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Correlation between VDR Expression and Antiproliferative Activity of Vitamin D_3 Compounds in Combination with Cytostatics

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Abstract. *Calcitriol is a potent antiproliferative agent against various tumour cells in vitro. Its biological activity is mediated by the vitamin D receptors (VDRs). Here, we present the results of a study on vitamin D_3 compounds (calcitriol and its analogue PRI-2191) as potential agents in combined antitumour therapy in vitro. Applying antiproliferative SRB and MTT assays, we measured the growth inhibitory effects of vitamin D compounds applied alone or in combination with either cisplatin or doxorubicin. Next, we examined the correlation of this effect with the presence of nVDR (nuclear VDR). The following cancer cell lines were applied: HL-60 (human leukaemia), SW707 (human colon cancer), A549 (human lung cancer), WEHI-3 (mouse leukaemia). The treatment of tumour cells with the combination of vitamin D compounds and cytostatics decreased the inhibitory concentration 50% (IC_{50}) values compared with the effects of cytostatics applied alone. The synergistic effect was positively correlated with nVDR expression.*

Calcitriol, the steroid hormone, and its numerous analogues have been proven to be potent antiproliferative agents against various normal and neoplastic cells *in vitro* (1). They also have been shown to induce differentiation of human promyelocytic leukaemia, breast cancer, colon adenocarcinoma and prostate cancer cells, as well as of normal or psoriatic keratinocytes (2-5). In addition to its antiproliferative and differentiation-inducing effects, calcitriol induces apoptosis in a number of different cancer cell lines *in vitro*. Moreover, inhibition of angiogenesis and regression of primary tumours have been observed in tumour-bearing animals treated with calcitriol (6).

The biological activity of calcitriol is mediated by the vitamin D receptor (VDR), which is the only nuclear receptor that binds the biologically most active vitamin D metabolite – calcitriol – with high affinity. This classifies the VDR into the classical endocrine receptor subgroup of the nuclear receptor superfamily (7). The VDR acts as a ligand-inducible transcription factor (1). It is localized mainly in the cell nucleus, however a portion of the receptor resides in the cytosol, where it co-localizes with the endoplasmic reticulum, Golgi complex and microtubules (8). The liganded VDR regulates the rates of transcription of key target genes involved directly or indirectly in calcium and phosphate regulation. This role of vitamin D_3 and of the VDR is well-established (1, 9). The VDR has also been detected in a wide variety of target tissues and cell types not involved in calcium and phosphate regulation, *e.g.* in the skin, muscle, prostate, breast, colon, thymus and haematopoietic cells (1).

Such biological properties suggest the potential therapeutic applications for calcitriol and its analogues, including antitumour therapy. Unfortunately, the calcemic activity of calcitriol excludes its use in clinics. One of the promising, low hypercalcemia-inducing analogues, PRI-2191, has been the object of our intensive studies (10). We have shown its antiproliferative and antitumour effects against numerous human and mouse cancer cell lines (11, 12).

Abbreviations: VDR, vitamin D receptor; PRI-2191, new analogue of vitamin D; IC_{50}, inhibitory concentration 50%; CISP, cisplatin; DOX, doxorubicin; SD, standard deviation.

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In this work, the immunocytochemical method of nuclear VDR (nVDR) detection with the use of monoclonal antibody was applied. The antiproliferative effect of calcitriol was positively correlated with nVDR expression in cancer cells.

Materials and Methods

Compounds. Calcitriol [1α,25$(OH)_2D_3$] and its side-chain modified analogue, PRI-2191, [(1*S*,24*R*)-1,24-$(OH)_2D_3$] were used. The compounds were obtained from the Pharmaceutical Research Institute, Warsaw, Poland. Samples of the compounds were stored in amber ampoules, under argon, at –20˚C. The amount of each compound was determined by UV spectrometry (Carl Zeiss spectrophotometer, Jena, Germany) at 265 nm. Prior to usage, the compounds were dissolved in absolute ethanol to the concentration of 10^{-4} M, and subsequently diluted in culture medium to reach the required concentrations.

