Combined Colonic Cancer Treatment with Vitamin D Analogs and Irinotecan or Oxaliplatin

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Abstract. *Aim: The aim of this study was the evaluation of the antitumor effect of two synthetic analogs of vitamin D, PRI-2191 and PRI-2205 in combined treatment with irinotecan or oxaliplatin on mouse (MC38) and human (HT-29) colon cancer cells. Materials and Methods: Mice bearing subcutaneous tumors were injected with vitamin D analogs and with irinotecan or oxaliplatin, according to various schedules. Results: Statistically significant inhibition of MC38 tumor growth by combined therapy was observed. When analogs were used in combined treatment with irinotecan, survival times of mice were significantly prolonged. We also observed improved antitumor effects in combined treatment with oxaliplatin in mice bearing HT-29 tumors, however, antagonism in life span prolongation was observed. Analog PRI-2191 increased the expression of vitamin D receptor (VDR), retinoic X receptor-α (RXRα) and phosphorylated extracellular signal regulated kinase 1/2 (p-ERK1/2) in HT-29 tumors when used alone. VDR and RXRα expressions were up-regulated by PRI-2191 analog, as compared to oxaliplatin alone. Conclusion: The obtained results suggest that vitamin D analogs could be used in combined colonic cancer treatment with irinotecan or oxaliplatin. However, the regulation of ERK1/2 expression by both analogs and oxaliplatin may explain the observed antagonistic interactions.*

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Data from experimental, clinical and epidemiological studies suggest that vitamin D and calcium may reduce colorectal cancer risk *via* various mechanisms, including binding of bile acids and long-chain fatty acids in the small intestine and protecting colon epithelial cells from mutagens. In addition, their influence on cell proliferation, differentiation, apoptosis, angiogenesis and cell-cycle regulation is considered (1-6).

Vitamin D and its analogs regulate gene expression by binding to specific vitamin D receptors (VDR). Upon ligand activation and dimerization with retinoid X receptor (RXR), VDR – RXR heterodimers bind specific nucleotide sequences, vitamin D response elements (VDREs), in target genes to activate or repress their expression (7). A number of vitamin D target genes have been identified in several tumor cell types: p21, E-cadherin, c-Jun N-terminal kinase (JNK), *c-Myc* oncogene, insulin-like transforming growth factor family and their receptors (8, 9).

The expression of VDR is low in normal colonic epithelial cells, increases with malignant transformation and then declines with progressive tumor growth, which is correlated with a decreasing level of VDR in the nucleus, as compared with the cytoplasm. Accordingly, high VDR expression is correlated with a favorable prognosis in patients with colorectal cancer, suggesting the important role of VDR in the pathogenesis of colonic cancer (1, 5, 6, 10). It has also been shown that proliferating HT-29 colonic cancer cells exhibit up-regulation of VDR and induction of 24-hydroxylase mRNA, whereas the differentiated cells fail to exhibit both of these biological responses, when exposed to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (11). Moreover, differentiation of colonic cancer cells induced by different treatments occurs *via* up-regulation of VDR (11, 12). Palmer *et al*. reported that vitamin D analogs promote differentiation only of colon cancer cells expressing VDR, and that this

process is related to induction of E-cadherin and inhibition of β-catenin signaling (13). A positive correlation has also been found between *E-cadherin* and *VDR* gene expression; moreover, expression of zinc-finger transcription factor (SNAIL) was associated with down-regulation of E-cadherin and VDR gene expression in patients with colorectal cancer. In addition, in human colonic cancer cells, SNAIL represses VDR gene expression and blocks the antitumor action of EB1089, a calcitriol analog (14-17).

Other experimental data have shown that dietary vitamin D significantly reduced the incidence of colonic tumors in rats treated with a carcinogen (18); moreover, it has been shown that in mice, vitamin D deficiency results in the aggressive growth of mouse MC-26 colonic cancer (19). These data may suggest that calcitriol or its analogs will be good candidates for combined treatment with chemotherapeutic agents of colonic cancer.

A number of studies on combined treatment with calcitriol or its analogs and different chemotherapeutic agents have been reported both *in vitro* (20-24) and *in vivo* (25;26). Our studies have indicated that a combination of cyclophosphamide or cisplatin with vitamin D analogs (PRI-2191, PRI-1906, PRI-2205 or PRI-2202) resulted in an increase in cytostatic antitumor effect (27, 28). On the basis of *in vitro* antiproliferative activity, the effect on the cell cycle *in vitro*, toxicity and antitumor activity *in vivo*, we selected new vitamin D analogs with favorable biological profiles. Selected analogs PRI-2191 and PRI-2205 are potent inhibitors of cancer cell proliferation both *in vitro* and *in vivo*, with relatively low toxicity (24, 27-30).

In the present study, we analyzed the effect of vitamin D analogs PRI-2191 and PRI-2205 on the *in vivo* antitumor activity of irinotecan and oxaliplatin in mice bearing mouse (MC38) or human (HT-29) colonic cancer.

Materials and Methods

Cells. The mouse colonic carcinoma cell line MC38, cultured *in vivo,* was obtained from the Tumor Bank of the Radiobiology Institute TNO, Rijswijk, the Netherlands. This cell line was adapted to growth *in vitro* as MC38/0 (31). The human colonic cancer cell line HT-29 was received from the Deutsches Krebsforschungszentrum, Heidelberg, Germany. The cell lines were maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

The cell lines were cultured *in vitro* as follows: MC38/0 in RPMI-1640 (IIET, Wroclaw, Poland) and HT-29 in RPMI 1640+Opti-MEM (1:1) (both from Gibco, Scotland, UK) both culture media were supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 5% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland). All cell lines were grown at 37˚C in a humidified atmosphere with 5% CO_2.

Compounds. Prior to usage, the compounds were dissolved in absolute ethanol to a concentration of 10^{-4} M, and subsequently diluted in culture medium to reach the required concentrations (ranging from 1 to 1000 nM).

For animal experiments, compounds were dissolved in 99.8% ethanol, then diluted in 80% propylene glycol to reach the required concentrations, and administered subcutaneously (*s.c.*) to mice in a volume of 50 μl per 10 g of body weight.

Irinotecan or oxaliplatin for *in vitro* (both from Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and oxaliplatin for *in vivo* research (Oxaliplatinum Medac, Hamburg, Germany) were diluted in water for injectionin order to reach the required concentrations, and were administered intraperitoneally (*i.p.*) to mice in a volume of 10 μl per gram of body weight.

