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The proapoptotic protein Bim is up regulated by 1α,25-dihydroxyvitamin D_3 and its receptor agonist in endothelial cells and transformed by viral GPCR associated to Kaposi sarcoma

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ABSTRACT

We have previously shown that 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] and its less calcemic analog TX 527 induce apoptosis via caspase-3 activation in endothelial cells (SVEC) and endothelial cells transformed by the viral G protein-coupled receptor associated to Kaposi sarcoma (vGPCR). In this work, we studied whether intrinsic apoptotic pathway could be activated by changing the balance between anti and pro-apoptotic proteins. Time response qRT-PCR analysis demonstrated that the mRNA level of anti-apoptotic gene Bcl-2 decreased after 12 h and increased after 48 h treatment with 1α,25$(OH)_2D_3$ or TX 527 in SVEC and vGPCR cells, whereas its protein level remained unchanged through time. mRNA levels of pro-apoptotic gene Bax significantly increased only in SVEC after 24 and 48 h treatment with 1α,25$(OH)_2D_3$ and TX 527 although its protein levels remained unchanged in both cell lines. Bim mRNA and protein levels increased in SVEC and vGPCR cells. Bim protein increase by 1α,25$(OH)_2D_3$ and TX 527 was abolished when the expression of vitamin D receptor (VDR) was suppressed. On the other hand, Bortezomib (0.25–1 nM), an inhibitor of NF-κB pathway highly activated in vGPCR cells, increased Bim protein levels and induced caspase-3 cleavage. Altogether, these results indicate that 1α,25$(OH)_2D_3$ and TX 527 trigger apoptosis by Bim protein increase which turns into the activation of caspase-3 in SVEC and vGPCR cells. Moreover, this effect is mediated by VDR and involves NF-κB pathway inhibition in vGPCR.

1. Introduction

Kaposi sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi sarcoma (KS) [1]. KSHV virally encoded G protein-coupled receptor (vGPCR) is a constitutively active lytic phase protein with significant homology to the human interleukin-8 receptor [2,3]. Transgenic expression of vGPCR induced angiogenic lesions similar to those observed in human KS lesions [4,5]. KS lesions are heterogeneous, composed of endothelial-derived spindle cells as well as multiple inflammatory and mesenchymal cells. Viral production occurs in a limited number (1–5%) of the KS cells that undergo lytic replication [2]. Expression of the vGPCR oncogene in these cells and in cells aberrantly expressing lytic gene programs may contribute to sarcomagenesis because of complex signaling network activation, including the NF-κB and Akt–mTOR pathways and multiple Rho GTPases and MAPKs, concomitant with the expression of potent proangiogenic, proinflammatory and chemo attractant factors [2]. Persistent expression and activity of vGPCR is required for tumor maintenance [6]. Thus, vGPCR and its regulated signaling pathways may represent suitable candidates for KS treatment [7].

1α,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] or calcitriol, the most active metabolite of vitamin D, has significant antineoplastic activity in different types of cancer cells [8–10]. Most of the

Abbreviations: TX 527, [19-nor-14,20-bisepi-23-yne-1,25$(OH)_2D_3$]; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; NF-κB, nuclear factor kappa B; vGPCR, viral G protein-coupled receptor; SVEC, SV-40 immortalized murine endothelial cells; qRT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; VDR, vitamin D receptor; KSHV, the Kaposi's sarcoma-associated herpes virus; KS, Kaposi sarcoma; vGPCR-shVDR or -shctrl, endothelial cells targeted with small hairpin RNA against mouse VDR or control.

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activity of $1\alpha,25(OH)_2D_3$ is mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily [11]. The VDR is present not only in cells and tissues involved in calcium regulation but also in a wide variety of other cells including neoplastic cells [12]. We have previously shown that vitamin D agonists, $1\alpha,25(OH)_2D_3$ and its less calcemic analog TX 527, inhibit the proliferation of endothelial cells (SVEC) and endothelial cells transformed by the viral G protein-coupled receptor associated to Kaposi sarcoma (vGPCR) *in vitro* and *in vivo* by a mechanism that depends on VDR expression [13]. In addition, the proteasome inhibitor Bortezomib as well as vitamin D agonists also inhibited the NF-κB pathway and controlled the proliferation and expression of pro-inflammatory genes [14].

