Figure 2 A, only data for day 16 is shown). Decreased tumor volume was also observed in mice treated with vitamin D analogs as compared to other groups. However, the differences in tumor inhibiting effect of the analogs used in the combined treatment were statistically significant only for day 15 and 16 of the experiments. Further analysis revealed that the mice in the groups which received PRI-2205 had statistically significantly smaller tumors at these particular days than groups received treatment containing PRI-2202 ($p<0.05$) or the mice treated without any vitamin D analogs ($p<0.01$). The average body weight of the mice was comparable, regardless of the therapy applied (data not shown).

The number of metastases in the lung of the mice on the last day of the experiment (22nd day) was also evaluated. The mice in all the groups, which received cyclophosphamide as a part of their therapeutic regimen had a statistically significant lower number of lung metastates as compared to animals from the groups which

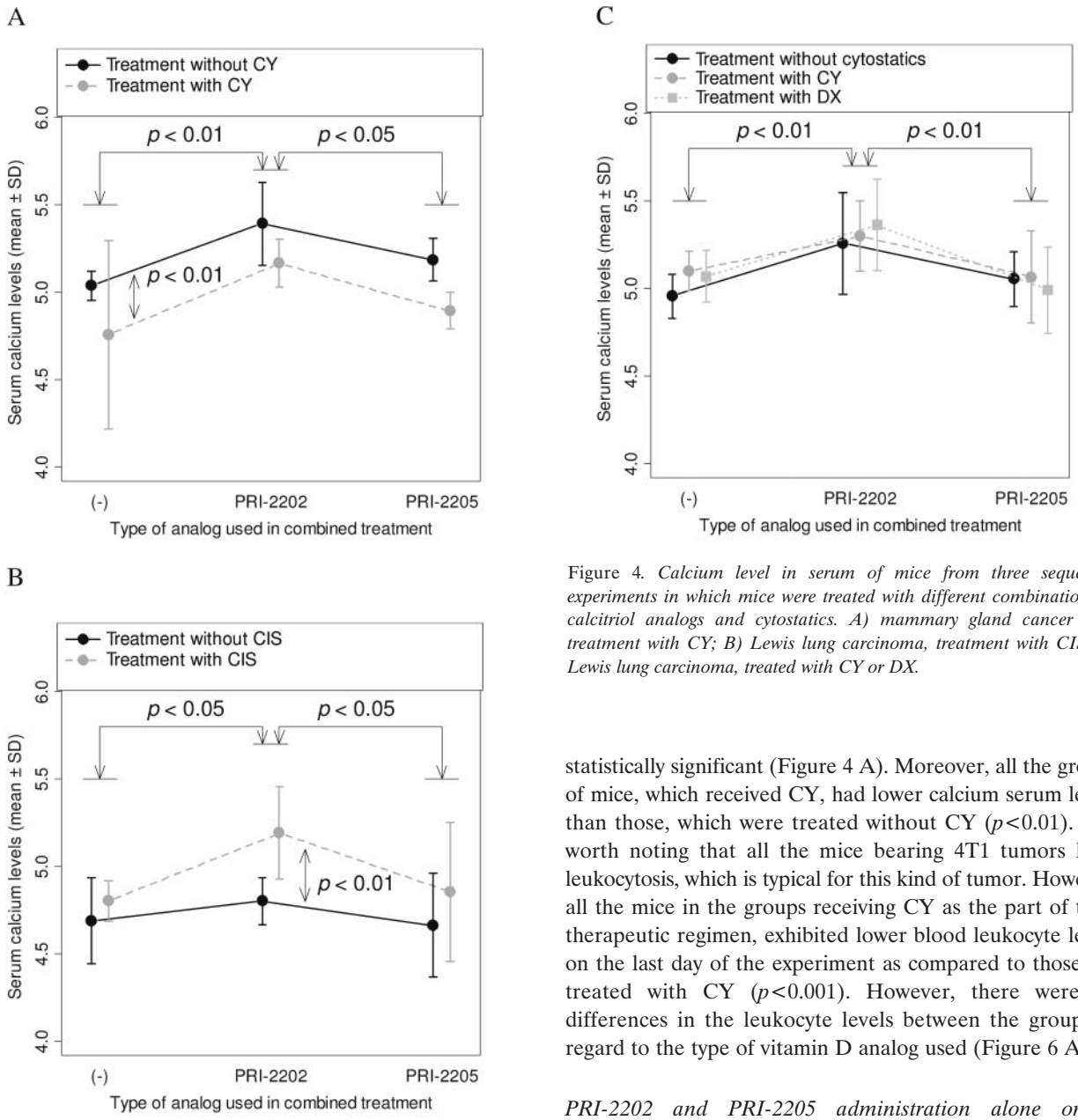


Figure 4. *Calcium level in serum of mice from three sequential experiments in which mice were treated with different combinations of calcitriol analogs and cytostatics. A) mammary gland cancer 4T1, treatment with CY; B) Lewis lung carcinoma, treatment with CIS; C) Lewis lung carcinoma, treated with CY or DX.*

had not received cyclophosphamide ($p<0.001$). However, there were no statistically significant differences in the number of metastases in the mice with regard to the type of vitamin D analog used in the treatment (Figure 3 A).