Mice. Female, 12-16 week old C57BL/6 mice, weighing 20-25 g, were supplied by the Medical University of Bialystok (Bialystok, Poland). Female 6-8 week-old NOD/SCID and Ncr-nu/nu mice, weighing 20-25 g, were supplied by the University Children's Hospital in Krakow (Poland) and the Medical University of Bialystok (Bialystok, Poland), respectively. Mice were maintained in specific pathogen-free (SPF) 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 (32) and were approved by the First Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland.

Design of the in vivo experiments. Mice were *s.c.* inoculated into the right flank region with 0.25 ml of a 33% suspension of homogenized MC38 tumor tissue in Hanks medium coming from *s.c.* tumors from another mouse.

Human colonic cancer HT-29 cells were harvested with the use of 0.05% trypsin/0.02% EDTA, washed twice with serum-free Minimum Essential Medium (α-MEM) and re-suspended in Hank's medium. A single-cell suspension (3.5×10^6/200 μl per mouse) with cell viability over 90% was inoculated *s.c.* Tumor volume and the body weight of animals were monitored three times a week.

Details of the treatment schedules used. In all experiments, vitamin D analogs were injected *s.c.*, at 1 μg PRI-2191 /kg/dose and 10 μg PRI-2205 /kg/dose.

Calcitriol analogs in combined treatment with irinotecan. Mice bearing MC38/0 mouse colon cancer cells: Irinotecan was injected *i.p.* at a dose of 50 mg/kg on days 8, 15 and 22 (total dose=150 mg/kg) after tumor cell inoculation. PRI-2191 was injected every two to three days from day 10 to 22 (total dose 6 μg/kg) and PRI-2205 from day 10 to 31 (total dose=100 μg/kg).

Mice bearing HT-29 human colon cancer cells: Depending on the experiment schedule, treatment was started on day 7 or 13. In the first experiment, irinotecan was injected *i.p.* at a dose of 10 mg/kg on days: 13, 17, 22, 27, 31, 36 (total dose=60 mg/kg) after tumor cell inoculation. PRI-2191 or PRI-2205 were injected every two to three days from day 13 to 41 (total dose=13 or 130 μg/kg, respectively). The experiment was ended on day 42.

In the second experiment, irinotecan was injected *i.p.* at a dose of 50 mg/kg on days: 7, 14, 21 (total dose=150 mg/kg) after tumor cell inoculation. PRI-2191 or PRI-2205 were injected every two to three days from day 10 to 40 (total dose=14 or 140 μg/kg, respectively). The experiment was ended on day 42.

Calcitriol analogs in combined treatment with oxaliplatin. Mice bearing MC38/0 mouse colon cancer cells: Oxaliplatin was

injected *i.p.* at a dose of 6 mg/kg on days 8, 15 and 22 (total dose=18 mg/kg), or at 12 mg/kg on day 8 after tumor cell inoculation. PRI-2191 was injected every two to three days from day 10 to 22 (total dose=6 μg/kg) or PRI-2205 from day 10 to 31 (total dose=100 μg/kg).

Mice bearing HT-29 human colon cancer cells: Depending on the experiment schedule, treatment was started on day 8 or 12. In the first experiment oxaliplatin was injected *i.p.* at a dose of 2 mg/kg (once a week, from day 8 to 43; total dose=12 mg/kg). PRI-2191 or PRI-2205 were injected every two to three days from day 8 to 50 (total dose=19 or 190 μg/kg, respectively).

In the second experiment oxaliplatin was injected *i.p.* at a dose of 2 mg/kg (three times-a-week, from day 12 to 52; total dose: 36 mg/kg) or at 6 mg/kg (once a week, from day 12 to 47; total dose 36 mg/kg). PRI-2191 or PRI-2205 were injected every two to three days from day 12 to 52 (total dose=18 or 180 μg/kg, respectively). The experiment was ended on day 54.

Some of the animals were scarified on day 27. Tumors were harvested for further analyses.

Evaluation of the therapeutic effect. Tumor volume was calculated using the formula $(a^2 \times b)/2$, where *a*=shortest tumor diameter in millimeters and *b*=longest tumor diameter in millimeters. Inhibition of tumor growth was calculated from the following formula: tumor growth inhibition TGI (%)=$[(W_T/W_C) \times 100] - 100\%$, where W_T is the median tumor volume of treated mice and W_C that of untreated control animals. The antitumor effect *in vivo* was also evaluated as the increase in life span (ILS) of treated mice over that of controls, calculated from the following formula: $[(MST_T/MST_C) \times 100] - 100$, where MST_T is the median survival time of treated animals, and MST_C is the median survival time of untreated control mice.

Evaluation of combination effects. The minimal expected inhibition (H) used to estimate the effect of combination of two compounds was evaluated using the formula: %H=100 – [(100 – E for cytostatic) × (100 – E for calcitriol analog)/100] (33), where E is the TGI or ILS.

Cell-cycle analysis. Cultured HT-29 cells were seeded at a density of 7.5×10^5 cells/ml of culture medium on 6-well plates (Sarstedt AG & Co., Nümbrecht, Germany) to a final volume of 4 ml. The cells were exposed to compounds at set concentrations for 120 h: PRI-2191 and PRI-2205 100 nM; irinotecan 0.75 μg/ml; oxaliplatin 3.8 μg/ml. Ethanol was used as a solvent for all compounds, diluted corresponding to its highest concentration applied to the compounds and it produced no toxicity. After 120 h of incubation, the cells were collected, washed in phosphate-buffered saline (PBS) and counted in a hemacytometer.

Cells (1×10^6) were then washed twice in cold phosphate buffered saline (PBS) and fixed for 24 h in 70% ethanol at –20˚C. The cells were then washed twice in PBS and incubated with RNAse (50 μg/ml, Fermentas GmbH, St. Leon-Rot, Germany) at 37˚C for 1 h. The cells were stained for 30 min with propidium iodide (50 μg/ml; Sigma-Aldrich Chemie GmbH) at 4˚C and the cellular DNA content was determined using a FACS Calibur instrument (Becton Dickinson, San Jose, CA, USA) and ModFit LT 3.0 program (Verity Software House, Topsham, ME, USA).

Westernblot analysis of VDR, RXRα, p27 and extracellular signal regulated kinase (ERK) expression. Specimens of the tumor tissue from euthanized animals were collected in liquid nitrogen, and stored at –80˚C. To determine protein expression by western blot, the collected tumors, frozen at –80˚C, were mechanically homogenized (Rotilabo, Carl Roth, Karlsruhe, Germany) in RIPA buffer (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and then kept on ice for 45 min, supplemented with a complete mixture of phosphatase and protease inhibitors (Sigma-Aldrich Chemie GmbH). Lysates were cleared by microcentrifugation at 17968 rcf ×g for 20 min.

