

The Novel Vitamin D Analog ZK191784 Inhibits Prostate Cancer Cell Invasion

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Abstract. *Background:* Low serum levels of $1,25(\text{OH})_2\text{D}_3$ ($1,25\text{D}$), have been associated with aggressive biologic behavior of prostate cancer (PCa). In the present study, we examined the effects of $1,25\text{D}$ and its novel, low-calcemic analog ZK191784 (ZK) on matrix metalloproteinases (MMPs), as well as on intercellular adhesion molecule-1 (ICAM-1) protein levels in human PCa cell lines LNCaP and DU-145. *Materials and Methods:* Cells were incubated with either vehicle (control), $1,25\text{D}$ or ZK. MMP-2 and MMP-9 activity was determined by gelatin zymography, while ICAM-1 levels were assessed by Western blot analysis and immunocytochemistry. *Results:* Compared to the controls, $1,25\text{D}$ and ZK caused a marked dose-dependent decrease in the gelatinolytic activity of the MMPs under study, particularly when ZK was used. Likewise, ICAM-1 was down-regulated in the cells incubated with $1,25\text{D}$ or ZK. *Conclusion:* Vitamin D analogs appear to be involved in the regulation of extracellular MMP activity and membrane adhesion molecule expression. Further studies, both *in vitro* and *in vivo*, are needed to define their role as potential therapeutic tools.

Prostate cancer (PCa) is one of the most common malignancies in Western countries and its incidence increases more rapidly with age than any other type of cancer (1, 2). The prognosis of PCa patients is highly variable and dependent on a variety of host, tumor and treatment parameters. Prognostic factors recommended for clinical use

include serum prostate-specific antigen (PSA), Gleason grade, pathologic stage and surgical margin status. Main treatment options are radical prostatectomy, external beam radiation, radioactive seed implants and androgen deprivation. There is no established role for chemotherapy. A major challenge of PCa treatment is the transition from hormone-sensitive localized disease to hormone-refractory metastatic cancer. The mechanisms responsible for this transition have not yet been elucidated (3).

Cancer cell metastasis involves proteolysis of the extracellular matrix and basement membrane, and requires the action of proteases. Matrix metalloproteinases (MMPs) are a large family of zinc-dependent, proteolytic enzymes that can cleave the components of the extracellular matrix. In particular, MMP-2 and MMP-9 are gelatinases (gelatinase-A and gelatinase-B, respectively) and represent key enzymes for degrading type IV collagen, a major component of the basement membrane (4). These secreted or transmembrane proteases need zinc for catalytic function and are produced as inactive zymogens requiring extracellular activation. The increased expression and activity of MMPs has been noted in almost all human cancer types and is associated with advanced tumor stage and poor prognosis (5, 6). MMPs are abundantly expressed by stromal cells in tumors and play a contributory role in cancer initiation and progression (7, 8). In PCa patients, analysis of MMP mRNA and protein levels in serum and tissue samples has shown that increased expression of MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9 is correlated with locally advanced and metastatic disease (9).

Intercellular adhesion molecule-1 (ICAM-1) is a transmembrane glycoprotein belonging to the immunoglobulin superfamily of adhesion molecules. It is constitutively expressed at low levels by endothelial cells and by some lymphocytes and monocytes, but is otherwise absent from the majority of cells in the human body (10), including the prostatic epithelium (11). ICAM-1 has been implicated in

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tumor prognosis and the progression of various types of cancer. It is expressed on the surface of many neoplastic cells and elevated plasma levels of its soluble form have been observed in cancer patients. Some studies have suggested that ICAM-1 may facilitate the spread of cancer cells to secondary sites (12).

Epidemiological studies suggest that calcitriol has preventive and therapeutic effects on PCa through several mechanisms, including induction of growth arrest, apoptosis and inhibition of metastasis and angiogenesis (13, 14). In addition, vitamin D metabolites have been shown to regulate MMP-2 and MMP-9 activity in human PCa cells (15, 16). Low serum levels of 1,25(OH)₂D₃ (1,25D), identified as the best indicator of vitamin D deficiency, have been associated with increased risk and a more aggressive behavior of PCa (17), while patients with well-differentiated, low Gleason grade PCa present high serum levels of vitamin D (18). Furthermore, calcitriol has antiproliferative and pro-differentiating effects on a variety of malignant cells, including human PCa cell lines (19), and has been seen to inhibit invasiveness of DU-145 PCa cells (15). These observations suggest that calcitriol may have relevant applications in the treatment of PCa. However, the side-effects of hypercalcemia and hypercalciuria at pharmacologic dosages constitute a major problem, and, consequently, low-calcemic analogs are under investigation (20).