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

Antibodies. Primary antibody: MoAb Anti-VDR 9A7 - rat anti-chick monoclonal anti-vitamin D receptor antibody - IgG_{2b} (Affinity BioReagents, Inc., Golden, USA) was used for immunocytochemical analysis (stock concentration 5 mg/ml). This antibody was found to cross-react with the calcitriol receptor of mammals, but does not show cross-reactivity with oestrogens and glucosteroids receptors (13). *Secondary antibody:* Biotinylated mouse anti-rat antibody IgG_{2b} (Sigma-Aldrich Chemie GmbH).

Immunocytochemical analysis of vitamin D receptors distribution. $1x10^5$/ml cells were incubated for 96 h with 100 nM of calcitriol or its analogue PRI-2191 or with culture medium. After that, cells were counted and $2.5x10^5$/ml cells per slide were centrifuged for 10 min in a Cytospin centrifuge (Shandon Southern, Astmoor, Cheshire, UK) and collected on glass slide (Fisher Scientific, Springfield, NY, USA), then fixed in 4% paraformaldehyde (POCH, Gliwice, Poland) with 0.1% Triton X-100 (Sigma-Aldrich Chemie GmbH) at room temperature for 15 min, before treatment with 0.1% Triton X-100 alone for 30 min. VDR was detected using the streptavidin-peroxidase technique described previously (14). Briefly, endogenous peroxidase was blocked by incubation with 1% H_2O_2 for 30 min and then the cells were incubated overnight at 4˚C with anti-VDR antibody (1:10-1:100). Later, they were washed and incubated with secondary biotinylated mouse anti-rat antibody (1:200) for 30 min, followed by peroxidase conjugated streptavidin (DAKO Corporation, CA, USA). Receptors were visualized by immersion in 0.1 M TRIS (POCH, Gliwice, Poland) containing (0.5%) diaminobenzidine (DAKO Corporation, CA, USA) with (1%) hydrogen peroxide. Control slides were incubated without primary anti-rat MoAb, but with mouse anti -IgG_{2b}, according to the manufacturer's instructions. Cells of human healthy ileum were used as a positive control.

Antiproliferative assays in vitro. The *in vitro* cytotoxic effect of all agents was examined after 72-h exposure of the cultured cells to varying concentrations of the tested compounds, using the SRB assay for adherent cells (SW707, A549), as described by Skehan *et al.* (15) or the MTT assay for leuakemia cells (HL-60, WEHI-3), as described by Marcinkowska *et al.* (16).

The SRB assay: The cells attached to the plastic were fixed by gently layering cold 50% trichloroacetic acid (TCA, Sigma-Aldrich Chemie Gmbh) on the top of the culture medium in each well. The plates were incubated at 4˚C for 1 h and then washed 5 times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (Sigma-Aldrich Chemie Gmbh) and dissolved in 1% acetic acid (POCH) for 30 min. Unbound dye was removed by rinsing (4x) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered TRIS base (POCH) for determination of optical density (at 540 nm wavelength) in a computer-interfaced, 96-well microtitre plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland).

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

The results were presented as an IC_{50} (inhibitory concentration 50%) – the concentration of tested agent which inhibits the proliferation of 50% of the cancer cell population. IC_{50} values were calculated separately for each experiment. Each compound at every concentration was tested in triplicate in a single experiment, which was repeated 3-7 times. Ethanol, used as a solvent, did not exert any inhibitory effect on cell proliferation ($p<0.05$).

Graphical data evaluation. The graphs presented in this work show inhibition of cell proliferation (%) caused by the cytostatic used alone (first line) or in combination with calcitriol or PRI-2191 (second line), according to Peters *et al.* (17). The third line on the graph expresses the hypothetical inhibition of proliferation by the two agents, calculated according to following formula:

$$\%H = \%CYT + (\%CYT \times (\%VIT/100)),$$

Table I. *Antiproliferative activity in vitro of 10 nM calcitriol and PRI-2191 correlated with the VDR expression.*

Cell line	VDR expression before incubation	VDR expression after incubation with:		% of cell growth inhibition after incubation with: ± SD	
		Calcitriol (10 nM)	PRI-2191 (10 nM)	Calcitriol (10 nM)	PRI-2191 (10 nM)
WEHI-3	+	++	+++	53.5±9.2	60.5±2.1
HL-60	+	++	+++	35.8±10.6	41.8±10.3
A549	–	+	++	1±1.5	1±0.6
SW707	–	+	++	1±0.5	0.3±1