Protein concentrations were determined using a protein assay (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (50 μg for detecting VDR and RXRα; 100 μg for p27; 40 μg for ERK and p-ERK; and 25 μg for actin) were separated in a 10% (VDR, RXRα, ERK1/2, p-ERK1/2, actin) or 15% (p27) sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (0.45 μm; GE Healthcare, Amersham, Little Chalfont, UK) or nitrocellulose membrane (0.22 μm; NitroBind, GE Water and Process Technologies, Osmonics, Hopkins, MN, USA). Protein loading and transfer efficiency were monitored *via* 0.1% Ponceau S-Red staining. Membranes were blocked overnight (4˚C) in 1% blocking reagent (Membrane Blocking Agent; GE Healthcare, Amersham) in PBS. On the following day the membrane was washed three times (×10 min) with 0.05% PBS/Tween-20 (PBST) and then incubated for 1 h at room temperature with primary antibody: rabbit anti-VDR, anti-RXRα, anti-ERK1/2, anti-p-ERK1/2 or anti-p27 polyclonal antibody (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or rabbit anti-actin (Sigma-Aldrich, Poznan, Poland). After incubation, the blot was washed three times with 0.1% PBST and incubated for 1 h with the secondary anti-rabbit immunoglobulins (GE Healthcare, Amersham). The membrane was finally washed three times with 0.1% PBST and incubated for 30 min with a fluorescent substrate for alkaline phosphatase-based detection (ECF; GE Healthcare, Amersham). Fluorescence was detected using a scanner (Typhoon scanner; GE Healthcare, UK). Densitometric analysis of the western blots was carried out using ImageJ 1.46r (National Institutes of Health, Bethesda, MA, USA).

Statistical evaluation. Statistical analysis was performed by employing STATISTICA version 7.1 (StatSoft, Inc., Tulsa, OK, USA). For tumor growth inhibition analysis, the Kruskal-Wallis ANOVA multiple comparison *p*-values (two-tailed) test was used. For survival analysis, the Peto & Peto modification of the Gehan – Wilcoxon test was used. For cell-cycle analysis HSD Tukey test for unequal N was used. *p*-Values less than 0.05 were considered significant.

Results

The effect of vitamin D analogs on the cell-cycle distribution of HT-29 cells treated with irinotecan in vitro. The effect on HT-29 cell-cycle distribution was analyzed after 120 h incubation of cells with irinotecan (0.75 μg/ml) and vitamin D analogs (100 nM). The results of the DNA analysis in FACS are summarized in Figure 1a.

The tendency to increase the percentage of HT-29 cells in the G_0/G_1 stage was observed after incubation with calcitriol or PRI-2191. Simultaneously, a decreased percentage of cells in S and G_2/M stages was observed. However, incubation of these cells with PRI-2205 did not influence the cell cycle. After incubation with irinotecan, a significant decrease in the

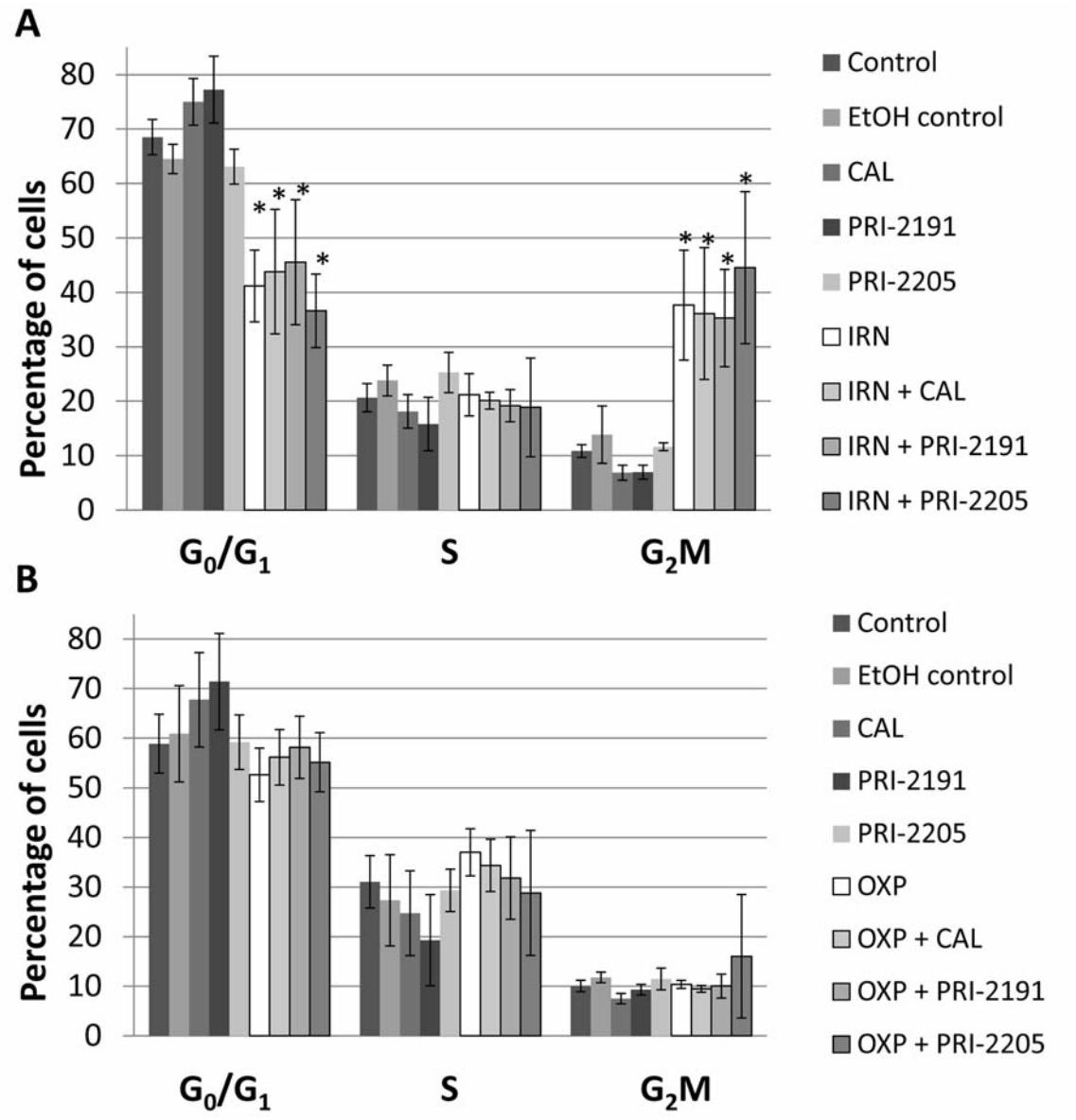

Figure 1. *FACS analysis of the cell-cycle distribution of HT-29 cells incubated with calcitriol or its analogs, alone and combined with irinotecan (IRN) or oxaliplatin (OXP). The results are presented as the mean (±SD) percentage of the cell population attributed to one of the cell-cycle phases: G_0/G_1, S, G_2/M. Analysis was performed using the ModFit LT 3.0 software. N=3-5 in each group. Arrows indicated statistically significant results as compared to control and EtOH group (HSD Tukey test for unequal N).*