ZK191784 (ZK) is a novel calcitriol derivative that inhibits lymphocyte proliferation and suppresses secretion of pro-inflammatory cytokines by monocytes (21). It has a therapeutic advantage over 1,25D by inducing immunosuppression at a concentration that does not cause hypercalcemia (21) and its anti-inflammatory properties have been also demonstrated in experimental colitis (22).

In order to substantiate the use of vitamin D derivatives in the treatment of advanced PCa, we examined the effects of ZK, compared to those of 1,25D, on MMPs and ICAM-1 in the androgen-dependent PCa cell line LNCaP and the androgen-independent PCa cell line DU-145. We also determined the action mechanisms of vitamin D derivatives and the expression of vitamin D receptor (VDR).

Materials and Methods

Antibodies and chemicals. Stock solutions of 4 mM 1,25D in isopropanol were donated by Leo Pharmaceutical Products (Ballerup, Denmark). ZK (1×10²) in ethanol was donated by Bayer Schering Pharma Aktiengesellschaft. The stock solutions were stored at -20°C, protected from light and freshly diluted in culture medium before each experiment.

Mouse monoclonal ICAM-1 antibody (G-5), rabbit polyclonal VDR antibody, goat polyclonal β-actin antibody and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (CA, USA). Mouse monoclonal MMP-2 (MMP2/8B4) and mouse monoclonal MMP-9 (56-2A4) antibodies

were obtained from Abcam (Cambridge, UK). RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), antibiotics and other non-specific reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell lines and cell culture. Human PCa cell lines DU-145 and LNCaP were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in Eagle's minimal essential medium (EMEM) and RPMI-1640 respectively, supplemented with 10% FBS, 100 U/ml penicillin, 10 mg/l streptomycin and 2 mM L-glutamine. All cultures were kept in an incubator at 37°C in a humidified atmosphere containing 5% CO₂. Experiments were performed by seeding 15×10⁴ cells in a culture dish, 60×15 mm, until subconfluence. After 48 h, LNCaP and DU-145 were incubated with ethanol and isopropanol vehicle controls, 1,25D or ZK under serum-free conditions for a further 48 h. The conditioned medium was then collected and used for the determination of MMP-2 and MMP-9 activity by gelatin substrate gel zymography and western blot analysis. The cells were scraped in PBS, centrifuged, re-suspended in lysis buffer (RIPA containing 50 mM Tris-HCl, 150 mM NaCl, 100 mM NaF, 2 mM EGTA 1% Triton X-100, 0.5 mM phenylmethanesulfonylfluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml aprotinin and 0.5 µg/ml pepstatin) for the determination of ICAM-1, MMP-2 and MMP-9 levels by western blot analysis.

Gelatin zymography. To analyze MMP-2 and MMP-9 activity in cell-conditioned medium, standard gelatin zymography was used. Briefly, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8%) containing 0.1% gelatin were overlaid with 4% stacking gels. Sample supernatants were mixed (10:1 volume) with a sample buffer consisting of 50 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol and 0.03% bromophenol blue. After loading the samples, electrophoresis was carried out at 125 V for 2 h. The gels were then soaked in 2.5% Triton X-100 on a shaker for 1 h at room temperature, changing the solution after 30 min to eliminate SDS. They were equilibrated at room temperature with gentle agitation for 30 min with the digestion buffer (50 mM Tris-HCl, pH 7.4, 10 mM CaCl₂, 150 mM NaCl), subsequently replaced with fresh digestion buffer and incubated at 37°C for 48 h. The gels were stained for 1-2 h with 0.5% Coomassie Brilliant Blue in 30% methanol and 10% acetic acid, and the destained with 30% methanol and 10% acetic acid, after which clear bands of digested gelatin were visible. The gels were scanned by Chemi-Doc, utilizing the Quantity One program (Bio-Rad Laboratories, Hercules, CA, USA).

Protein determination. Protein concentration was determined by the Bradford method (23), using bovine serum albumin as the standard.

Western blot analysis. After protein measurement, aliquots of cell lysates containing equal amounts of protein (25 µg), or aliquots of conditioned medium (25 µl), collected from the cells treated for 48 h with vehicle, 1,25D or ZK were mixed with 4× Laemmli's reducing sample buffer, boiled at 95°C for 5 min and loaded onto 10% polyacrylamide-bis-acrylamide gels. Following SDS-PAGE, proteins were transferred to pure nitrocellulose membranes (Bio-Rad Laboratories), according to Laemmli (24) and incubated overnight at 4°C with the specific primary antibody, washed, and then incubated for 60 min with the relative secondary antibody. After washing with phosphate-buffered saline containing 0.1% Tween 20 (pH 7.4) (TPBS),

