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Influence of vitamin D_3 analogues in combination with budesonid R on proliferation of nasal polyp fibroblasts

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Vitamin D (VD) and its different analogues, besides their classic role as regulators of calcium and phosphor homeostasis, have emerged as a large family of antiproliferative agents. Such properties suggested VD potential as a therapy for chronic inflammatory diseases, including nasal polyposis (NP). NP growth involves both an inflammatory process and the proliferation of fibroblast as an important factor inducing aberrations in the phenotype of the epithelium. The aim of this study was to investigate the possible influence of 1α,25-dihydroxyvitamin D_3 (calcitriol) and 1α,24(*R*)-dihydroxyvitamin D_3 (tacalcitol) in monotherapy and in combination with budesonid R (BR) on NP fibroblast proliferation. Material and methods: The study involved 26 samples of NP. NP cells were cultured on 96-well plates beginning with a concentration of 5×10^3 cells per well with RPMI 1640 medium supplemented with antibiotics and 10% foetal bovine serum. After the fourth to sixth passage the medium was replaced with a nutrient medium with calcitriol or tacalcitol in a defined concentration (from 10^{-9} M to 10^{-3} M) alone or in combination with BR in 1:1, 1:3 or 3:1 ratios, each at concentrations from 10^{-5} M to 10^{-3} M. Results: Growth inhibition of nasal fibroblasts exposed to calcitriol or tacalcitol was noted. Significant antiproliferating activity was observed at calcitriol concentrations of 10^{-4} M and 10^{-3} M after 48 h, and at a concentration of 10^{-3} M after 72 h with the percentage of proliferating cells reduced to 30% compared to the control samples ($P<0.05$). In cells treated with tacalcitol the maximal effect was seen at 10^{-4} M after 48 h and at 10^{-3}M after 72 h with a 60% inhibition with respect to the control ($P<0.05$). The inhibition of fibroblast proliferation reached the maximal level when they were exposed to calcitriol: BR (1:1) or tacalcitol: BR (1:1), each at a concentration of 10^{-4} M, after 72 h (82% and 69%, respectively). Conclusions: The antiproliferative activity of calcitriol and tacalcitol in NP cultures was confirmed. Because of its lower toxicity and higher activity tacalcitol seems to be the more promising agent in NP therapy, both as a single medication and in treatment protocols with BR.

Keywords: budesonid R, calcitriol, fibroblast, nasal polyps, proliferation, tacalcitol, vitamin D3

INTRODUCTION

Vitamin D_3 is a secosteroid hormone which is synthesized in the skin or derived from nutritional sources, primarily metabolized in the liver to 25-hydroxyvitamin D_3 and subsequently in the kidney into a hormonally-active form, 1α,25-dihydroxyvitamin D_3 (calcitriol) (Holick *et al*., 1984). The classic role for vitamin D (VD), i.e. the regulation of calcium and phosphor homeostasis, is achieved by the conversion to 1,25(OH)2D3, the most active metabolite of VD. Increasing evidence from animal, cellular and molecular studies has revealed, however, more subtle actions of VD. The biological effects of VD derivates are mediated through a specific nuclear receptor (vitamin D receptor; VDR), a phospho-protein which functions as a transcription factor (Evans, 1998). The presence of VDR has been observed in almost

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Abbreviations: BR, budesonid R; NP, nasal polyps; VD, vitamin D; VDR, vitamin D receptor.

every cell type in the human body, including cancer cells and some activated cells of the immune system (Hansen & Mäenpää, 1997). Through the binding motifs in the promoter regions of target genes, VD is involved in a modulation of the transcription of more than 60 genes (MacDonald *et al*., 2001). Thus VD is involved in essential cell regulatory processes such as proliferation, differentiation, apoptosis, angiogenesis and inhibition of pro-growth/pro-survival signalling pathways in a wide variety of cell types (Johnson *et al*., 2002).