percentage of HT-29 cells in G_0/G_1 and an increase in the G_2/M stage was observed in all protocols used. Combined incubation with irinotecan and PRI-2191 showed a tendency for an increase in HT-29 cells in the G_0/G_1 stage, in comparison to irinotecan alone. A similar, but less marked tendency was observed in the case of calcitriol. PRI-2205 increased the percentage of cells in the G_2/M stage compared to irinotecan (Figure 1a). The percentage of cells in sub-G1 did not exceed 4% (data not shown).

The effect of vitamin D analogs on the cell-cycle distribution of HT-29 cells treated with oxaliplatin in vitro. The effect on HT-29 cell-cycle distribution was analyzed after 120 h of cell incubation with oxaliplatin (3.8 μg/ml) and vitamin D analogs (100 nM). The results of DNA analysis in FACS are summarized in Figure 1b.

After incubation with oxaliplatin, a decrease in the percentage of HT-29 cells in the G_0/G_1 stage and an increase in the S stage were observed in all protocols used. Combined

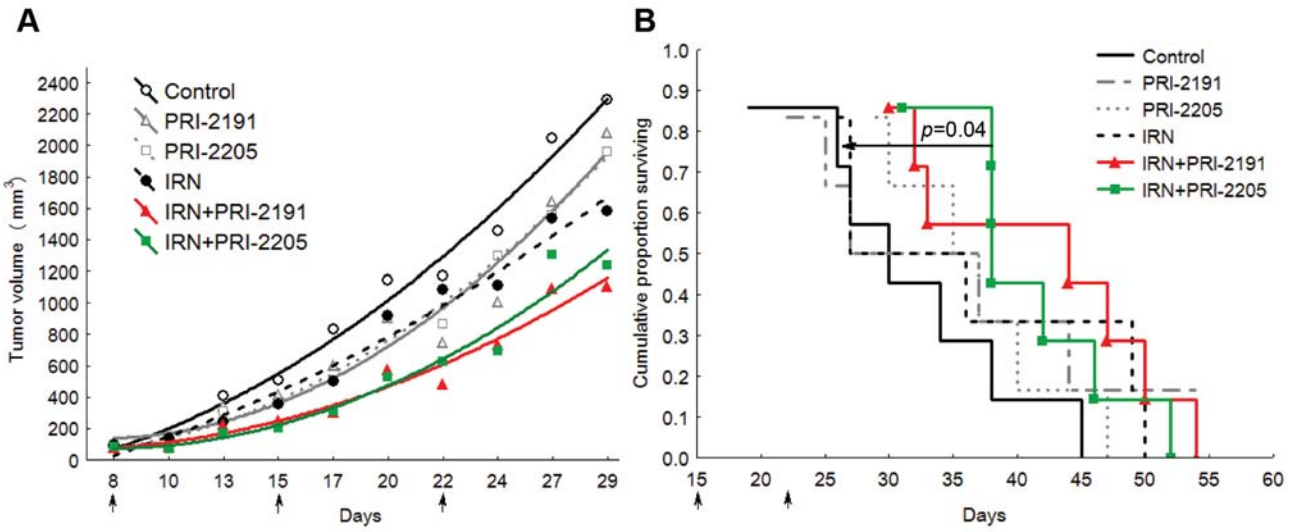

Figure 2. *Kinetics of tumor growth (A) and survival analysis (B) of mice bearing MC38 colonic cancer treated with a combination of irinotecan (IRN) with PRI-2201 or PRI-2205. Black arrows indicate the days of IRN injection. Vitamin D analogs were injected s.c., three times per week, PRI-2191 was injected from day 10 to 22 (total dose=6 μg/kg) or PRI-2205 was injected from day 10 to 31 (total dose=100 μg/kg). IRN was injected i.p. at a dose of 50 mg/kg/dose (total dose=150 mg/kg) after tumor cell inoculation. Statistical analysis: (A) Kruskal-Wallis. (B) Peto and Peto modification of the Gehan - Wilcoxon test (p<0.05, IRN+PRI-2205 as compared to control). The initial number of mice was 7 in the control, IRN+PRI-2191 and IRN+PRI-2205 groups and 6 in the PRI-2191, PRI-2205 and IRN groups.*

incubation with oxaliplatin and calcitriol or its analogs showed a tendency for an increase of HT-29 cells in the G_0/G_1 stage in comparison to oxaliplatin alone. Similarly, a tendency to for a decrease in cells in the S phase, as compared to oxaliplatin-alone was observed in the case of calcitriol and its analogs (Figure 1b). The percentage of cells in sub-G1 phase did not exceed 5% (data not shown).

The effect of PRI-2191 and PRI-2205 administration alone and in combination with irinotecan. One mouse colonic cancer MC38 growth *in vivo*, both analogs retarded tumor growth as compared to the untreated control and the irinotecan-treated group. For example, on the 24th day of the experiment, the TGI of irinotecan-alone was 24%, whereas combined treatment with PRI-2191 inhibited tumor growth by 41% as compared to the control, and that with PRI-2205 by 52%. The difference in tumor volume between mice treated with irinotecan combined with PRI-2205 as compared to the control was statistically significant from day 17 to 24. Analysis of interaction between these compounds showed there to be an additive effect, which became changed to synergic from the 24th day of the experiment to the end of the measurement (Figure 2a).

Irinotecan combined with PRI-2191 prolonged the survival time of mice by 47%, while for that when combined with PRI-2205 by 27%, whereas used alone it prolonged survival by 5%. This indicated synergy and an additive effect, respectively, in life span prolongation by vitamin D analogs (Table I). The cumulative proportion of surviving mice treated with irinotecan combined with PRI-2205 was significantly higher than that of control mice (Figure 2b).