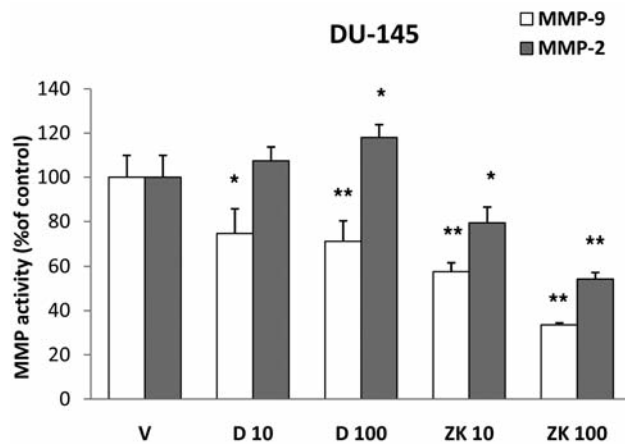


Figure 1. $1,25(\text{OH})_2\text{D}_3$ ($1,25\text{D}$) and ZK191784 (ZK) regulate matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase (MMP-9) activity in DU-145 cell lines. Activity of gelatinases measured by zymography after 48 h culture. MMP-9 and MMP-2 were detected in culture medium of DU-145 cells. Serum-starved cells were incubated with isopropanol vehicle control (Vis), ethanol vehicle control (Vet), 10 nM $1,25\text{D}$ (D 10), 100 nM $1,25\text{D}$ (D 100), 10 nM ZK (ZK 10), or 100 nM ZK (ZK 100). Each densitometric value, expressed as a percentage of the value recorded when the cells were incubated with vehicle control alone, represents the mean \pm SD of four separate experiments. * $p < 0.05$, and ** $p < 0.01$ in comparison to the respective vehicle treatment.

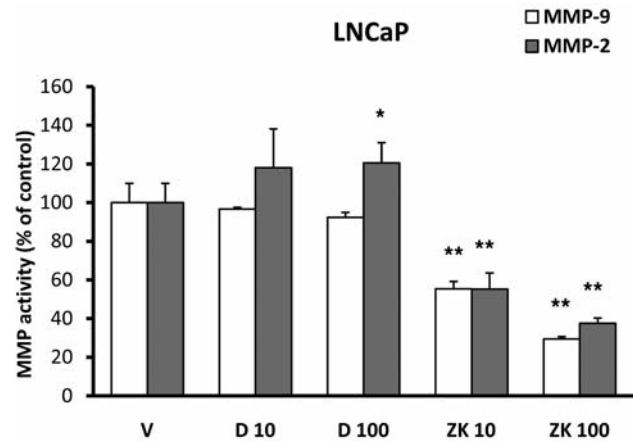


Figure 2. $1,25(\text{OH})_2\text{D}_3$ ($1,25\text{D}$) and ZK191784 (ZK) regulate matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase (MMP-9) activity in LNCaP cell lines. Activity of gelatinases measured by zymography after 48 h culture. MMP-9 and MMP-2 were detected in culture medium of LNCaP cells. After serum starvation, the cells were incubated with isopropanol vehicle control (Vis), ethanol vehicle control (Vet), 10 nM $1,25\text{D}$ (D 10), 100 nM $1,25\text{D}$ (D 100), 10 nM ZK (ZK 10), or 100 nM ZK (ZK 100). Each densitometric value, expressed as a percentage of the value recorded when the cells were incubated with vehicle control alone, represents the mean \pm SD of four separate experiments. * $p < 0.05$ and ** $p < 0.01$ in comparison to the respective vehicle treatment.

the membranes were incubated with enhanced chemiluminescence detection reagent (Amersham ECL Plus kit; GE Healthcare, Amersham, UK). Image acquisition and densitometric analysis were performed by Chemi-Doc (Bio-Rad), using the Quantity One program (Bio-Rad). Densitometric values, normalized with the use of the respective β -actin protein band, are reported as the percentage of the control value (cells treated with ethanol or isopropanol vehicle controls, for stimulation respectively with ZK or $1,25\text{D}$).

Immunocytochemistry. DU-145 and LNCaP cells were cultured on electrostatic slides and were grown to 70–80% confluence. The cells were stimulated and incubated with vehicle, 100 mM $1,25\text{D}$ or ZK for 48 h, then fixed in 95% alcohol for 10 min at room temperature, dried and stored at 4°C until immunostained. Immunocytochemical analysis was performed on slides of DU-145 and LNCaP cells, using the streptavidin-biotin peroxidase complex (ABC) method. Endogenous peroxidase activity was quenched by pre-treatment with 3.0% hydrogen peroxide in distilled water for 10 min. Cells were then washed in PBS, incubated first with 10% normal horse serum (UltraVision, LabVision, Fremont, CA, USA) functioning as a blocking agent for non-specific binding, and then with primary antisera under the following conditions: anti-ICAM-1 at 1:100 dilution for 45 min at room temperature, after antigen retrieval in thermostatic bath at 97°C with 1 mM EDTA buffer (pH 9) for 7 min; anti-human MMP-9 at a concentration of 5 $\mu\text{g}/\text{ml}$ for 1 h at room temperature, after antigen retrieval in thermostatic bath at 97°C in 1 mM EDTA buffer (pH 9) for 5 min; anti-human MMP-2 at a concentration of 5 $\mu\text{g}/\text{ml}$ for 1 h at 37°C , after antigen retrieval in thermostatic bath at 97°C in 1 mM EDTA buffer (pH 9) for 5 min. The antibodies were diluted in antibody diluent (Ventana Medical