These observations suggest the potential of active VD and its different derivatives and analogues as a therapy for chronic inflammatory diseases, including nasal polyposis (NP). The pathophysiology of NP is considered to be the ultimate manifestation of chronic inflammation of the upper respiratory tract of unknown etiology (Norlander *et al*., 1999). From the histopathologic viewpoint, polyps are characterized by squamous metaplasia of the respiratory epithelium, increased subepithelial fibrosis with an accumulation of extracellular matrix, associated with marked edema, dilated capillary, venous channels and eosinophile infiltrates (Pawankar, 2003). To date, fibroblasts have been considered mainly a physical barrier but recent studies have shown their significance in the complex mechanism of polyp genesis. The present theory is that stroma abnormalities, with fibroblast thought to play an important role, may induce aberrations in the phenotype of epithelium (Rinia *et al*., 2007). Upon stimulation, nasal fibroblasts, *via* production of collagens and fibronectin, are known to promote extracellular matrix generation and tissue remodelling. These interrelations play a crucial role in maintaining chronic inflammation and subsequent polyp formation.

At present, steroids are most commonly used in the therapy of NP, but due to their well-known side effects other agents suitable for NP therapy are under intensive investigation. Although, most ongoing research and trials concentrate on the anti-inflammatory properties of VD, we decide to assess the anti-proliferating feature of these substances. Topical VD and related compounds have been shown recently to be effective in the treatment of psoriasis and other hyperkeratotic skin disorders (Lebwohl *et al*., 2007).

The purpose of this study was to determine the possible influence of 1α,25-dihydroxyvitamin D_3 (calcitriol) and 1α,24(*R*)-dihydroxyvitamin D_3 (tacalcitol) on proliferation of NP fibroblasts. The inhibitory effect of BR (22R-16α,17α-butylidenodioxy-11 β,21-dihydroxy-1,4-pregnadien-3,20-dion) on fibroblast proliferation revealed by our group in both *in vitro* and *in vivo* studies (Rostkowska-Nadolska *et al*., 2002; 2005) inspired us to analyze the effectiveness of a combination of VD analogues and BR in various proportions, doses and duration of treatment.

MATERIAL AND METHODS

Twenty-six patients (19 males and 7 females) with NP treated surgically at the Department of Otolaryngology, Wroclaw Medical University were included in the study. All the subjects met the diagnostic criteria for chronic rhinosinusitis as established by the Task Force on Rhinosinusitis (AAO-HNS). Patients had been free of any medication for at least 4 weeks before surgery and had bilateral polyps in the nasal cavities on endoscopic examination and Computed Tomography. The presence of comorbidity or smoking history was also excluded. The subjects underwent polypectomy for nasal obstruction with subsequent tissue sampling for further examination.

The control group consisted of 9 healthy persons (6 males and 3 females). The absence of NP was assessed by clinical history, endoscopic examination and imaging. A history of other diseases was also excluded. Control tissue samples were taken from unchanged middle turbinate mucosa. The study was approved by the Local Ethics Committee of Wroclaw Medical University.

NP specimens and control mucosa were immediately disinfected with Betadine, rinsed in phosphate-buffered saline (PBS), cut into small fragments and placed into a sample tube containing 1 ml PBS. The tubes were directly transported on ice to the laboratory for further investigations. A part of each sample was fixed in 10% buffered neutral formalin, processed routinely, and embedded in paraffin wax for subsequent immuno-histochemical examination to establish diagnosis and to exclude other pathologies.

The tissue was milled mechanically and then washed in a solution containing 0.25% trypsin and EDTA. Suspended cells were cultured in Nunc Easy Y Flaks (25 cm^2) and after six to nine passages were sown onto 96-well plates beginning with a concentration of 5×10^3 cells per well in RPMI 1640 medium supplemented with penicillin (1000 U/ml), streptomycin (10 mg/ml) and enriched with 10% foetal bovine serum. The culture was kept in an incubator (ASSAB) at 37°C under a 5% CO_2 atmosphere and 100% humidity. After the fourth to sixth passage the medium was replaced with a nutrient medium with calcitriol or tacalcitol alone or in combination with BR.

The final concentrations of calcitriol and tacalcitol ranged from 10^{-9} M to 10^{-3} M; VD analogue: BR in 1:1, 1:3 and 3:1 ratios, each at a concentration from 10^{-5} M to 10^{-3} M. Subsequently, the cultures were incubated under the same conditions. Cell

counts were performed after 24, 48 and 72 h using the CyQUANT Cell Proliferation Assay Kit (Molecular Probes). Cells were washed with PBS and fixed with methanol and frozen at 78°C for 24 h. After defrosting to room temperature 200 µl of CyQUANT-GR was added. Measurements were conducted with a WALLAC 1420 VICTOR TM scanner (Perkin Elmer) with filters for the λ=480 nm (excitation) and λ=535 nm (emission) to measure cell fluorescence. Control cultures consisted of cells from unchanged middle turbinate mucosa were incubated only with vehicle in the same conditions.