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

Cell line	IC_{50} for cisplatin (μg/ml ± SD)		
	alone	with calcitriol	with PRI-2191
WEHI-3	0.055±0.005	0.002±0.001*	0.001±0.000*
HL-60	0.230±0.060	0.126±0.040*	0.011±0.050*†
A549	1.725±0.392	1.907±0.416	1.855±0.643
SW707	1.840±1.138	1.744±1.107	1.768±1.098

* $p<0.05$ combined treatment *versus* cisplatin alone.
† $p<0.05$ combined treatment with calcitriol *versus* combined treatment with PRI-2191.

where: %H = hypothetical inhibition of cell proliferation by both agents applied together; %CYT = inhibition of proliferation by cytostatic alone; %VIT = inhibition of proliferation by calcitriol or PRI-2191 alone.

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

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

Results and Discussion

Immunocytochemical analysis of vitamin D receptors distribution. As demonstrated by light microscopy, nVDR is localized in the cell nucleus of healthy human ileum cells (MoAb 1:100), keratinocytes and T-cells (18, 19). The presence of nVDR in cancer cells was detected at significantly higher concentrations (1:10) of monoclonal antibody. In our research, the specific reaction of the calcitriol receptor was also evidenced in the nucleus of cancer cells. Further, the influence of calcitriol, or its analogue PRI-2191, on the expression of nVDR was examined. The increase of the VDR expression after incubation with either of the tested vitamin D compounds was observed in all cell lines. The results are summarized in Table I.

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

Cell line	IC_{50} for doxorubicin (μg/ml ± SD) applied		
	alone	with calcitriol	with PRI-2191
WEHI-3	0.058±0.070	0.011±0.001	0.020±0.068
HL-60	0.084±0.010	0.040±0.010*	0.022±0.002*
A549	0.213±0.045	0.166±0.059	0.209±0.040
SW707	0.941±0.422	1.160±0.607	1.023±0.551

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

Treatment with either calcitriol or PRI-2191 applied alone. The results of the studies on the antiproliferative activity of either calcitriol or PRI-2191 applied alone are summarized in Table I. HL-60 and WEHI-3 cells, which are VDR-positive, were the most sensitive to the inhibitory effects of both calcitriol and its analogue. This fact suggests that higher antitumour activity *in vitro* is positively correlated with the VDR expression in tumour cells.

Combined treatment with cytostatics and either calcitriol or PRI-2191. The results of the combined treatment with vitamin D compounds and cisplatin or doxorubicin are presented in Tables II and III, respectively. A few-fold decrease of both cytostatics' IC_{50} values was observed after pre-incubation of the VDR-expressing cells (WEHI-3 and HL-60) with both calcitriol and its analogue. The most significant decrease of IC_{50} value was observed for the combination of cisplatin with PRI-2191 against HL-60 and WEHI-3 cells (Figures 1, 2). Combined treatment with PRI-2191 and doxorubicin was synergistic only at lower concentrations of the cytostatics (Figures 3, 4) applied on both cell lines. These data show that significant synergistic effects occur in cells with a high level of VDR expression. It should be stressed that PRI-2191 was more effective in the combined treatment with cytostatics than calcitriol (Table II).

On the other hand, there were no changes in IC_{50} values for the combined therapy against the A549 and SW707 cell lines, which means that there is no synergistic action between vitamin D compounds and cytostatics on VDR-negative cells.

These results suggest that the synergistic effect of cytostatics and vitamin D compounds on inhibition of cell proliferation depends on the sensitivity of the cells to the