Apoptosis can be initiated via two alternative signaling pathways, the death receptor-mediated extrinsic apoptotic pathway and the mitochondrial-mediated intrinsic apoptotic pathway [15–17]. Mitochondria play critical roles in the regulation of various apoptotic processes, including drug-induced apoptosis [17,18]. In several cancer cells, $1\alpha,25(OH)_2D_3$ induces apoptosis by activation of the intrinsic pathway. $1\alpha,25(OH)_2D_3$ suppresses the expression of anti-apoptotic genes, such as Bcl-2 [19,20], and increases the expression of the pro-apoptotic gene Bax [12,21–23], however, not all cancer cells respond to this mechanism [24]. Evasion of cell death is fundamental to the development of cancer and its metastasis; the role of the pro-apoptotic BH3-only protein Bim in the suppression of metastasis in breast tumor cells has been recently reported [25].

In a previous work, we have shown that $1\alpha,25(OH)_2D_3$ and its less calcemic analog TX 527 inhibit the proliferation of endothelial cells (SVEC) and endothelial cells transformed by the viral G protein-coupled receptor associated to Kaposi sarcoma (vGPCR) by inducing cell cycle arrest and apoptosis through a mechanism that involved the activation of caspase-3 [26]. In this work, we further explore the effects of $1\alpha,25(OH)_2D_3$ and TX 527 in SVEC and vGPCR on the apoptotic pathway by studying the mRNA and protein expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic proteins Bax and Bim.

2. Experimental

2.1. Chemicals and reagents

The vitamin D analog TX 527 [19-nor-14,20-bisepi-23-yne-1,25 $(OH)_2D_3$], originally synthesized by M. Vandewalle and P. De Clercq (University of Ghent, Ghent, Belgium), was provided by Théramex (Monaco). $1\alpha,25(OH)_2D_3$, Immobilon P (polyvinylidenedifluoride; PVDF) membranes and the antibiotic G418 were supplied by Sigma–Aldrich (St. Louis, MO, USA). Puromycin was provided by Invivogen (San Diego, CA, USA). The antibodies used: rat monoclonal anti-VDR (Affinity Bioreagents, Golden, CO, USA), rabbit polyclonal anti-Bcl-2 and anti-Bim (Cell Signaling Technology, Danvers, MA, USA); anti-Bax (Thermo Fisher Scientific Inc., Rockford, IL USA); anti-tubulin, anti-rabbit, anti-mouse and anti-rat horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). High Pure RNA Isolation Kit was provided by Roche Applied Science (Indianapolis, IN, USA). Superscript II Reverse transcriptase was acquired from Invitrogen (Gent, Belgium). The SYBR Green PCR Master Mix reagent and TaqMan Fast Univ. PCR Master Mix were from Applied Biosystems (Foster City, CA, USA) and PCR primers and fluorogenic probes for mouse β-actin and Bax primers were received from prof. C. Mathieu (KU Leuven, Leuven, Belgium). TX 527 and $1\alpha,25(OH)_2D_3$ were used at 10 nM because this concentration consistently showed antiproliferative effects [13,24]. Other chemicals used were of analytical grade.

2.2. Cell lines and transfections

SV-40 immortalized murine endothelial cells (SVEC) and stably expressing viral G protein-coupled receptor full-length (vGPCR) were used. Stable over expression of vGPCR promotes tumor formation when injected into immune-suppressed mice and induces angiogenic lesions similar to those developed in Kaposi sarcoma [4,13]. Transfected cells were selected with 500 μg/ml G418. Stable SVEC and vGPCR endothelial cells targeted with small hairpin RNA against mouse VDR (SVEC-shVDR and vGPCR-shVDR) or control shRNA (SVEC-shctrl and vGPCR-shctrl) were obtained by transduction of lentiviral particles generated in HEK293T cells following the manufacturer's protocols as reported before [13]. The stable cell lines were selected with 2 μg/ml puromycin and the medium was freshly changed every other day. VDR knock-down was monitored by Western blot analysis.

2.3. Quantitative real-time PCR

Total RNA for real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was isolated using the High Pure RNA Isolation Kit (Roche). RNA (0.5–1 μg) was reverse transcribed using the Superscript II Reverse transcriptase and qRT-PCR reactions were performed on the resulting cDNA (2 μl of cDNA; dilution 1/10) in an ABI 7500 Real Time PCR system (Applied Biosystems, CA, USA). Specific primers were used to detect Bcl-2, Bax, Bim levels. The real time PCR data was analyzed by 2-delta delta Ct method using β actin as reference parameter. Reactions were carried out using the SYBR Green PCR Master Mix reagent. Sequences of forward primers (Fw), and reverse primers (Rv) were as follows: Bcl-2: TCATCCACAGGGCGATGTT (Fw), CGGTGGTGGAGGAACTCTTC (Rv); Bim: GCTCTGCACTGTGTCGATG TG (Fw), GCCCTCCCTTGTTTACATTCAC (Rv). Sequences of forward/reverse primers, and detection probes for β actin and Bax were designed by prof. C. Mathieu (Laboratory of Clinical and Experimental Endocrinology, KU Leuven, Leuven, Belgium) and the reactions were carried out using TaqMan Fast Univ. PCR Master Mix (Applied Biosystems).