After culturing, cell viability was evaluated by staining with trypan blue and assessed with light microscopy. Apoptotic cell death was examined by Annexin-V-FLUOS Kit (Roche Diagnostics, Mannheim, Germany) according the manufacturer's specifications.

Chemicals. Calcitriol (1α,25-dihydroxycholecalciferol), tacalcitol (1α,24(*R*)-dihydroxyvitamin D_3) and budesonid R were from Pharmaceutical Institute (Poland); penicillin, streptomycin, trypsin and EDTA from Aldrich-Sigma; FBS from PAA The Cell Culture Company; RPMI 1640 medium from Biomed (Poland); PBS from Aldrich-Sigma.

Statistics. The percentage of proliferating cell was calculated in cultures exposed to the VD analogues compared to the untreated control. The analysis was performed using the Statistica 5.0 package (Statsoft, Poland). The differences in cell proliferation between NP fibroblasts and the control with respect to the reagent used were analyzed with the Student's *t*-test. All values were expressed as means ±S.D. For each experiment, each drug concentration was tested in triplicate. Significance level was assumed for $P<0.05$.

RESULTS

The rates of fibroblast proliferation after treatment with tested substances are presented in Figs. 1–4.

Treatment with calcitriol or tacalcitol applied alone

A growth inhibition of nasal fibroblasts exposed to calcitriol at concentrations between 10^{-4} M and 10^{-3} M was observed at each time interval. The strongest antiproliferating activity was observed at 10^{-4} M and 10^{-3} M after 48 h ($P<0.05$), and at 10^{-3} M after 72 h where the percentage of proliferating cells decreased to 30% when compared to the control ($P<0.05$). There was no growth inhibition at concentrations below 10^{-5} M (Fig. 1).

Tacalcitol at the higher concentrations (10^{-4} M and 10^{-3} M) decreased fibroblast proliferation after 24, 48 and 72 h. The maximal effect was observed at 10^{-4} M after 48 h and at 10^{-3} M after 72 h, reaching an inhibition of about 60% with respect to the control ($P<0.05$). Similarly to the action of calcitriol, the growth of fibroblasts was not reduced by tacalcitol at a concentration below 10^{-5} M (Fig. 2).

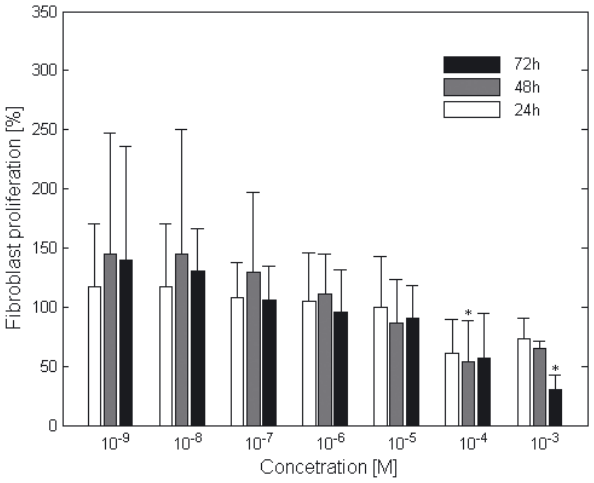


Figure 1. Effect of calcitriol on inhibition of fibroblast proliferation after 24, 48 and 72 h of incubation, expressed as percentage of control cell cultures (untreated). Values are expressed as mean ±S.E. Different from the control: *$P\leq0.05$.

Combined treatment with budesonid R and tacalcitol or calcitriol

The proliferation of fibroblasts was not substantially changed by the tacalcitol:BR combination in the concentration range of 10^{-9} M to 10^{-6} M. Thus, further measurements were performed with higher concentrations only.