Serum calcium levels were measured in all mice on the last day of the experiment (22nd day). Mice in the groups, which received PRI-2202, had significantly higher serum calcium levels as compared to mice which had received the treatment including PRI-2205 ($p<0.05$) or without any analog ($p<0.01$). The difference between groups without addition of vitamin D analogs and groups treated with PRI-2205 was not statistically significant (Figure 4 A). Moreover, all the groups of mice, which received CY, had lower calcium serum levels than those, which were treated without CY ($p<0.01$). It is worth noting that all the mice bearing 4T1 tumors have leukocytosis, which is typical for this kind of tumor. However, all the mice in the groups receiving CY as the part of their therapeutic regimen, exhibited lower blood leukocyte levels on the last day of the experiment as compared to those not treated with CY ($p<0.001$). However, there were no differences in the leukocyte levels between the groups in regard to the type of vitamin D analog used (Figure 6 A).

PRI-2202 and PRI-2205 administration alone or in combination with cisplatin in mice bearing Lewis lung carcinoma. Mice in all the groups receiving CIS as part of their treatment regimens had statistically significant lower tumor volumes than animals from the groups which received no CIS, but only after the 13th day of the experiment ($p<0.01$, Figure 2 B, only data for day 20 is shown). A decrease in tumor growth in the groups administered with CIS in combination with vitamin D analogs, was also observed. Tumor growth inhibition (TGI) on the 20th day of the experiment was 39% for cisplatin administered alone, and 52 and 62% for cisplatin administered in combination with PRI-2202 and PRI-2205, respectively. However, these differences were not statistically significant.