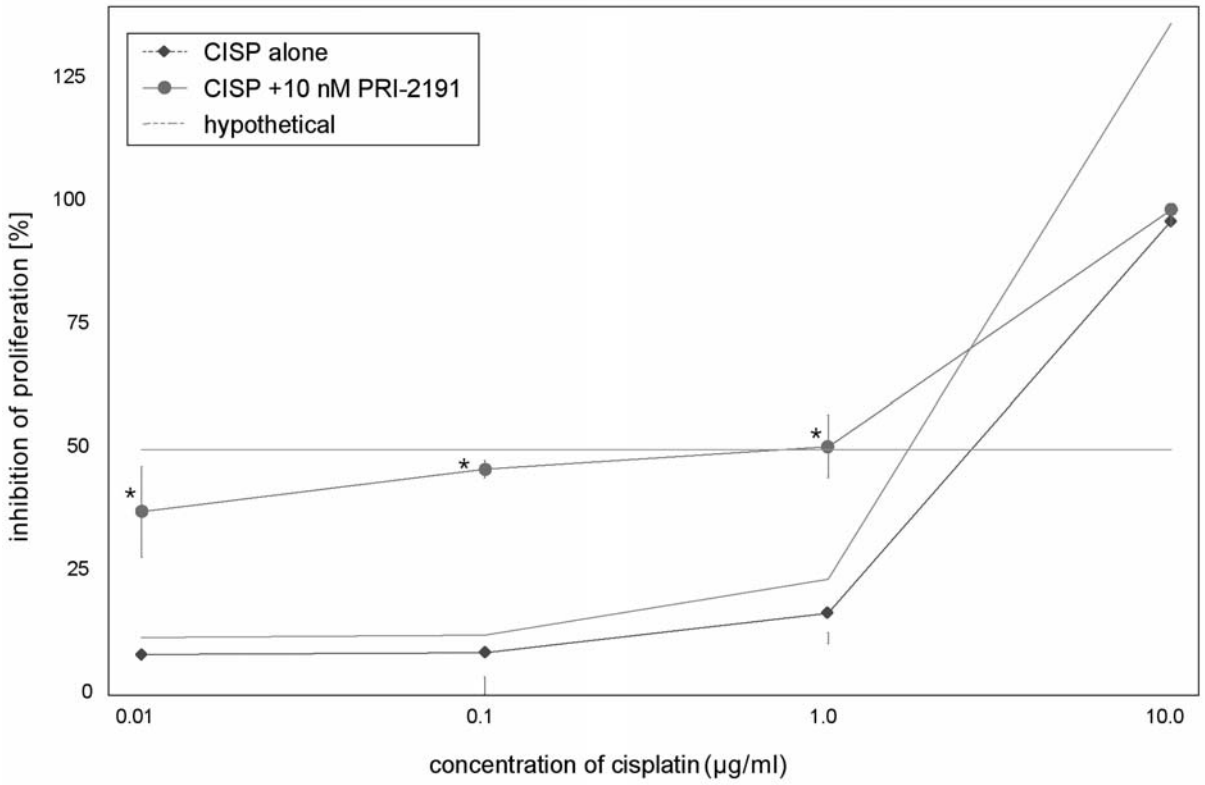

Figure 1. *Synergism between cisplatin and PRI-2191 in HL-60 cells in vitro model. *- statistically significant, p<0.05, combined treatment versus cisplatin alone.*