System, Tucson, AZ, USA) and staining was achieved using a biotin-conjugated anti-rabbit and anti-mouse secondary antibody (UltraVision) together with streptavidin-peroxidase (UltraVision). Bound antibodies were visualized using 3,3'-diaminobenzidine (Dako, Glostrup, Denmark) as the chromogen. Cells were counterstained with Mayer hematoxylin, dehydrated in graded ethanol, then cleared with coverslips applied. In negative controls, the specificity of the antisera was tested by substituting the primary antibody with non-immune serum at the same concentration. Tissue samples of human colonic carcinoma, human breast carcinoma and schwannoma were used as positive controls for the antibodies against ICAM-1, MMP-9 and MMP-2, respectively.

Statistical methods. Statistical significance was determined either by one-way ANOVA followed by Bonferroni *t*-test, or by Student's *t*-test. One-way analysis of variance was used to determine significance among groups, after which the modified *t*-test with Bonferroni correction was used for comparison between individual groups. Differences were considered significant at $p < 0.05$.

Results

$1,25\text{D}$ and ZK regulate MMP-2 and MMP-9 activity in DU-145 and LNCaP cell lines. The levels of secreted MMP-2 and MMP-9 activity in conditioned medium were examined by zymography. When culturing DU-145 cells with ZK (Figure 1), we noted a remarkable reduction in the gelatinolytic activity of both MMP-2 (79.4% under ZK 10, $p < 0.05$, and 54.3% under ZK 100, $p < 0.01$), and MMP-9

(57.6% under ZK 10, $p<0.01$, and 33.5% under ZK 100, $p<0.01$). Similarly, in LNCaP cultured cells, ZK significantly reduced the gelatinolytic activity of both MMP-2 (55.2% under ZK 10, $p<0.01$, and 37.6% under ZK 100, $p<0.01$) and MMP-9 (55.4% under ZK 10, $p<0.01$, and 29.4% under ZK 100, $p<0.01$), as shown in Figure 2.

On adding 1,25D to DU-145 cell cultures (Figure 1), a lower reduction in the gelatinolytic activity of MMP-9 (74.6% under D 10, $p<0.05$, and 71.3% under D 100, $p<0.01$) was obtained, whereas an increase in MMP-2 proteolytic activity was observed (118% under D 100, $p<0.05$). In LNCaP cells, the gelatinolytic activity of MMP under 1,25D was only slightly reduced as regards to MMP-9 and was increased with regard to MMP-2 (120% under D 100, $p<0.05$), as shown in Figure 2.

When the highest dosage of ZK was used, densitometric analysis of the zymograms demonstrated that the reduction of MMP activity was dose-dependent in both cell lines ($p<0.01$).

Western blot and immunocytochemistry confirmed the notable decrease of MMP-2 and MMP-9 expression in DU-145 and LNCaP cell lines after ZK stimulation, and the slight decrease of MMP-9 in DU-145 after 1,25D stimulation (Figure 3).

1,25D and ZK influence VDR protein levels in DU-145 and LNCaP cell lines. Figure 4 shows VDR protein levels determined by Western blot analysis in total cell lysates, obtained after culturing the cells for 48 h with 1,25D or ZK at two different dosages. The stimulation of cells with 1,25D and ZK induced a marked increase of VDR levels in both cell lines. The increase of VDR protein levels after stimulation with both 1,25D and ZK was more evident in LNCaP than in DU-145 cells. VDR protein levels were, respectively, in DU-145 cells, 165.5% (D 100, $p<0.01$), 134.7% (ZK 10, $p<0.01$), 193% (ZK 100, $p<0.05$) and, in LNCaP cells, 248.3% (D 10, $p<0.01$), 304.7% (D 100, $p<0.01$), 236.7% (ZK 10, $p<0.01$) and 230.5% (ZK 100, $p<0.01$).