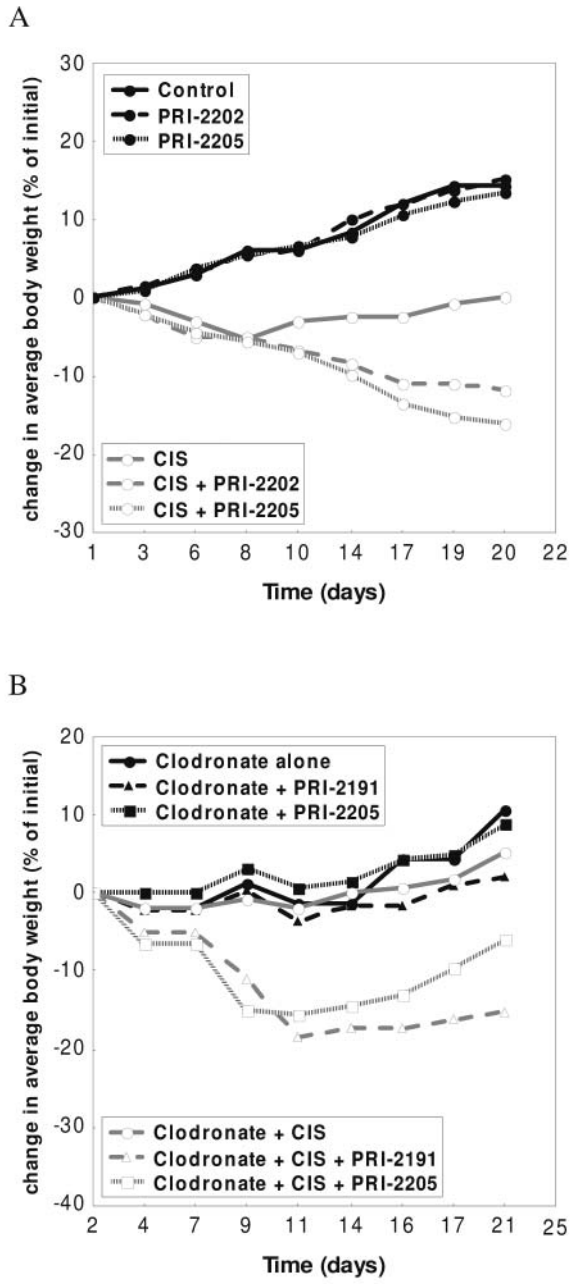


Figure 5. *Changes in average body volume of mice bearing Lewis lung carcinoma and treated with different combinations of cisplatin and calcitriol analogs. A) Lewis lung carcinoma, treatment with CIS; B) Lewis lung carcinoma, treatment with CIS together with administration of clodronate.*

All mice receiving CIS had a lower body weight during the course of the experiment than those not receiving the CIS and this effect was even more pronounced in the mice from the groups in which chemotherapy was combined with the addition of vitamin D analogs (Figure 5 A).

Mice in the groups which were treated with PRI-2202 had significantly higher serum calcium levels on the last day of the experiment (21st day) as compared to mice which received PRI-2205 ($p<0.05$) or without any analogs ($p<0.05$, Figure 4 B). In addition, all the mice receiving CIS, had higher serum calcium levels than mice from the groups which were not administered with this agent ($p<0.01$).

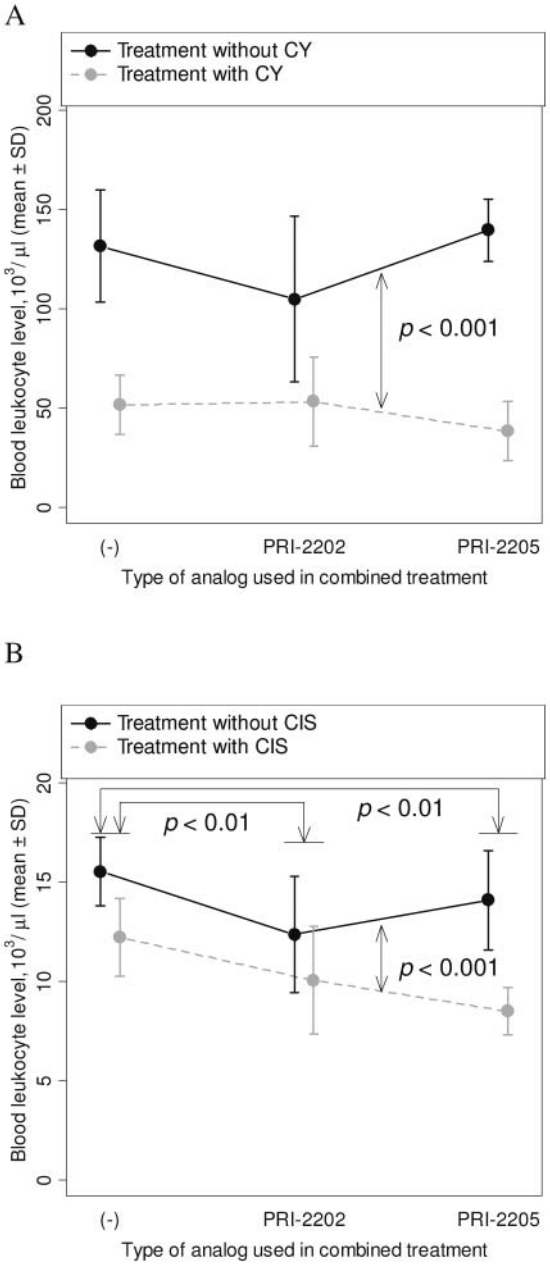
The groups which received CIS had significantly lower blood leukocyte levels than groups without administration of the cytostatic ($p<0.001$). The application of both PRI-2202 and PRI-2205, lowered leukocyte levels as compared to the groups without addition of vitamin D analogs ($p<0.01$, Figure 6 B).