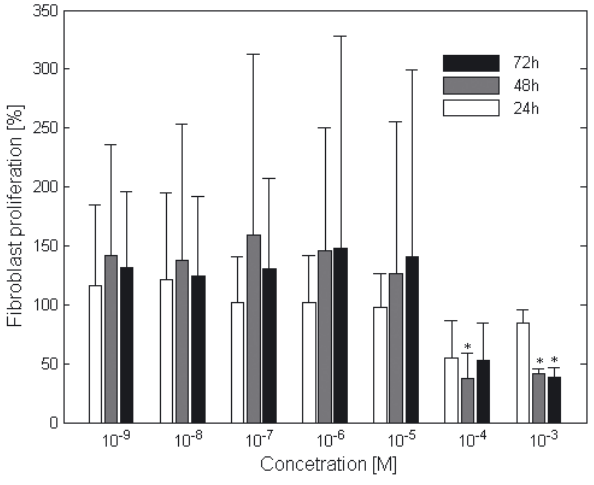


Figure 2. Effect of tacalcitol on inhibition of fibroblast proliferation after 24, 48 and 72 h of incubation, expressed as percentage of control cell cultures (untreated). Values are expressed as mean ±S.E. Different from the control: *$P\leq0.05$.

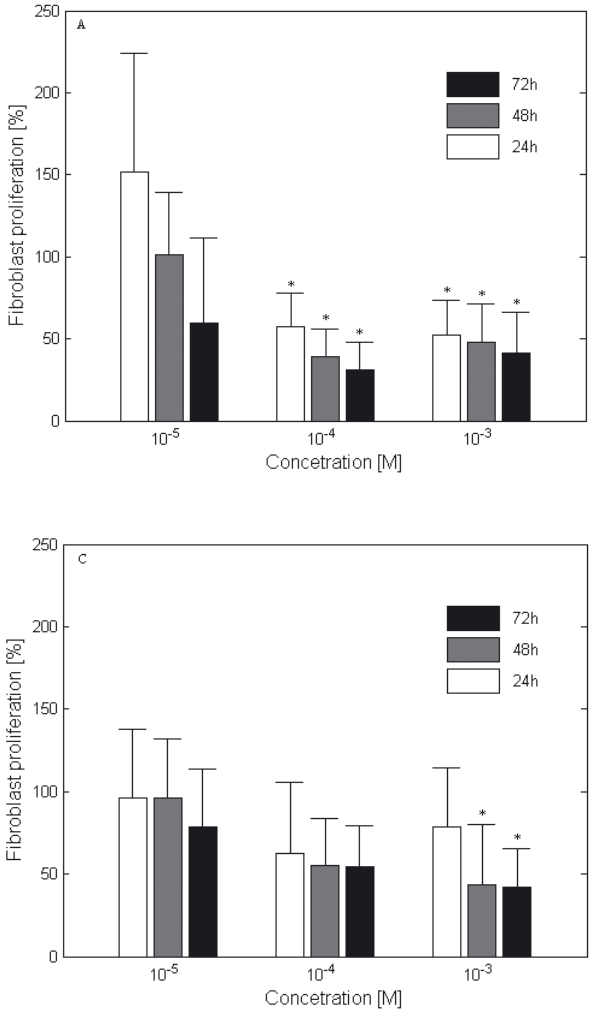


Figure 3. Influence of tacalcitol: budesonid R in 1:1 (A), 1:3 (B) and 3:1 (C) ratio on fibroblast proliferation, expressed as percentage of control cell cultures (untreated). Values are expressed as mean ±S.E. Different from the control: *$P \leq 0.05$

Tacalcitol:BR administered in the ratio of 1:1 and each used at the concentration of 10^{-5}–10^{-3} M affected cell proliferation. The maximal effect was observed at 10^{-4} M and 10^{-3} M after 72 h with an inhibition of approx. 70% and 60% compared to the control ($P<0.05$). The growth of fibroblasts was reduced insignificantly by 10^{-5} M solution of the two agents after 72 h of treatment (Fig. 3A).

The addition of tacalcitol:BR at a ratio of 1:3 and each used at concentrations of 10^{-5}–10^{-3} M, inhibited cell proliferation with a significant decrease in the percentage of proliferating cells to 40% at 10^{-3} M compared to the control ($P<0.05$) (Fig. 3B).

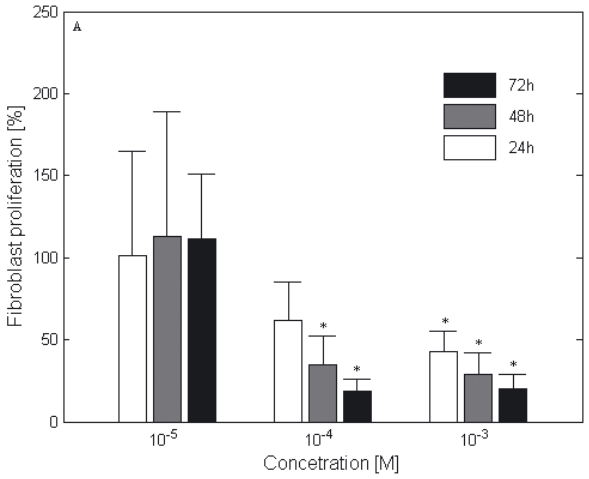
Tacalcitol:BR at a 3:1 ratio and each used at concentrations from 10^{-5} M to 10^{-3} M also affected cell proliferation. Statistically significant effects were obtained at 10^{-3} M after 48 h and 72 h of exposure with an inhibition of growth of about 60% with respect to the control ($P<0.05$) (Fig. 3C).