1,25D and ZK inhibit ICAM-1 protein expression in DU-145 cells. Western blot analysis was performed to detect the effects on ICAM-1 in total cell lysates, obtained after culturing the cells for 48 h in 1,25D and ZK at two different dosages. Stimulation of the cells with vitamin D derivatives induced a considerable decrease in ICAM-1 protein levels, clearly evident at the highest dosage, compared to the ethanol or isopropanol treated control in DU-145 cell line (Figure 5). Recorded data were, respectively, 78.1% and 60.9% for D 10 and D 100 ($p<0.01$) and 71% and 50.7% for ZK 10 and ZK 100 ($p<0.01$). On the other hand, basal expression of ICAM-1 protein was not detectable in LNCaP cells.

Immunocytochemical expression of ICAM-1 in DU-145 cells was decreased, confirming the results achieved by western blot analysis (Figure 5).

Discussion

In the current study, we showed that the novel calcitriol analog ZK can reduce MMP-2 and MMP-9 levels in both DU-145 and LNCaP cell lines in a statistically significant manner. In line with Bao *et al.* (16), we found that 1,25D slightly reduces MMP-9 activity in DU-145 cells, and even less so in LNCaP cells. In contrast to Schwarts *et al.*, who demonstrated reduced levels of both MMP-2 and MMP-9 in DU-145 cells after 1,25D treatment (15), our results indicate that the gelatinolytic activity of MMP-2 is increased by adding 1,25D to cell culture medium in both DU-145 and LNCaP cells.

It has been reported that 1,25D is a potent inhibitor of epithelial cell growth by inducing cell cycle arrest, differentiation or apoptosis (25), and can also influence tumor progression, by reducing metastatic spread that involves regulation of proteases (26, 27). Clinical use of calcitriol has been limited because it has been proven to cause hypercalcemia and hypercalciuria at pharmacologic dosage (28). An alternative strategy for developing a calcitriol-based therapy is to design novel vitamin D analogs which share the immunosuppressive properties of calcitriol, though not the hypercalcemic profile. ZK is devoid of calcitriol side-effects and could represent a therapeutic option for PCa.

In our study, both cell lines responded to ZK stimulation with a reduction in MMP activity and with an increase in VDR protein levels. Meanwhile, under 1,25 D stimulation, both cell lines showed an increase in VDR protein levels, more evident in LNCaP cells than in DU-145 cells. Nevertheless, this increase in DU-145 cells corresponded to a more remarkable reduction in MMP-9 activity than was observed in LNCaP cells. This may be due to the difference in the activation of the VDR target gene expression in those particular PCa cell lines. Other studies reveal that LNCaP have about 2-3 fold the VDR content of DU-145 cells and yet much higher than physiologic concentrations of 1,25D are required to inhibit cell growth (29).

Although the mechanisms responsible for transition to androgen independence are still unclear, androgen-independent PCa is characterized by a higher invasive potential (30). In the present study, we used the androgen-independent cell line DU-145 and the androgen-dependent cell line LNCaP. We demonstrated that ICAM-1 is expressed in DU-145 cells alone, and is absent from LNCaP cells. ICAM-1 has been implicated in cancer metastasis. The regulation of ICAM-1 expression by cancer cells may facilitate the metastatic process, whereby invasive cells dissociate from the proper tumor, enter the circulation, avoid being targeted by immune cells, and invade distal sites (12). Aalinkel *et al.* reported that the constitutive gene expression of proangiogenic factors, such as ICAM-1,