2.4. SDS–PAGE and Western blot analysis

Total proteins from whole cell lysates were subject to Western blot analyses as reported before [27]. Briefly, cell lysates were prepared using lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 0.5 mM PMSF. The protein concentration of each sample was determined by Bradford method [28]. Equal amounts of protein (15–20 μg) from each sample were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA) and incubated with appropriately diluted specific primary antibodies after blocking unspecific sites on the membrane with 5% non fat dry milk. Antibodies dilutions used include monoclonal rat anti-VDR (1:1,500), rabbit anti-Bcl-2 (1:1,000), anti-Bax (1:1,000) anti-Bim (1:500), cleaved caspase-3 (1:500) and mouse tubulin (1:2000) combined with anti-rat (1:5000), rabbit (1:10,000) and anti-mouse (1:5000) horseradish peroxidase–conjugated secondary antibodies respectively. Immunoreactive bands were detected by Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Argentina) and quantified using image J software, a public domain program, developed at the National Institutes of Health.

2.5. Statistical analysis

Quantitative results were expressed as means ± SD. Results from qRT-PCR and Western blot were analyzed by one way ANOVA followed by Bonferroni test to evaluate differences between control (vehicle) and treated conditions ($1\alpha,25(OH)_2D_3$, TX 527) at each time point and between ctrl and Bortezomib (0.25–1 nM) in doses response studies, $^{*}p < 0.05$. The Western blots shown are representative of at least three analyses performed on samples from at least three independent experiments.

3. Results and discussion

Apoptosis or programmed cell death is a key regulator of physiological growth control and regulation of tissue homeostasis. One of the most important advances in cancer research in recent years is the recognition that cell death mostly by apoptosis is crucially involved in the regulation of tumor formation and also critically determines treatment response [29].

The Bcl-2 gene has been implicated in a number of cancers, including B- and T-cell lymphomas, cervical, lung, breast, prostate, and colorectal cancers [30,31] and has also been involved in resistance to conventional cancer treatment [32,33]. Bcl-2 expression is up regulated in endothelial cells expressing vGPCR being a survival advantage and suggesting an important role for Bcl-2 in the sarcomagenesis induced by KSHV [34]. Bcl-2 family proteins regulate mitochondria-dependent apoptosis with the balance of anti- and pro-apoptotic members. Thus, inhibition of Bcl-2 expression or changing the balance between pro- and anti-apoptotic proteins could be an important mechanism to trigger apoptosis in vGPCR cells. In this work, we further explore the effects of $1\alpha,25(OH)_2D_3$ and TX 527 in SVEC and vGPCR cells on the apoptotic pathway studying the regulation of Bcl-2, Bax and Bim expression at mRNA and protein level. First, SVEC and vGPCR cells were plated for 1 day, then starved for 24 h and treated with 10 nM of $1\alpha,25(OH)_2D_3$ or TX 527 or vehicle (0.01% ethanol) in the presence of 2% FBS for different periods of time (3–72 h). Total RNA was extracted and reverse transcribed. At each time point, gene