Table I. *Median survival time (MST) of mice bearing MC38 tumors, treated with vitamin D analogs alone or combined with irinotecan (IRN).*

Groups	MST (days)	ILS			N
		%	%H	Effect	
Control	30	-			7
PRI-2191	32	7			6
PRI-2205	36	20			6
IRN	32	5			6
IRN + PRI-2191	44	47	11	Synergistic	7
IRN + PRI-2205	38	27	24	Additive	7

ILS: Increase in life span. The minimal expected inhibition (H): %H=100-[(100-E for cytostatic) × (100-E for calcitriol analog)/100], where E represents the ILS; N: number of mice.

Transient impairment of body weight by up to 14% was observed only in mice treated with PRI-2191 alone or combined with irinotecan (data not shown).

Human colonic cancer HT-29 growth in vivo. In this series of experiments, we did not confirm the observations from *in vitro* studies. Analogs of vitamin D used, did not improve the

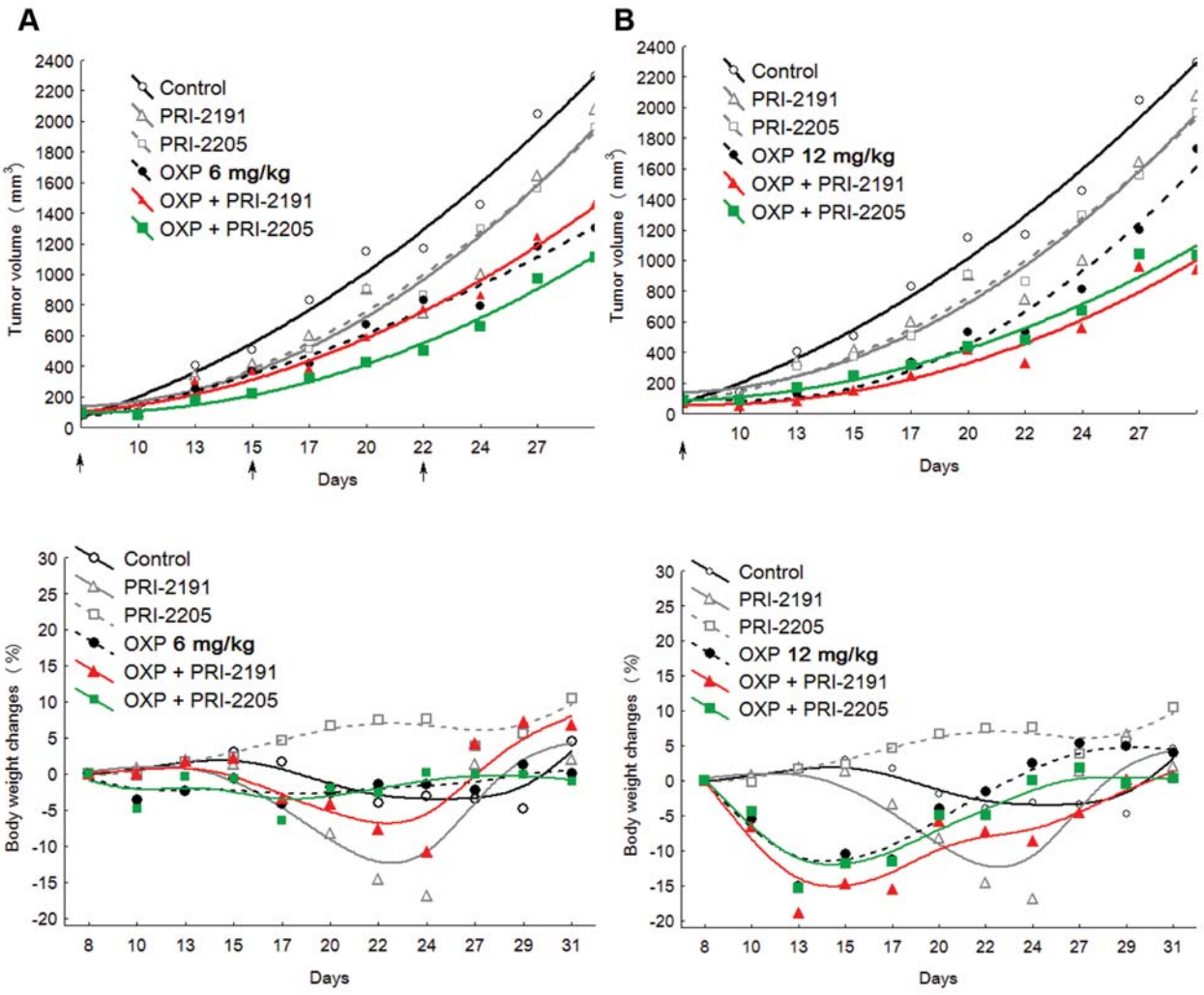

Figure 3. *Kinetics of tumor growth in mice bearing MC38 colon cancer treated with a combination of oxaliplatin (OXP) and vitamin D analogs. The lower panels illustrate body weight changes of animals. A: Multiple-dose administration of OXP. B: Single-dose treatment with OXP. Black arrows indicate the days of OXP injection. OXP was injected i.p. at a dose of 6 mg/kg/dose (total dose=18 mg/kg) (A) or 12 mg/kg on day 8 after tumor cell inoculation (B). Analog PRI-2191 was injected s.c. three times per week from day 10 to 22 (total dose=6 μg/kg) and PRI-2205 from day 10 to 31 (total dose=100 μg/kg). Statistical analysis: Kruskal-Wallis multiple comparison test. Number of mice were: 7 in the control, OXP, OXP+PRI-2191 and OXP+PRI-2205 groups and 6 in the PRI-2191 and PRI-2205 groups.*

antitumor potency of irinotecan in the human colon cancer model (data not shown).

The effect of PRI-2191 and PRI-2205 administration alone and in combination with oxaliplatin. Mouse colonic cancer MC38 growth in vivo: PRI-2191 and PRI-2205 improved oxaliplatin anticancer activity only in a single dose of 12 mg/kg/day. PRI-2205 also improved the antitumor activity of oxaliplatin at the lower, multiple dose. This effect was calculated as being additive. However, the treatment protocol using a single 12 mg/kg dose caused toxicity, manifested by body weight loss reaching 15% in mice treated with oxaliplatin alone, and 19% or 15% when combined with PRI-2191, and PRI-2205, respectively (Figure 3). Statistical analysis showed that significant ($p<0.05$) inhibition of tumor growth was observed only in mice treated with oxaliplatin at 12 mg/kg combined with PRI-2191 (on days 10-17 and 24) or with PRI-2205 on days 20 and 22. Further statistical analysis was not valid because on the 27th day, only four out of seven control mice remained alive.