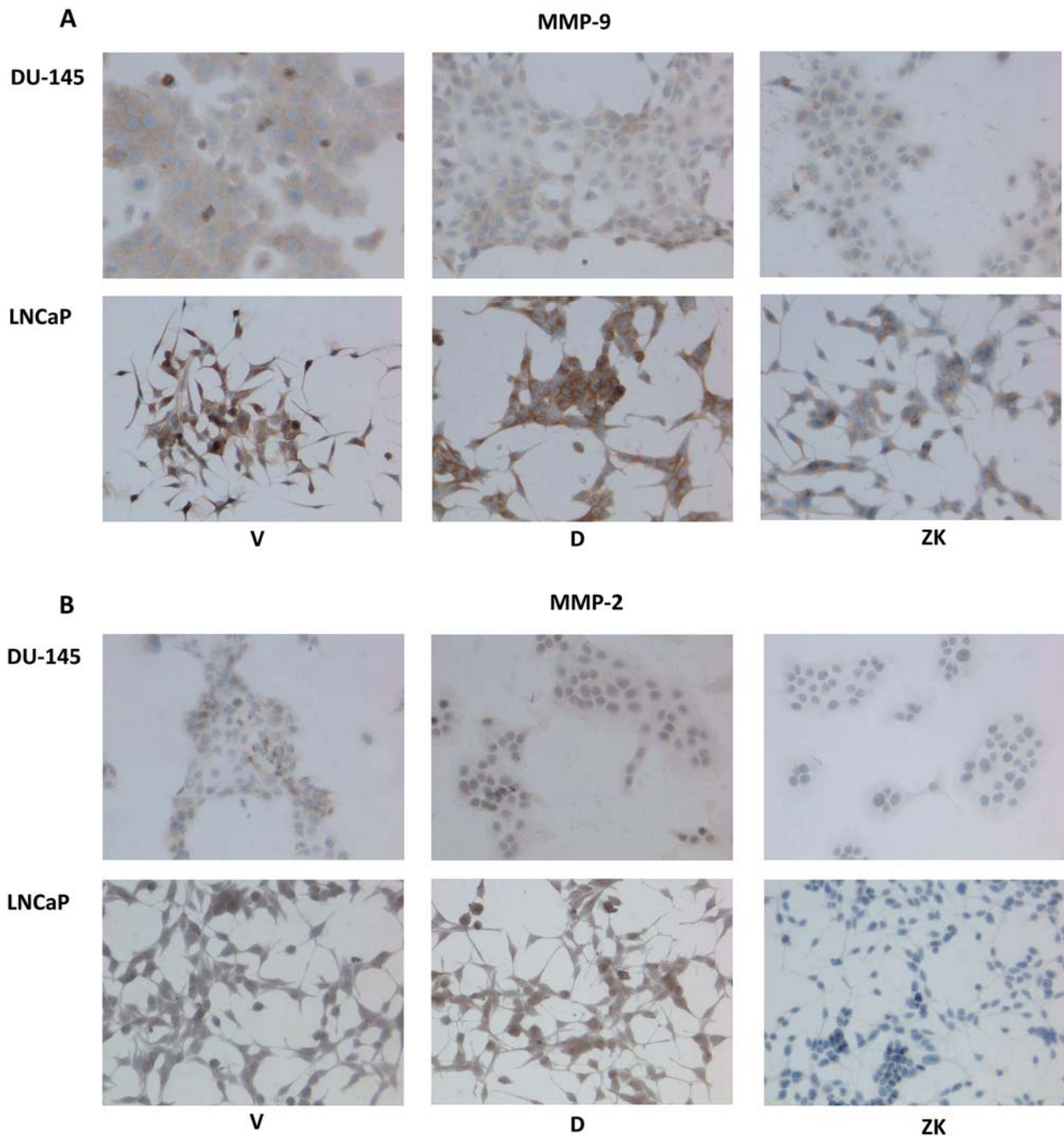


Figure 3. Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase (MMP-9) expression in DU-145 and LNCaP cell lines after ZK191784 (ZK) and 1,25(OH)₂D₃ (1,25D) stimulation. Photomicrographs of immunocytochemical analysis for MMP-9 and MMP-2 in DU-145 and LNCaP cells cultured on electrostatic slides, grown to 70-80% confluence and incubated with vehicle control alone (V), 100 mM 1,25D (D) or 100 mM ZK (ZK) for 48 h. Each panel is representative of at least two similar experiments.

vascular endothelial growth factor (VEGF), interleukin-8 and transforming growth factor β 2 (TGF- β 2), is significantly greater in DU-145 and PC3 cells than in LNCaP cells (31).

Our study demonstrated that the vitamin D analog ZK can remarkably reduce MMP activity both in LNCaP cells and in DU-145 cells, where it also inhibits ICAM-1 protein expression. Indeed, the metastatic potential of prostate