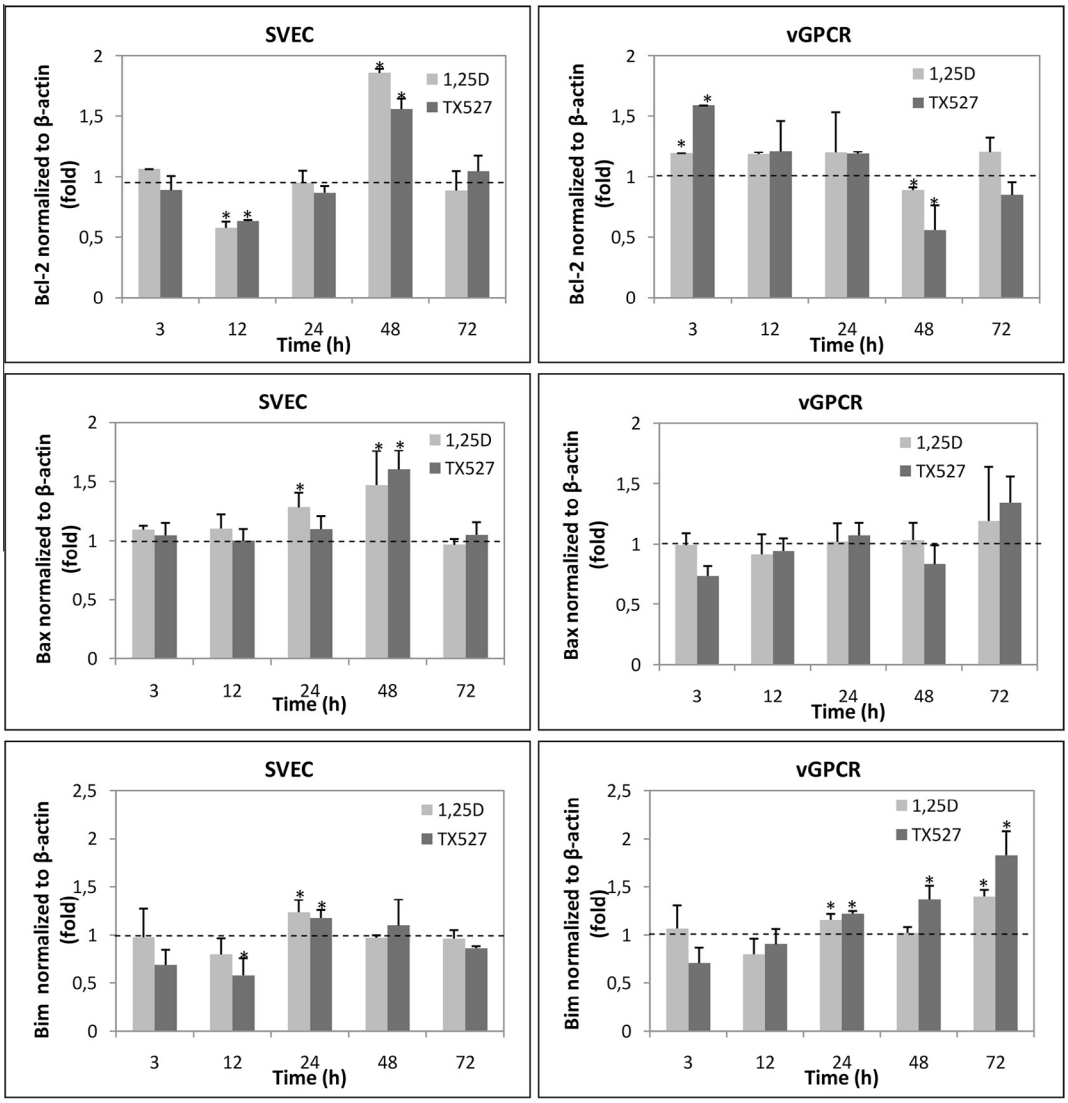

Fig. 1. Regulation of Bcl-2, Bax and Bim mRNA expression by $1\alpha,25(OH)_2D_3$ and TX 527. SVEC and vGPCR cells were cultured and treated with 10 nM of $1\alpha,25(OH)_2D_3$ (1,25D) and TX 527 or vehicle (0.01% ethanol) for 3–72 h. Total RNA was extracted and reverse transcribed. Gene expression of Bcl-2, Bax, Bim and β-actin was assessed by qRT-PCR analysis at each time point. Bar graphs show quantitative results expressed as a ratio between treated (1,25D or TX 527) versus control (vehicle) samples normalized to β-actin RNA levels. The statistical significance of the data from three independent experiments at each time point was evaluated using one way-ANOVA followed by Bonferroni test $^{*}p < 0.05$.

expression of Bcl-2, Bax, and Bim was assessed by qRT-PCR analysis using specific primers and β-actin mRNA was used to normalize gene expression. The results shown in Fig. 1 indicated that Bcl-2 gene expression decreased after12 h and increased after 48 h treatment with $1\alpha,25(OH)_2D_3$ or TX 527 in SVEC cells. On the contrary, a rapid increase at 3 h and a significantly decrease at 48 h in vGPCR cells was observed. Bax mRNA expression significantly increased in SVEC cells 24 h and 48 h after treatment with $1\alpha,25(OH)_2D_3$ or TX 527, while no effects were found in vGPCR after vitamin D agonists' stimulation. Bim mRNA expression increase was observed only at 24 h in SVEC, whereas Bim gene expression levels increase was sustained up to 72 h after $1\alpha,25(OH)_2D_3$ or TX 527 treatment in vGPCR. To further investigate whether the changes in mRNA levels were reflected in changes in protein expression, Bcl-2, Bax and Bim protein levels were analyzed by Western blot. To that end, time-response studies (12–48 h) with $1\alpha,25(OH)_2D_3$ (10 nM), TX 527 (10 nM), or vehicle in SVEC and vGPCR cells were performed. Total proteins from whole cell lysates were prepared and subject to Western blot analysis with Bcl-2, Bax and Bim. Protein bands quantification were done using Image J program and normalized with tubulin. The results in Fig. 2 revealed that neither Bcl-2 nor Bax protein levels were significantly changed at each time point. In contrast, Bim protein levels were up regulated in SVEC and vGPCR cells (Fig. 3). Bim, a BH3 only protein member of the Bcl-2 family proteins, causes apoptosis by disrupting mitochondrial integrity. Its role in inducing apoptosis in cancer cells has recently been demonstrated [35–38]. According to our results, the compound 5,7-dihydroxy-8-nitrochrysin, achrysin analog, sufficiently induced Bim protein expression to drive cells into apoptosis in MDA-MB-453 breast cancer cells while Bcl-2, Bcl-xl and Bax protein levels did not substantially change [17]. Moreover, in colorectal cancer cells, Bim contributes to reactive oxygen species to induce apoptosis under selenite treatment [39].