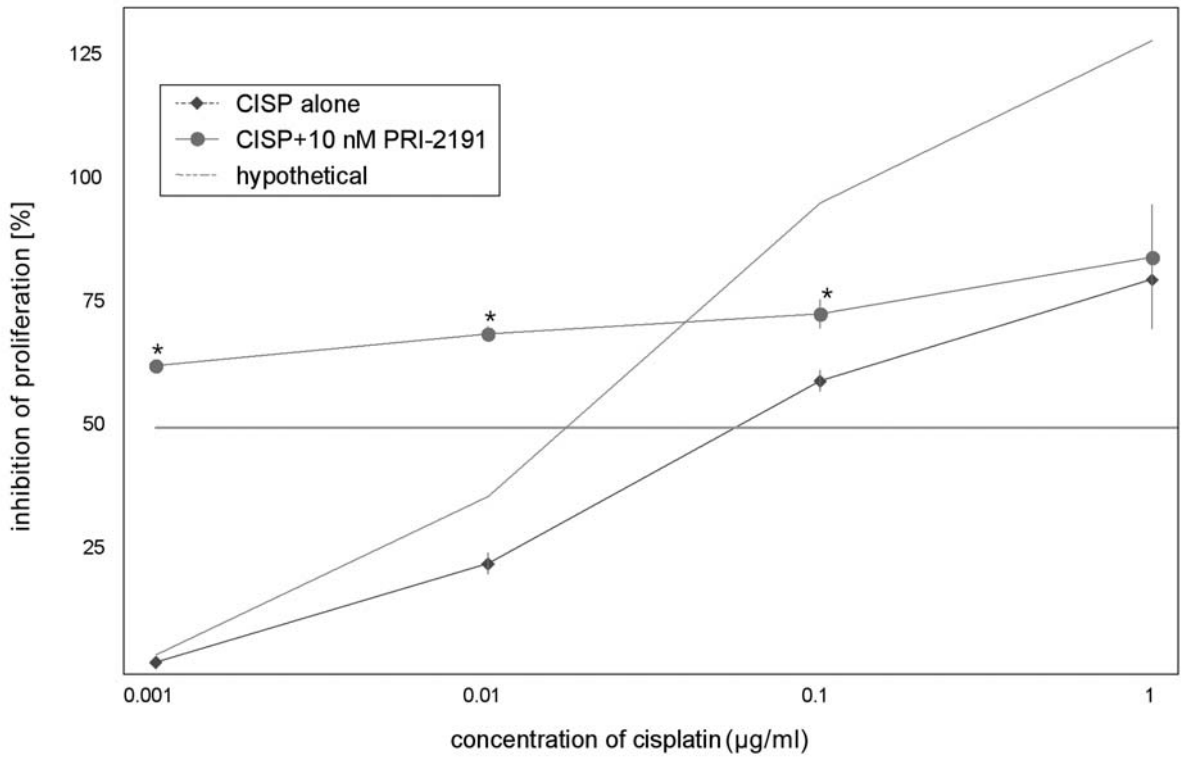

Figure 2. *Synergism between cisplatin and PRI-2191 in WEHI-3 cells in vitro model. *- statistically significant, p<0.05, combined treatment versus cisplatin alone.*

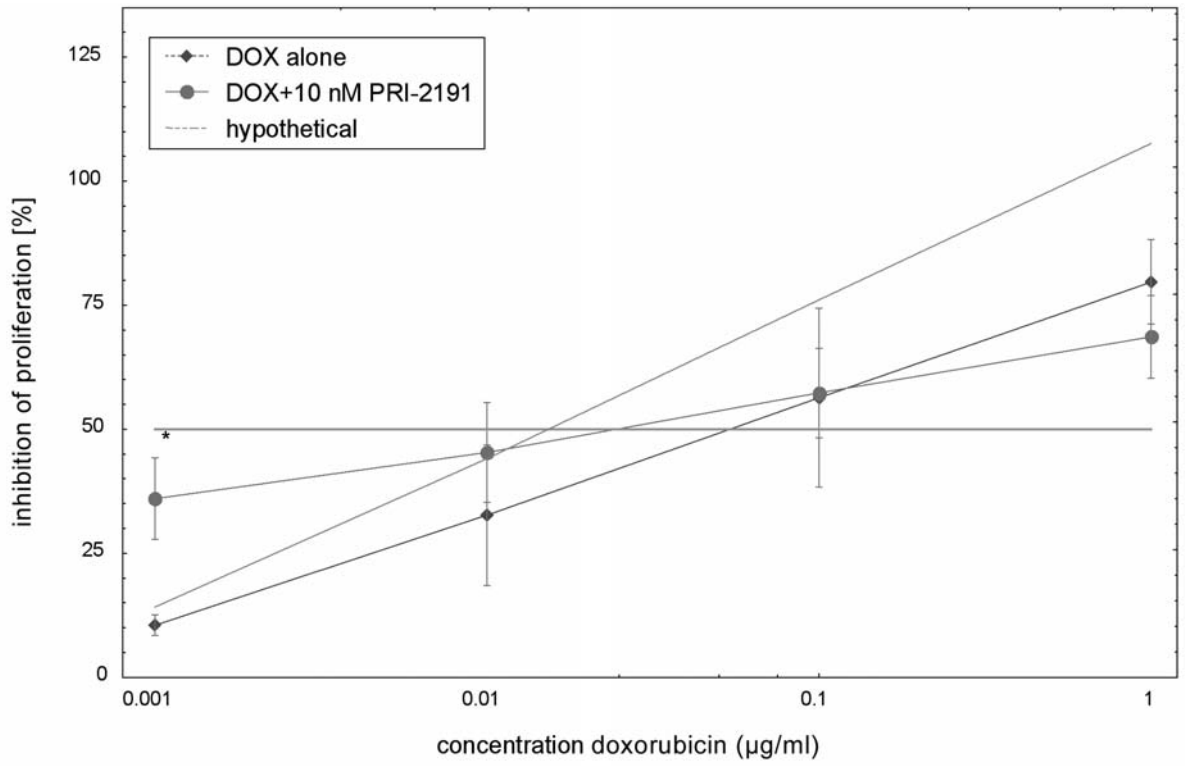

Figure 3. *Synergism between doxorubicin and PRI-2191 in WEHI-3 cells in vitro model. *- statistically significant, p<0.05, combined treatment versus doxorubicin alone.*