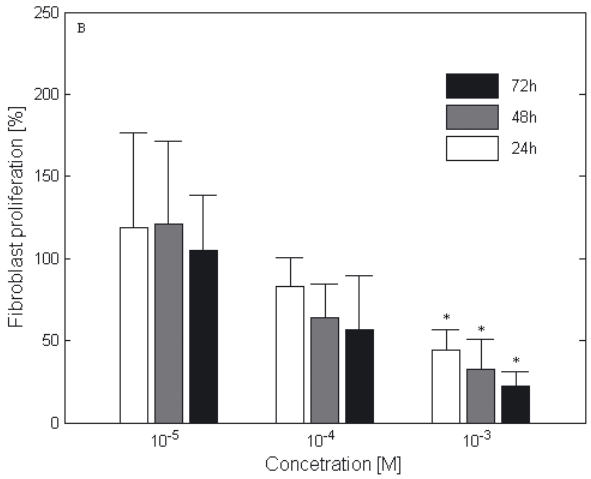
Compound of calcitriol:BR (1:1), each at 10^{-4} M and 10^{-3} M, strongly reduced fibroblast proliferation after each time interval. A maximal inhibition of approx. 80% was observed after 72 h ($P<0.05$) (Fig. 4A). The two agents in combination (1:3) affected fibroblast number when used at 10^{-4} M and 10^{-3} M with significant cell proliferation reduction to 40% compared to the control after 72 h ($P<0.05$) (Fig. 4B). Cells responded to calcitriol:BR (3:1) administered at 10^{-3} M and 10^{-4} M each, with the maximal effect of 60% inhibition observed at the latter after 48 h and 72 h of treatment ($P<0.05$) (Fig. 4C).

Among the examined compounds the maximal inhibition of proliferation was obtained when fibroblasts were exposed to calcitriol:BR (1:1) and tacalcitol:BR (1:1) each at the concentration of 10^{-4} M after 72 h (82% and 69%, respectively) ($P<0.05$).

DISCUSSION

Due to their potential therapeutic significance, VD and its analogues are intensely being examined in different hyperproliferative disorders (e.g. psoriasis), immune dysfunction and endocrine disorders (Ruzicka &Trompke, 2004; Lebwohl *et al*., 2007). Below we present the influence of VD derivates on the proliferation of fibroblast from NP. Both calcitriol and tacalcitol were found to substantially inhibit fibroblast proliferation. The most significant cell response was observed if either was used at a concentration of 10^{-4} M or 10^{-3} M alone or in combination with BR. The antiproliferative effects of VD analogues were dose- and time-dependent, which is consistent with the literature (Takahashi *et al*., 2003). The lower concentration used was mostly without any effect but in some cases cell growth was also affected by 10^{-5} M and even 10^{-6} M solutions. It has





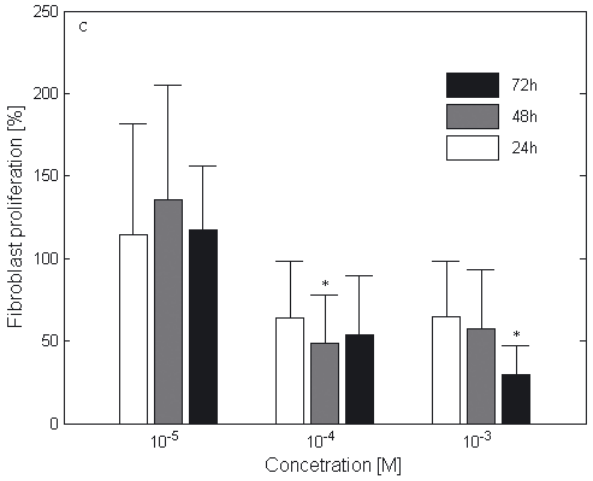


Figure 4. Influence of calcitriol:budesonid R in 1:1 (A), 1:3 (B) and 3:1 (C) ratio on fibroblast proliferation, expressed as percentage of control cell cultures (untreated). Values are expressed as mean ±S.E. Different from the control: $*P \leq 0.05$