Analyzing ILS with the use of the formula computing the degree of influence of combined treatment, antagonism between vitamin D analogs and oxaliplatin in both protocols was demonstrated (Table II).

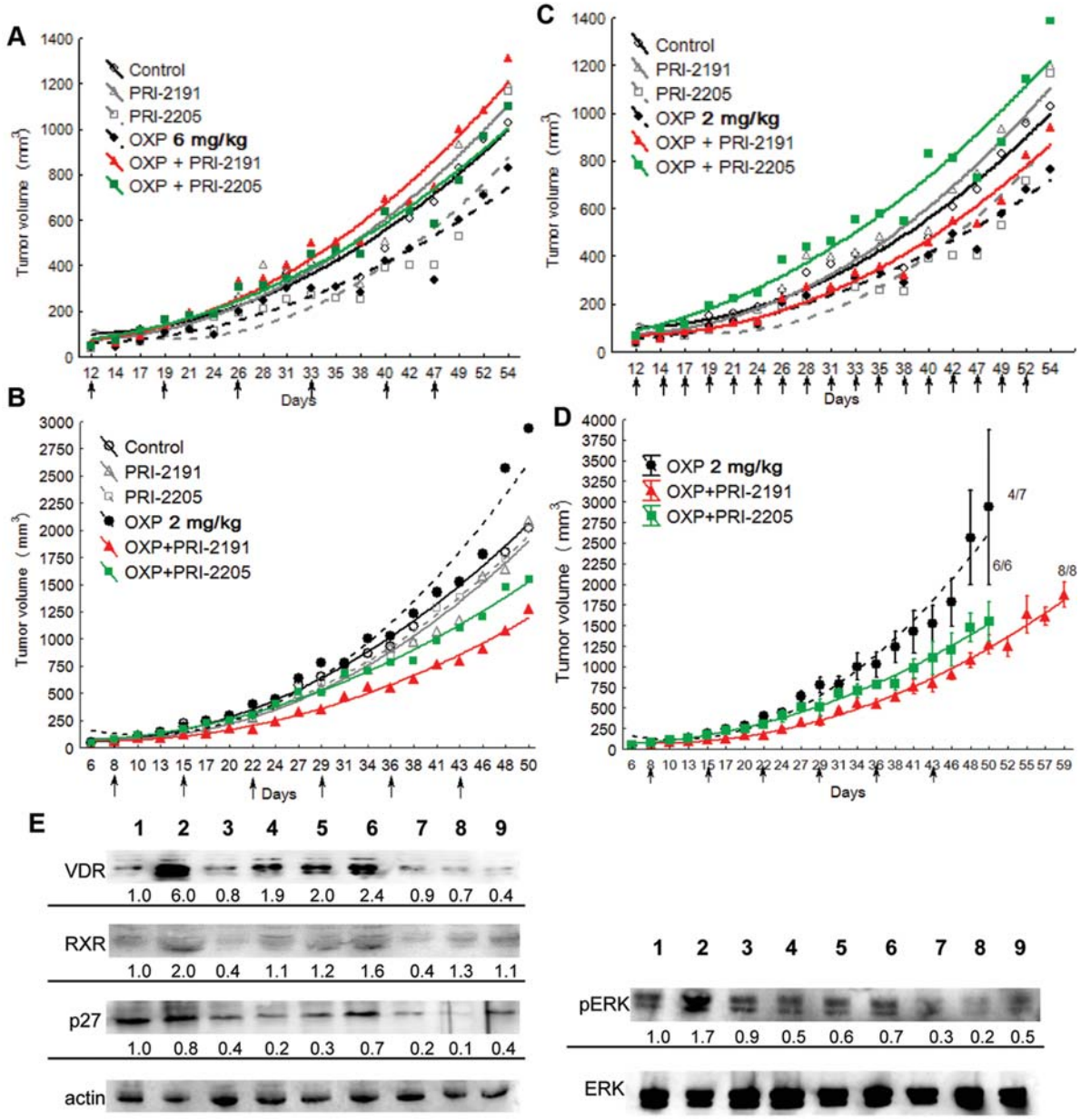

Figure 4. *Kinetics of tumor growth in mice bearing HT-29 human colonic cancer, treated with a combination of oxaliplatin (OXP) and vitamin D analogs. A: Once-a-week administration of 6 mg/kg of oxaliplatin. B: Once-a-week administration of 2 mg/kg of oxaliplatin. C: Three times-a-week administration of 2 mg/kg of oxaliplatin. Black arrows indicate the days of oxaliplatin injection. D: Effect of combined treatment with oxaliplatin and PRI-2191 or PRI-2205 on HT-29 tumor growth, selected groups from data in Figure 4B. E: Vitamin D receptor (VDR), retinoid X receptor α (RXRα), p27 and extracellular signal regulated kinase (ERK) protein expression in tumors harvested from mice treated with oxaliplatin at doses of 2 (three times-a-week) or 6 mg/kg (once-a-week). Depending on the experiment schedule, treatment was started on day 12 or 8. A: Oxaliplatin was injected i.p. at the dose of 6 mg/kg (once-a-week, from day 12 to 47; total dose=36 mg/kg). Analogs PRI-2191 and PRI-2205 were injected three times a week from day 12 to 52 (total dose=18 or =180 μg/kg, respectively). B: Oxaliplatin was injected i.p. at 2 mg/kg (once a week, from day 8 to 43; total dose=12 mg/kg). Analogs PRI-2191 and PRI-2205 were injected three times a week from day 8 to 50 (total dose=19 or =190 μg/kg, respectively). C: Oxaliplatin was injected i.p. at a dose of 2 mg/kg (three times-a-week, from day 12 to 52; total dose=36 mg/kg). Analogs PRI-2191 or PRI-2205 were injected three times-a-week, from day 12 to 52 (total dose=18 or =180 μg/kg, respectively). The numbers in Figure 4D (4/7, 6/6, 8/8) indicate the number of mice which were alive on the last day of observation for each group. After day 50, mice with tumors which exceeded a volume of 2 cm^3 were sacrificed. The mice from groups treated with oxaliplatin and PRI-2191 were sacrificed on day 71. For western blot analysis, the cells were prepared from HT-29 tumors growing subcutaneously in mice (Figure 4A and C). Representative immunoblots are presented. Densitometric analysis was carried out using ImageJ 1.46r. (http://imagej.nih.gov/ij/). All blots were normalized to that of actin, except for pERK which was normalized to ERK, and the fold-change protein level expression (under each blot) is reported in comparison to that of the control (lane 1). 1: Control; 2: PRI-2191; 3: PRI-2205; 4: OXP 6 mg/kg; 5: OXP 6 mg/kg+PRI-2191; 6: OXP 6 mg/kg + PRI-2205; 7: OXP 2 mg/kg; 8: OXP 2 mg/kg + PRI-2191; 9: OXP 2 mg/kg + PRI-2205.*

Human colon cancer HT-29 growth in vivo. Once a week administration of oxaliplatin. When oxaliplatin was used at the higher dose (6 mg/kg), starting from day 12 of the experiment, neither analog affected tumor growth (Figure 4a). Oxaliplatin-alone used at this dose retarded tumor growth, but not in a significant manner. Maximal body weight decrease in animals treated by oxaliplatin was 10%, combined with PRI-2205 – 12% and with PRI-2191 – 14% (data not shown).