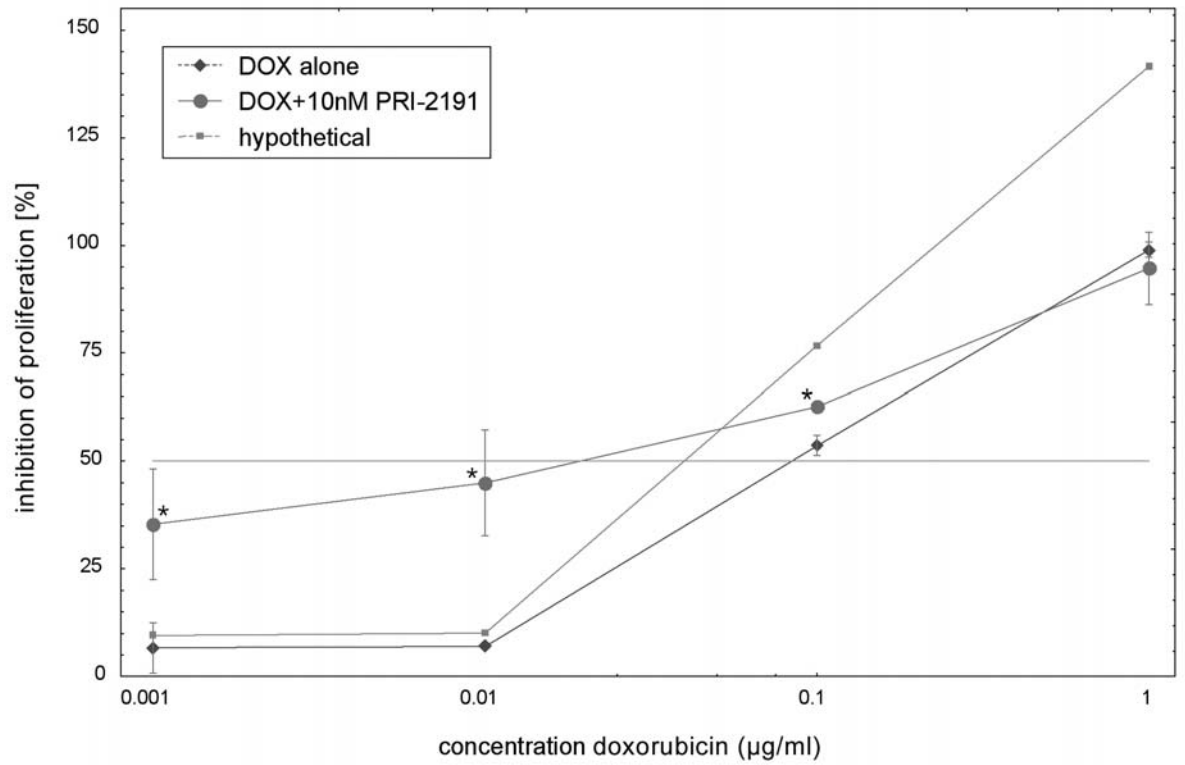

Figure 4. *Synergism between doxorubicin and PRI-2191 in HL-60 cells in vitro model. *- statistically significant, p<0.05, combined treatment versus doxorubicin alone.*

antiproliferative activity of vitamin D_3 compounds, as well as on the VDR expression.

In conclusion, both calcitriol and its analogue revealed strong antiproliferative activity against the VDR-positive cell lines, applied either as a single-drug or in combination with cytostatics. Moreover, the combinations of cytostatics with PRI-2191 had more potent synergistic effects than combinations with calcitriol. Thus, because of its favourable biological properties (high antitumour and low calcemic activity), PRI-2191 seems to be a good candidate for further preclinical and clinical studies, especially as a partner compound in combined treatments. Moreover, the expression of VDR in cancer cells could be a significant indicator for the potential application of vitamin D compounds in single as well as in combined with cytostatics antitumour treatments.

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