PRI-2202 and PRI-2205 administration alone or in combination with CY or DX in mice bearing Lewis lung carcinoma. There were statistically significant effects of the type of cytostatic agent on the tumor volume on the 6th, 8th, 10th, 15th and 21st days of the experiment. On the 6th day, mice treated with CY had statistically significant higher tumor volume than mice treated with DX or without either of the cytostatic agents. However, starting from the 8th day the differences in tumor volume were statistically significant only between the groups treated with either CY or DX. Moreover, on the 21st day, the tendency reversed, since by that time the groups treated with CY had statistically significant lower tumor volumes than the mice in the other groups (Figure 2 C, only data for day 21 is shown). As regards the effect of the type of vitamin D analog, no significant differences in tumor volume between groups were found.

The animals in all groups, which received CY as a part of their therapeutic regimens, had a statistically significantly lower number of metastases on the last day of the experiment (21st day) as compared to mice from groups receiving DX ($p<0.05$) and groups not treated with cytostatics ($p<0.01$). There were no statistically significant differences in the number of metastases between groups regarding the type of vitamin D analog applied (Figure 3 B).

The average body weight of mice in the experimental groups did not differ significantly in this model, regardless of the therapy applied (data not shown). No significant effect of the type of cytostatic used in the treatment (CY, DX) was observed in terms of serum calcium levels measured on the last day of the experiment (21st). However, the mice treated with PRI-2202 exhibited significantly higher serum calcium levels as compared to the mice which were treated with PRI 2205 ($p<0.01$) or without any analogs ($p<0.01$). The differences between the groups treated with PRI-2205 and without any analogs was not statistically significant (Figure 4 C).

Both the cytostatics used in this experiment significantly lowered the blood leukocyte level in those groups as



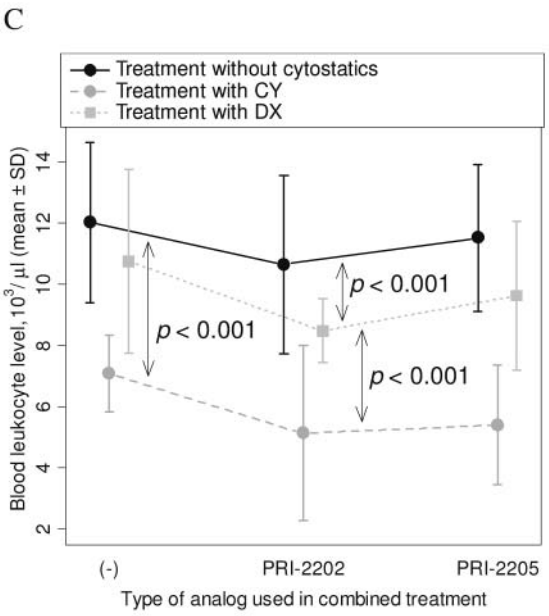


Figure 6. *Leukocytes in the blood samples from mice bearing. A) mammary gland cancer 4T1, treatment with CY; B) Lewis lung carcinoma, treatment with CIS; C) Lewis lung carcinoma, treatment with CY or DX.*

compared to the groups without cytostatics ($p<0.001$). Moreover, further analysis revealed that leukopenia was more pronounced in mice receiving CY as compared to DX ($p<0.001$). The type of vitamin D analog used in the combined therapy did not significantly influence the blood leukocyte level (Figure 6 C).

PRI-2191 and PRI-2205 administration alone or in combination with CIS and clodronate in mice bearing Lewis lung carcinoma. Statistical analysis revealed that starting from Day 11 the mice treated with CIS, had lower tumor volumes as compared to groups that received treatment without this cytostatic ($p<0.05$, Figure 2 D, only data for day 18 is shown). The effect of the analog type used in the treatment on the tumor volume was also statistically significant until the 18th day of the experiment. Mice receiving PRI-2205 had statistically significant lower tumor volumes during this period than mice receiving PRI-2191 or mice not exposed to vitamin D analogs ($p<0.05$, days 7-18, Figure 2 D).