We have recently reported that $1\alpha,25(OH)_2D_3$ and TX 527 induces apoptosis in SVEC and vGPCR cells by a mechanism that depends on VDR expression [26]. Therefore, we investigated whether $1\alpha,25(OH)_2D_3$ or TX 527 induced Bim protein levels through the participation of VDR. VDR knock-down SVEC and vGPCR cells, SVEC-shVDR and vGPCR-shVDR respectively, or control SVEC-shctrl and vGPCR-shctrl, were cultured and treated with both vitamin D agonists ($1\alpha,25(OH)_2D_3$ and TX 527 at 10 nM) or vehicle for 48 h, since 48 h was the maximum observed Bim increase in both SVEC and vGPCR cells (Fig. 3). Total proteins from whole cell lysates were subject to Western blot with anti VDR, Bim and tubulin antibodies. First, we monitored VDR expression to corroborate VDR down expression (Fig. 4 upper panel). Both agonist, $1\alpha,25(OH)_2D_3$ and TX 527, increased VDR expression at 48 h as we have demonstrated before in these cells lines [13,26]. Bim protein levels, detected by Western blot increase significantly in both, SVEC and vGPCR after 48 h treatment with $1\alpha,25(OH)_2D_3$ or TX 527. This effect was suppressed when the VDR was knocked down (Fig. 4 lower panel).VDR down regulation also impairs caspase-3