Oxaliplatin used at a dose of 2 mg/kg/day from day 8 after tumor inoculation did not affect HT-29 tumor growth. However, when vitamin D analogs were included in the treatment protocol, tumor growth retardation was statistically significant, as compared to oxaliplatin-alone in days 17, 22, 24, 27, 29 for PRI-2191. On the 46th day of the experiment, combined treatment with oxaliplatin and PRI-2191 led to a TGI of 49%, and with PRI-2205 of 32%. Analyzing the type of interaction between oxaliplatin and PRI-2191, we found synergism, but between oxaliplatin and PRI-2205, only a sub-additive effect (Figure 4b). For better analysis of this experiment, Figure 4d shows curves of tumor growth in groups treated with oxaliplatin alone and in combination with vitamin D analogs. Body weight did not change during the whole experiment.

Three times a week administration of oxaliplatin. Oxaliplatin at a dose of 2 mg/kg/dose was also administered in the same schedule as vitamin D analogs. Unfortunately, such a treatment schedule failed to improve oxaliplatin activity *via* vitamin D analogs (Figure 4c).

The effect of PRI-2191 and PRI-2205 administration alone and in combination with oxaliplatin on VDR, RXRα, p27 and ERK expression in tumors of human colonic cancer HT-29 cells. Some of the animals from experiments shown in Figure 4a and c were euthanized on the 27th day and the tumors were obtained for further analyses.

The results of the western-blot studies showed that in tumors from mice treated with PRI-2191 alone, the expression of VDR, RXRα and phosphorylated ERK1/2 (p-ERK1/2) was increased as compared to tumors from control animals. PRI-2205 alone seemed to have a different activity, reducing the expression of RXRα. In tumors from mice treated with oxaliplatin at a dose of 6 mg/kg starting at day 12, we observed only a tendency for an increase in the expression of VDR and RXRα in tumors from mice treated with oxaliplatin and both analogs. Different results were observed when oxaliplatin was used at a dose of 2 mg/kg and starting earlier, at day 8. In this group, the expression of VDR, RXRα and p-ERK1/2 was diminished as compared to the control. However, when oxaliplatin was used in combined treatment with PRI-2191 or PRI-2205, the tendency to increase RXRα expression was observed. In the case of p-ERK1/2 the two analogs seem to act differently: PRI-2191 reduced, while PRI-2205 slightly increased expression, as compared to oxaliplatin-alone (Figure 4e).

Table II. *Median survival time (MST) of mice bearing MC38 tumors, treated with vitamin D analogs alone or combined with oxaliplatin (OXP) (at a dose of 6 or 12 mg/kg/day).*

Groups	MST (days)	ILS		N
		%	Effect	
Control	30	-		7
PRI-2191	32	7		6
PRI-2205	36	20		6
OXP 6	45	50		7
OXP 6 + PRI-2191	43	43	Antagonistic	7
OXP 6 + PRI-2205	42	40	Antagonistic	7
OXP 12	42	40	-	7
OXP 12 + PRI-2191	37	23	Antagonistic	7
OXP 12 + PRI-2205	36	20	Antagonistic	7

ILS: Increase in life span; N: number of mice.

The expression of cyclin-dependent kinase inhibitor (CDKI) p27, was also analyzed. Both analogs used alone reduced its expression. Moreover, the expression of this protein was diminished in tumors from mice treated with oxaliplatin. PRI-2205, in contrast to PRI-2191, increased the level of p27 in mice treated with combined treatment protocols, as compared to oxaliplatin alone (Figure 4e).

Discussion

Apart from the activity of calcitriol analogs as single agents, they exert anticancer activities by synergizing with chemotherapy drugs (20, 34). Our present experiments were conducted in mice bearing human or mouse colonic cancer cells. To choose the best experimental conditions, we performed a series of experiments with single and multiple doses of irinotecan and of oxaliplatin. We concluded that under certain experimental conditions, calcitriol analogs can interact synergistically with irinotecan and oxaliplatin. Moreover, we observed the importance of the schedule, as well as the time of oxaliplatin administration. Better results were observed when the oxaliplatin administration was started earlier (from day 8 after tumor transplantation) and continued longer (until the 43rd day), once a week.

Recently published epidemiological studies have shown a correlation between the season in which certain types of cancer are diagnosed or resected and subsequent survival. Diagnosis in the summer and autumn months has been associated with better survival in most of these studies (35, 36). It has been suggested that exposure to sunlight and the subsequent higher levels of cutaneous vitamin D synthesis at

the time of diagnosis and/or treatment might be the basis of the improved survival of patients (35-37). Under our experimental conditions, we observed a 7% and 20% prolongation of life span in mice treated with PRI-2191 and with PRI-2205, respectively. Moreover, when combined treatment with irinotecan was applied, both analogs prolonged the survival time of mice as compared to irinotecan alone (Table I, Figure 2b). Unfortunately, oxaliplatin seems not to be such a good partner for combined anticancer treatment with vitamin D analogs, especially when used at higher dosages. Although under optimal experimental conditions we were able to observe improved retardation of tumor growth using calcitriol analogs (Figure 4d), for example at the last day of tumor measurement (59th day), all animals from the group treated with oxaliplatin combined with PRI-2191 had survived (Figure 4d), at higher doses we observed antagonism in ILS between oxaliplatin and vitamin D analogs.