been shown by others that the range of concentrations of VD analogues needed for the reduction of cell number differs depending on the cell origin, growth pattern, conditions of the experiment, and the mode of drug administration. Pourgholami *et al.* (2000) noted a significant antiproliferative effect of calcitriol on a liver cancer cell line at a concentration of 10^{-9} M. Similarly, the growth of prostate epithelial cells and cardiac myocytes was significantly inhibited when pre-occluded with a lower concentration of 1α,25-(OH)2D3 (Sprenger *et al.*, 2001; Nibbelink *et al.*, 2007). In contrast, it was revealed in human melanoma cells that only the higher concentration of VD3 derivates, i.e. 10^{-6} M, influenced cell proliferation (Gruber & Januszewska, 2002). Gniadecki *et al.* (1996) reported increased keratinocyte proliferation by 10^{-11} M 10^{-9} M 1α,25(OH)2D3 which is consistent with our observation.

The application of potentially effective doses of calcitriol in topical treatment is limited by the effects on calcium metabolism, hypercalcinuria, hypercalcaemia, nephrocalcinosis, nephrolithiasis and soft tissue calcinosis. These undesirable side effects have motivated the synthesis of analogues presenting lower toxicity with subsequent good antiproliferating and immunomodulating properties. Tacalcitol is such a synthetic analogue of VD which structurally differs from calcitriol only by the hydroxylation in position 24 instead of position 25. Its biological properties are common with those of VD and it has a similar effectiveness in the regulation of cell proliferation and differentiation, as has been shown in some models (Takahashi *et al.*, 2003). Tacalcitol decreased human keratinocyte proliferation in a concentration-dependent manner with the maximal effect observed at 10^{-7} M (Takahashi *et al.*, 2003). In our study the inhibitory response of fibroblasts derived from

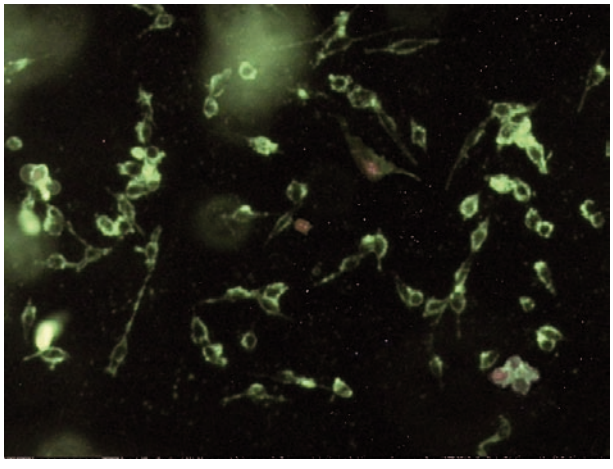


Figure 5. Cell death after tacalcitol at 10^{-4} M. Low number of necrotic cells (pinkish cells) and prevalence of apoptosis (green cells).

NP to tacalcitol exceeded the response to calcitriol used at the same concentration.

Numerous long term clinical trials have confirmed the efficacy, safety and tolerability of topical tacalcitol in the treatment of vulgar psoriasis (Leone & Pacifico, 2005). It was formulated as an ointment with a concentration of 4 μg/g to be applied once a day for 8 weeks (Ruzicka & Trompke, 2004). The side effects that were observed during the treatment included local irritations such as pruritus, a burning sensation and erythema. No systemic side effects were reported, no changes in mean values of serum calcium, parathyroid hormone, calcipotriol and in urinary excretion was noted even in those cases requiring the largest amounts of ointment (Ruzicka & Trompke, 2004). It is known that even repeated applications of tacalcitol leads to systemic absorption through the skin of less than 0.1% of the drug dose applied.