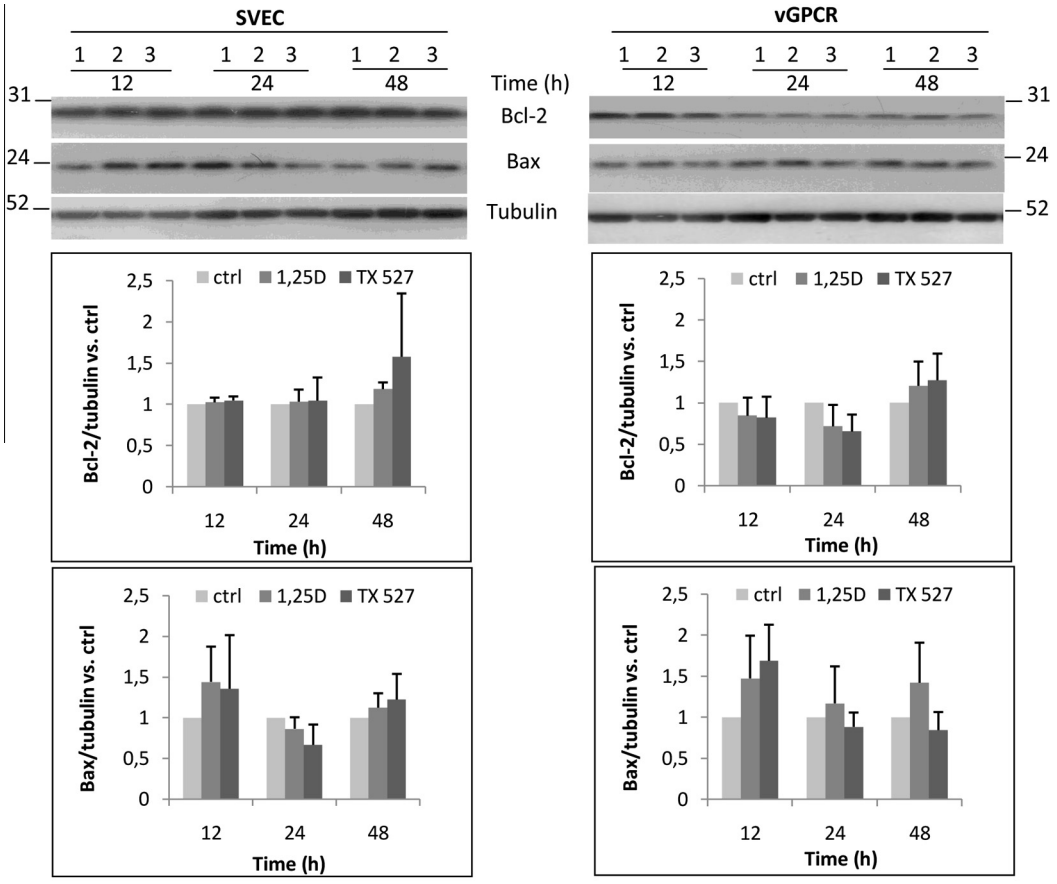

Fig. 2. Bcl-2 and Bax protein levels remain unchanged upon $1\alpha,25(OH)_2D_3$ and TX 527 treatment. SVEC and vGPCR cells were cultured and treated with 10 nM of $1\alpha,25(OH)_2D_3$ (lane 2) and TX 527 (lane 3) or vehicle (lane 1, 0.01% ethanol) for 12–48 h. Western blots were performed with anti-Bcl-2, anti-Bax and anti-tubulin antibodies. A representative blot from three independent experiments is shown. Protein bands quantification from three independent experiments was done using Image J program. The results were then represented in bar graphs as a radio of Bcl-2 or Bax protein normalized with tubulin from treated conditions versus the control. Significant differences between control (vehicle) and stimulated cells at each time point were analyzed by one way ANOVA followed by Bonferroni test, $^{*}p < 0.05$.

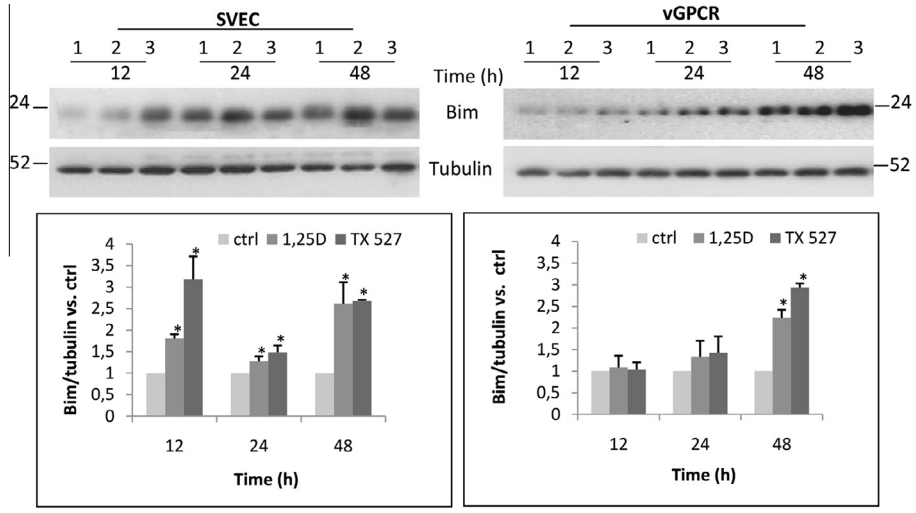

Fig. 3. $1\alpha,25(OH)_2D_3$ and TX 527 increase Bim protein levels. SVEC and vGPCR cells were cultured and treated with 10 nM of $1\alpha,25(OH)_2D_3$ (lane 2) and TX 527 (lane 3) or vehicle (lane 1, 0.01% ethanol) for 12–48 h. Total proteins from cell lysates were subject to Western blots with anti-Bim and anti-tubulin antibodies. A representative blot from three independent experiments is shown. Protein bands quantification from three experiments was done as shown in Fig. 2. The results represented in bar graphs were expressed as a radio of Bim protein normalized with tubulin from treated conditions versus the control. Significant differences between control (vehicle) and stimulated cells at each time point were analyzed by one way ANOVA followed by Bonferroni test, $^{*}p < 0.05$.