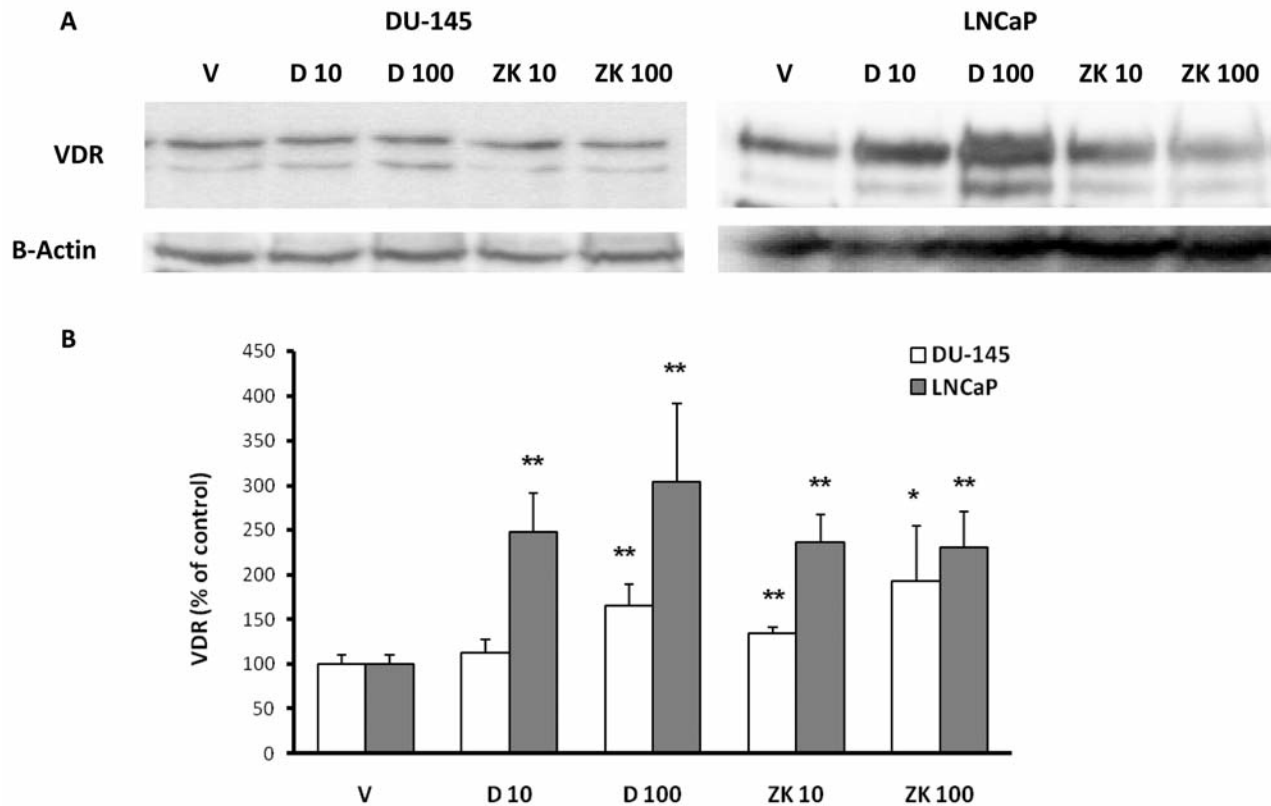


Figure 4. $1,25(\text{OH})_2\text{D}_3$ (1,25D) and ZK191784 (ZK) influence the vitamin D receptor (VDR) protein levels in DU-145 and LNCaP cell lines. Effects of 10 nM 1,25D (D 10), 100 nM 1,25D (D 100), 10 nM ZK (ZK 10), or 100 nM ZK (ZK 100) on vitamin D receptor (VDR) protein levels determined by western blot analysis in starved DU-145 and LNCaP cells after 48 h culture. Each densitometric value, expressed as a percentage of the value recorded when the cells were incubated with vehicle control alone, represents the mean \pm SD of four separate experiments. A: Representative western blot. B: Quantitative data. To detect possible differences in protein loading, the membrane was stripped and reprobed with anti- β -actin antibody. * $p < 0.05$ and ** $p < 0.01$ in comparison to vehicle treatment.

cancer correlates with the expression of ICAM-1. This finding is of particular note considering a recent study by Yin *et al.*, who proposed the therapeutic use in PCa of low-dose calcitriol, combined with a vitamin E analog, in order to avoid hypercalcemic side-effects (32).

Microarray analysis of PCa cells demonstrated the association of the detrimental transition from androgen-dependent to androgen-independent phenotype, with enhanced malignant potential (33). The flow-cytometric results presented by Berry *et al.* indicate a similar association, with minimal or no surface expression of ICAM-1 on the androgen-dependent cell line LNCaP, but elevated expression on the androgen-independent cell line DU-145 (34). Our results are in accordance with these findings and demonstrate that ICAM-1 is down-regulated by vitamin D analog in DU-145 cells. A large genome-wide characterization of gene expression variations and DNA copy number changes in PCa cell lines, confirmed that ICAM-1 is associated with an enhanced malignant

potential (33). Conrad *et al.* have recently suggested the use of human antibodies to target cell surface antigens overexpressed by hormone-refractory metastatic PCa cells (35). The authors also reported that ICAM-1 is differentially expressed during the transition from a hormone-sensitive to a hormone-refractory phenotype and that it mediates prostate cell invasion, thus playing an important role in tumor metastasis to distal sites such as the bone (35).

In conclusion, our study shows that ZK inhibits ICAM-1 expression and considerably reduces MMP activity in both DU-145 and LNCaP cell lines. Moreover, ICAM-1 has been identified as a cell surface antigen that is differentially expressed in the DU-145 androgen-independent PCa cell line. Since vitamin D analogs seem to be involved in the regulation of extracellular MMPs and membrane adhesion molecules, our results encourage further studies with the aim of defining clearly the effects, both *in vitro* and *in vivo*, of these potential therapeutic tools.