Moreover, the addition of PRI-2205 to the combined therapy substantially improved the antitumor effect of CIS. The mice receiving cytostatics alone had higher tumor volumes than mice treated by cytostatic in combination with PRI-2205.

It is worth noting that clodronate did not reveal any detectable antitumor effect (data not shown). All the mice administered with CIS showed a lower average body weight during the course of the experiment and this effect was even more pronounced in the animals which received combined treatment with CIS and vitamin D analogs. The animals started to recover after stopping the administration of CIS (Figure 5 B).

Discussion

Vitamin D or its analogs combined with cytostatics are able to enhance the antiproliferative and antitumor activity of CIS, carboplatin, DX, docetaxel, paclitaxel, genistein, etoposide or tamoxifen both *in vitro* and *in vivo* (7, 30-32, 34, 35, 38, 44-49).

In the present study the combined treatment with CY and vitamin D analogs did not significantly affect body weight of the treated mice, but caused leukopenia. CY revealed an advantageous effect on the hypercalcemia, lowering the calcium serum level. However, the serum calcium level was significantly raised in the mice treated with the PRI-2202. The growth of the mouse mammary gland cancer 4T1 causes hypercalcemia during tumor progression (50). Hence, the reduction of the tumor burden brought about by the CY could be responsible for decreasing the hypercalcemia observed in the mice. Moreover, it has been shown that CY caused a reduction of the hypercalcemia which develops during the progression of Wegener's granulomatosis (51, 52) or refractory multiple myeloma in man (53).

The present data indicated that CY can be considered as a promising candidate for combined treatment with vitamin D or its analogs. To obtain a more pronounced antitumor effect further studies are necessary to find the optimal doses and treatment protocol.

Our results were in accordance with the literature showed that vitamin D enhanced the antitumor effect of CIS (32, 45, 49, 54). However, the combined treatment with CIS and vitamin D analogs, caused an unexpected toxicity manifested by body weight loss, leukopenia and hypercalcemia. Kawai *et al.*, have previously suggested that the toxicity of CIS is caused, among other things, by oxidative stress in renal tubular epithelial cells resulting from release of intracellular calcium (55). Therefore, the toxicity of the combined treatment observed in our studies, could be due to hypercalcemia caused by the combined effects of CIS and vitamin D analogs on the calcium serum level. Even though the PRI-2205 did not significantly raise the serum calcium level. However, toxicity was not observed previously when the parallel administration of clodronate bisphosphonate, reduced the hypercalcemia caused by vitamin D analogs and prevented deaths of the animals (32, 56). Therefore, in the present work the experimental protocol was changed in the fourth experiment as compared to the second one, so that the increased dose of CIS was only administered three times and was accompanied by the parallel use of clodronate bisphosphonate. Both CIS and PRI-2205 demonstrated notable antitumor activity in this model and their combined administration has also resulted in the additive therapeutic effect. Interestingly, the toxicity of this treatment protocol was not diminished by clodronate. Another vitamin D analog, tacalcitol (PRI-2191), causing hypercalcemia in treated animals, which can be successfully diminished by clodronate bisphosphonate (32), was used as an additional control. However, toxicity of the combined treatment was also observed in the case of this analog. A properly designed experimental protocol could diminish the joint hypercalcemic effect caused by combined treatment with the CIS and vitamin D analogs. In our experiments, continuous administration of CIS resulted in a steady decrease of animal body weights, which gradually returned to normal after discontinuation of drug administration.

Our *in vitro* results showed that the potentiation of the CIS antitumor effect by the vitamin D analogs was more pronounced when the cytostatics were used in rather low concentrations. That was the reason for using low doses of cytostatics in our *in vivo* experiment. One aim was to discover an effective treatment program compensating for the decrease of the cytostatic dose by the addition of a vitamin D analog. The current conclusion based on these initial experiments with the combination of vitamin D analogs and cytostatics in low doses is was that such treatment is not effective *in vivo*, despite the encouraging results from *in vitro* studies. Nevertheless, combined treatment with vitamin D analog and CIS appeared to be more effective than for CIS applied alone, even when the higher dose of cytostatics was used. Further studies examining the optimal doses and schedules of combined treatment with vitamin D analogs and the unexpected toxicity observed in such combined treatment with CIS are currently in progress.

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