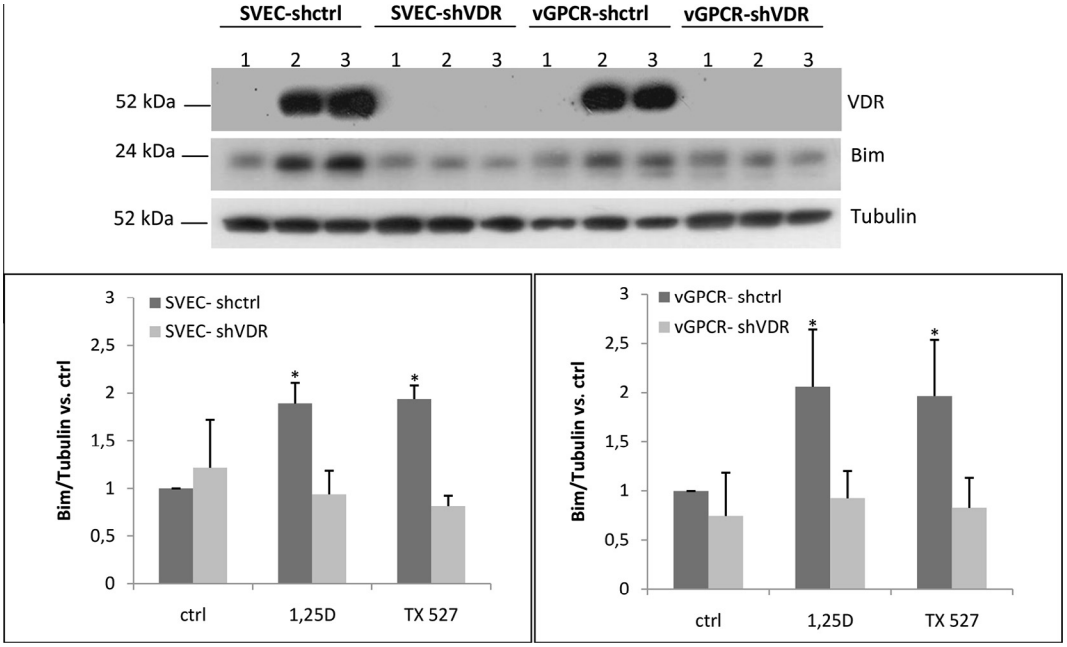

Fig. 4. Bim up regulation by vitamin D agonist is VDR dependent. Stable SVEC and vGPCR cells targeted with small hairpin RNA against mouse VDR (SVEC-shVDR and vGPCR-shVDR) or control shRNA (SVEC-shctrl and vGPCR-shctrl) respectively were treated with vehicle (lane 1), $1\alpha,25(OH)_2D_3$ (lane 2), or TX 527 (lane 3) at a concentration of 10 nM for 48 h. Total proteins from cell lysates were subject to Western blot analysis with anti-VDR, Bim and tubulin antibodies. A representative Western blot from three independent experiments is shown. Bim protein bands from three experiments were quantified, normalized using tubulin and represented in bar graphs as shown in Fig.2. Significant differences between control (vehicle) and stimulated cells were analyzed by one way ANOVA followed by Bonferroni test, $^{*}p < 0.05$.

cleavage in SVEC and vGPCR upon $1\alpha,25(OH)_2D_3$treatment [26] supporting the idea that Bim can trigger apoptosis by a mechanism dependent on caspase-3 activation.

Down regulation of the NF-κB pathway is part of the mechanism involved in the antiproliferative effects of $1\alpha,25(OH)_2D_3$ on endothelial cells transformed by vGPCR [40]. Also, the inhibition of NF-κB pathway by Bortezomib or vitamin D agonist TX 527decreased the proliferation and expression of pro-inflammatory genes in vGPCR cells [14]. Hence, we investigated whether the inhibition of NF-κB induced changes on Bcl-2, Bax and Bim expression in SVEC and vGPCR cells. In addition, since Bortezomib has shown to decrease cell proliferation and to induce cell cycle arrest in vGPCR cells upon TX 527 treatments [13], we investigated whether Bortezomib induced caspase-3 cleavage in SVEC and vGPCR cells. To that end, SVEC and vGPCR cells were cultured in DMEM 2% FBS and treated with Bortezomib (0.25–1 nM), or vehicle (0.1% DMSO) for 24 h. Western blotting was performed to detect Bim, Bax and Bcl-2 and cleaved caspase-3 protein levels. The results shown in Fig. 5 demonstrated that Bim and caspase-3 protein levels significantly increased in a dose-dependent manner,