In the clinical practice therapeutic benefits are often obtained by applying in combination two or even more agents of different mechanisms of action. Therefore, here the attempt was made to assess the presence of synergistic effects of the combination of VD analogues and BR in various proportions. Since the VD receptor, and the glucocorticoid receptor belong to the nuclear receptor superfamily, the two hormones may act through similar pathways. Dexamethasone, for example, potentiates the anti-proliferating effects of 1α,25(OH)2D3 *in vitro* and *in vivo* (Bernardi *et al*., 2001). In SCC cells, the effect appears to be associated with increases in VDR protein and in ligand binding (Yu *et al*., 1998). It has been observed that VD-induced cell cycle arrest and apoptosis are increased by an addition of dexamethasone (Yu *et al*., 1998; Hershberger *et al*., 1999; Bernardi *et al*., 2001). A joint action of the antiproliferative VD and BR of mainly anti-inflammatory properties might have intensified the nasal fibroblast growth inhibition. In our study tacalcitol : BR in a 1 : 1 ratio and concentration of each at 10^{-4} M had better inhibitory properties than 10^{-4} M tacalcitol alone after 72 h of incubation (53% *vs.* 31%). At the other concentrations used the addition of BR did not substantially enhance the action of VD.

After 48 h and 72 h of incubation a significantly greater response was noted to calcitriol : BR in 1 : 1 and 3 : 1 ratio at the concentration of each 10^{-4} M when compared to calcitriol alone in the same concentration. Similarly, an improved inhibitory effect was observed at the concentration of 10^{-3} M each. In cultures pre-occluded with either calcitriol or tacalcitol at a concentration of 10^{-3} M each also in compounds with BR the number of dying cells increased substantially. The relatively quick cell death after exposure to those agents and the cell morphology indicated strong contribution of necrosis in that process. This observation does not agree with the principle that an ideal anti-proliferating agent should lead to cell death *via* apoptosis rather than necrosis. An opposite effect was noted in the case of tacalcitol at the concentration of 10^{-4} M. In those compounds containing tacalcitol the low number of necrotic cells with subsequent reduced proliferation suggests prevalence of apoptosis (Fig. 5). In those tests the best results were seen when cells were treated with tacalcitol : BR in 1 : 1 ratio each at a concentration of 10^{-4} M when lowered proliferation rate and cell number were the most apparent.

The mechanism underlying the antiproliferative effects of VD analogues has yet to be clarified; however, several mechanisms of activity have been proposed. The inhibitory effects could be mediated by inducing the expression of cyclin-dependent kinase inhibitors such as $p21^{WAF1/CIP1}$ (James *et al*., 1996), $p27^{KIP1}$ (Wu *et al*., 1997) and subsequent G0/G1 cell cycle arrest. Other extensively studied mechanisms include inhibition of DNA synthesis, promotion of apoptosis and regulation of pro-growth/pro-survival signal transduction pathways such as the mitogen-activated protein kinase (MAPK) and Akt pathways (McGuire *et al*., 2001). Other potential molecular effectors of the antiproliferative actions of 1α,25-(OH)2D3 could be TGF-β1, which exerts antiproliferative actions on epithelial cells (Koli & Keski-Oja, 1995). In addition, it was shown that the c-*MYC* protooncogene could be down-regulated by 1α,25-(OH)2D3 in breast cancer cells (Saunders *et al*., 1993).

VD has also been demonstrated to induce apoptosis, although the underlying processes are only beginning to be elucidated. It could be connected with up-regulation of genes associated with apoptosis such as TRPM-2/clusterin, cathepsin B and TNFα as well as with down-regulation of antiapoptotic genes such as *BCL-2* (Rocker *et al*., 1994; James *et al*., 1996). A unique influence of calcitriol on apoptosis was shown in a xenograft model of human retinoblastoma, where a VD metabolite had no significant effect on cell proliferation but induced apoptotic cell death (Audo *et al*., 2003).

In conclusion, the current study presents potential applicability of active vitamin D_3 analogues in the prevention and treatment of NP. Our study showed that only higher concentrations of VD analogues influenced fibroblast proliferation. However, tacalcitol, because of its weak interference with the phosphocalcine homeostasis, lower toxicity and better antiproliferative activity when compared to calcitriol, seems to be the more promising in NP therapy. Moreover, our study indicated the potential for NP

treatment with topical tacalcitol : BR compound for the first time.

Tacalcitol suppressed in a dose-dependent manner RANTES and other chemokines which are produced by fibroblasts and keratinocytes (Fukuoka *et al*., 1998). Downregulation of RANTES, a potent chemokine for eosinophils, and of other VDR-dependent chemokines which play an important role in NP formation may significantly improve VD efficacy in NP treatment. Due to the very limited published information on similar studies further research is mandatory.

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