Our previous results, as well as literature data, have shown that vitamin D enhanced the antitumor effect of cisplatin (27, 29, 38-40). However, combined treatment with cisplatin and vitamin D analogs caused an unexpected toxicity manifested by body weight loss, leukopenia and hypercalcemia (27). Toxicity of the combined treatment with oxaliplatin was observed in our studies only at the highest doses of oxaliplatin. Oxaliplatin is a drug that is better tolerated than cisplatin, especially in terms of nephrotoxicity, but it displays a characteristic pattern of neurotoxicity (41). This agent is a component of FOLFOX (5-fluorouracil, folinic acid and oxaliplatin) and FOLFOXIRI (5-fluorouracil/ leucovorin, oxaliplatin, and irinotecan) colon cancer treatment protocols used in clinics (42). Some clinical findings showed that parallel use of calcium and magnesium infusions were able diminish the neurotoxic effects of oxaliplatin (43). One of the mechanisms leading to neurotoxicity of oxaliplatin treatment is calcium chelation by the oxaliplatin metabolite oxalate and subsequent blocking of calcium-sensitive voltage-gated sodium channels (44). Although in our previous studies PRI-2205 did not significantly raise the serum calcium level (30), PRI-2191 administration led to a moderate increase in its level (29). A result of the up-regulation of serum calcium by PRI-2191 may be the better general condition of mice and prolonged anticancer effect of combined treatment by this analog and oxaliplatin, particularly at the optimal dose and schedule, by which mice treated with both agents survive until day 71 of the experiment, whereas the mice from the remaining groups were euthanized earlier because of large tumors and general poor condition (Figure 4d).

Increases in the sensitivity of colonic cancer cells to the cytotoxic effect of irinotecan or oxaliplatin by calcitriol or its analogs could be related to various activities of calcitriol, used as a single agent balanced between pro- and anti-apoptotic pathways described earlier (30, 45-47). In particular, calcitriol increased the level of the pro-apoptotic protein Bak (Bcl-2 family member) (45), and reduced the anti-apoptotic activity of β-catenin (13). It has also been shown that the target gene of *p53 – p21*$^{WAF1/CIP1}$ is a primary calcitriol-responding gene with VDR-binding promoter regions, in which p53 also co-localizes (8). Moreover, the p27 protein levels were observed to be significantly enhanced in mammary gland tumors derived from mice treated with the PRI-2191 analog (29). A previous study by Chen *et al*. on Caco-2 human colonic cancer cells demonstrated that calcitriol increased *c-JUN* gene expression and induced rapid protein kinase C (PKC)-dependent activation of ERK2 and JNK1 (Jun N-terminal kinase). Moreover, calcitriol increased transcription factors, and activator protein-1 (AP-1) transcriptional activities in an ERK- and JNK-dependent manner, which played an important role in stimulating cell differentiation (48).

Analysing HT-29 tumors, harvested from mice treated with oxaliplatin and vitamin D analogs, we can conclude that the expression of VDR and RXRα is present in cells from all tumors independently of treatment schedule. However, it has been shown that in tumors from mice treated with PRI-2205, the expression of both receptors is lower, especially when compared with the expression in tumors from mice treated with PRI-2191. Moreover, the analog PRI-2191 increased the levels of p-ERK1/2, which is not observed in tumors from mice treated with PRI-2205 (Figure 4e). This could suggest different mechanisms of action for these two analogs. Previously, we showed that PRI-2205 appeared to be less potent in the induction of cancer cell differentiation *in vitro,* as compared to calcitriol and PRI-2191 (30). PRI-2205 caused apoptosis of HL-60 cells at a dose of 10 nM, but at a higher dose (100 nM) it caused cell differentiation; however, prostate cancer LNCaP cells accumulated in the G_0/G_1 stage after incubation with PRI-2205. Moreover, in the case of MCF-7 breast cancer and CCRF/CEM leukemia cell lines, PRI-2205, in contrast to all other analogs, increased the accumulation of cells in the G_2/M stage (30). Our present *in vitro* studies on HT-29 cells showed that PRI-2205 did not influence the cell cycle, whereas PRI-2191, similarly to calcitriol, increased the number of cells in the G_0/G_1 phase (Figure 1), which is in accordance with the results of ERK1/2 expression from *in vivo* studies, and suggests that this mechanism of action of PRI-2191 is related to cell differentiation. In *in vitro* culture regarding combined treatment with PRI-2191, similarly to control vitamin D compounds and oxaliplatin, there was a tendency for an increase in the number of cells in G_0/G_1, and a decrease in S phase cells, as compared to oxaliplatin-alone. In contrast, PRI-2205 diminished the number of cells in the S phase and increased those in G_2/M phase. However, analyzing tumors from mice treated with both agents in combined treatment

protocols, and unlike PRI-2191, PRI-2205 somewhat abrogated the oxaliplatin-induced reduction of the level of the p27 protein, but in a manner not exceeding control values. Interestingly, when we analyzed the results of protein expression in tumors from mice treated with oxaliplatin-alone and combined with both analogs, we observed that the reduction of expression of RXRα by oxaliplatin was reversed by the use of both analogs. A tendency to increase VDR expression was only observed in groups treated with oxaliplatin at 6 mg/kg/day and both analogs. However, the phosphorylation of ERK1/2, reduced by oxaliplatin, was further reduced by PRI-2191. This is rather surprising in the context of the results of p-ERK1/2 levels in tumors from mice treated with PRI-2191 alone. PRI-2205 slightly increased the phosphorylation level of this protein as compared to oxaliplatin-alone. Mitogen-activated protein kinases are serine/threonine kinases that play an important role in signal transduction from the cell surface to the nucleus. Amongst them, the ERK cascade is mainly involved in the regulation of cell proliferation in a variety of cells. Studies by Wang *et al*. suggest that oxaliplatin-induced ERK inactivation is involved in oxaliplatin-induced apoptosis (49). On the other hand, the function of VDR and RXRα are modulated by phosphorylation mediated through ERK1/2, and there are several possible mechanisms by which phosphorylation of these receptors could modulate transcription (50). This could be a reason for the observed lower expression of VDR, as well as of the RXR receptor, in tumors from mice treated with oxaliplatin. Moreover, inhibitors of ERK1/2 signaling can affect the activity of calcitriol on gene transcription. This could explain the antagonistic effects observed in some combination protocols.

Conclusion

Based on the above observations, we conclude that calcitriol analogs could, under specific conditions, be good partners for irinotecan or oxaliplatin in the treatment of colonic cancer. However, differences in the sensitivity of particular cancer cells to such treatment, as well as the effects of particular protocols, need further analyses. It seems possible that the balance between the inhibitory activity of oxaliplatin on ERK phosphorylation and the pro-differentiating activity of PRI-2191 could be responsible for differences in their antitumor activity in the various treatment protocols used. However, based on the results presented here, we are not able to propose the mechanism of interaction between oxaliplatin and PRI-2205.

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