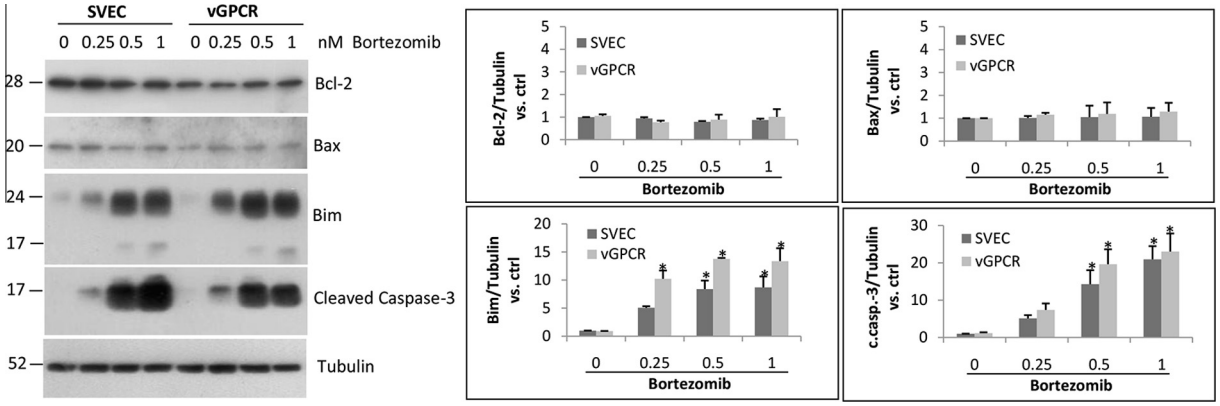

Fig. 5. The proteasome inhibitor Bortezomib induces Bim expression and caspase-3 cleavages in a dose dependent fashion. SVEC and vGPCR cells were cultured and treated with the proteasome inhibitor Bortezomib (0.25–1 nM) or vehicle (0.1% DMSO) for 24 h. Western blots were performed with anti-Bim, anti-Bcl-2, anti-Bax, anti-cleaved caspase-3 and anti-tubulin antibodies. A Representative blot from three independent experiments is shown. Protein bands quantification from three independent experiments was performed as shown in Fig. 2. The results were then represented in bar graphs as a radio of Bcl-2, Bax, Bim or cleaved caspase-3 protein normalized with tubulin from Bortezomib (0.25–1 nM) conditions versus the control. Significant differences between control and Bortezomib conditions were analyzed by one way ANOVA followed by Bonferroni test, $^{*}p < 0.05$.

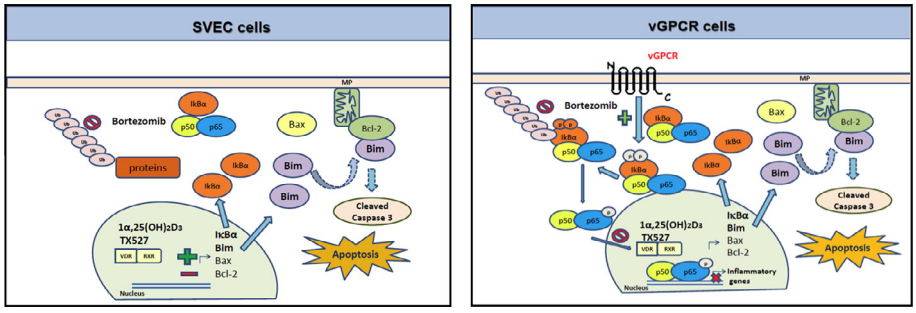

Fig. 6. Proposed model of apoptosis induced by vitamin D agonists and Bortezomib in SVEC and vGPCR cells. Either 1α,25(OH)$_2$D$_3$ or TX 527 through VDR binding induce an increase of Bim protein and caspase 3 cleavage without changing the levels of Bcl-2 nor Bax proteins in SVEC and vGPCR cells. Similarly to vitamin D agonist, Bortezomib induces and increases IκB protein inhibiting NF-κB pathway. Besides, the expression of inflammatory genes in vGPCR cells as well as an increase in Bim expression occurs in both cell lines. Bim protein could associate to Bcl-2 at the mitochondria membrane and induce caspase 3 cleavage.

while no changes were observed in Bcl-2 and Bax protein levels in both SVEC and vGPCR cells. In agreement with our results, it was observed that Bim transcription is repressed due to the kit mutation in human mast cells expressing the Kit D816V mutation, but when the proteasome is inhibited, Bim is upregulated and induces caspase-3-dependent apoptosis [41]. Apoptosis mediated by Bim protein has recently been reported under glucose and oxygen-deprivation conditions in cardiomyocytes [42] and cytokine deprivation in mouse mast cells [43]. Our results show that for the first time vitamin D agonists 1α,25(OH)$_2$D$_3$ or TX 527 induce a change in the balance of pro and anti-apoptotic proteins in SVEC and vGPCR cells. We have found that the expression of the pro-apoptotic protein Bim increased without changing the levels of Bcl-2 nor Bax proteins. Although the apoptosis induction mechanism has not yet been elucidated, Bim protein could associate to Bcl-2 at the mitochondria membrane and induce caspase-3cleavage. Similarly to vitamin D agonist, Bortezomib also induced Bim expression and caspase-3 cleavage suggesting that the inhibition of NF-κB pathway may be involved in the effects mediated by the agonists in vGPCR cells (Fig. 6).

4. Conclusions

Taken together, these results demonstrate that the increase of the pro-apoptotic BH3-only protein Bim by 1α,25(OH)$_2$D$_3$ or TX 527 in endothelial cells and cells transformed by vGPCR is sufficient to induce apoptosis by a caspase-3 dependent mechanism. Moreover, this effect is mediated by VDR and involves the inhibition of the NF-κB pathway in vGPCR transformed cells.

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