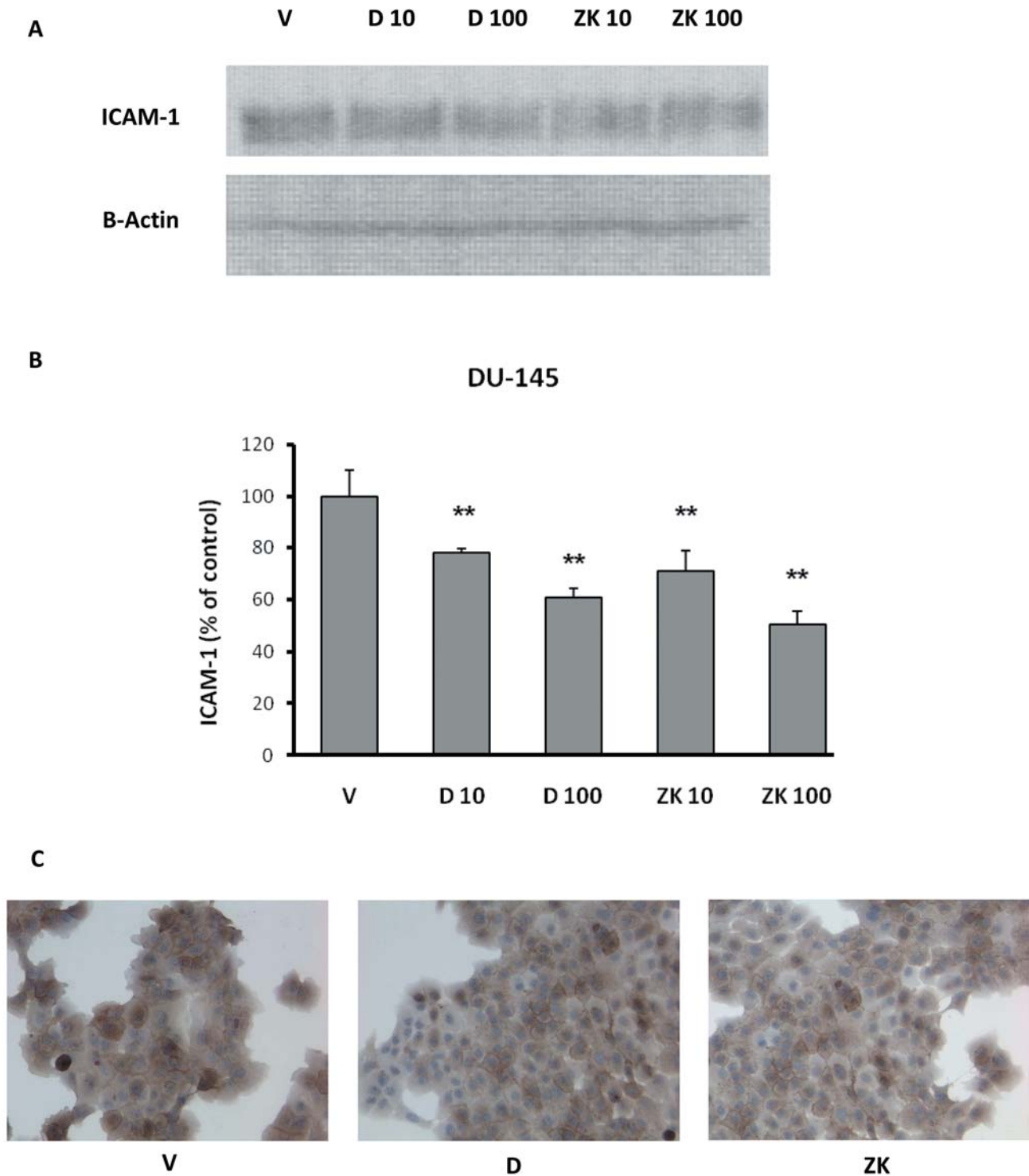


Figure 5. $1,25(\text{OH})_2\text{D}_3$ ($1,25\text{D}$) and ZK191784 (ZK) influence ICAM-1 protein levels in DU-145 cell line. Effects of 10 nM $1,25\text{D}$ (D 10), 100 nM $1,25\text{D}$ (D 100), 10 nM ZK (ZK 10), or 100 nM ZK (ZK 100) on ICAM-1 protein levels determined by western blot analysis in starved DU-145 cells after 48 h culture. Each densitometric value, expressed as a percentage of the value recorded when the cells were incubated with vehicle control alone, represents the mean \pm SD of four separate experiments. A: Representative western blot. B: Quantitative data. In order to detect possible differences in protein loading, the membrane was stripped and reprobed with anti- β -actin antibody. C: Photomicrographs of immunocytochemical analysis for ICAM-1 in DU-145 cells cultured on electrostatic slides, grown to 70-80% confluence and incubated with vehicle control alone (V), D 100 or ZK 100 for 48 h. Each panel is representative of at least two similar experiments. ** $p < 0.01$ in comparison to vehicle treatment.

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