Vitamin D: proteases, protease inhibitors and cancer

Silvia Álvarez-Díaz¹, María Jesús Larriba¹, Ph. D., Carlos López-Otín², Ph. D. and Alberto Muñoz¹*, Ph. D.

¹Instituto de Investigaciones Biomédicas "Alberto Sols", Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Arturo Duperier 4, E-28029 Madrid, Spain.
²Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, Julián Clavería s/n, E-33006 Oviedo, Spain.

*Correspondence to: Alberto Muñoz; Instituto de Investigaciones Biomédicas "Alberto Sols", Arturo Duperier 4, E-28029 Madrid, Spain; E-mail: amunoz@iib.uam.es
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**Abbreviations:** DUB, deubiquitinating enzyme; ECM, extracellular matrix; KLK, kallikrein; MMP, matrix metalloprotease; NES1, normal epithelial cell-specific 1; PAI, plasminogen activator inhibitor; RXR, retinoid X receptor; TIMP, tissue inhibitor of metalloproteases; tPA, tissue plasminogen activator; TNFα, tumor necrosis factor α; VDR, vitamin D receptor; VDRE, vitamin D response element; UB, ubiquitinating enzyme; uPA, urokinase plasminogen activator; UPS, ubiquitin-proteasome system; 1,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃.
Abstract

The active vitamin D metabolite 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, Calcitriol) is a major regulator of gene expression in higher organisms. Protein abundance is an endpoint of gene expression that results from the balance between induction and degradation and is essential for adequate cell function. Proteins are degraded by proteases whose activity is in turn controlled by a number of endogenous protease inhibitors. 1,25(OH)₂D₃ regulates several proteases and protease inhibitors in different cell types, putatively contributing to its regulatory effects of cell physiology. We have recently shown that 1,25(OH)₂D₃ strongly induces the expression of cystatin D, an inhibitor of several cysteine proteases of the cathepsin family. Cystatin D induction may contribute to the antitumor effect of 1,25(OH)₂D₃ against colon cancer by mechanisms that are both dependent and independent of cathepsin inhibition. Transcriptomic studies suggest that 1,25(OH)₂D₃ also modulates the function of the ubiquitin-proteasome system. Thus, proteases and protease inhibitors are candidates to mediate to a certain extent the complex action of 1,25(OH)₂D₃ in cancer cells.

**Running title:** Vitamin D regulates cellular protein degradation.
Protein degradation: proteases and protease inhibitors

In healthy cells and organisms the balance between protein production and degradation is of crucial importance. Proteins are degraded by proteolytic enzymes (proteases). They are classified in five major classes based on the specific chemical groups responsible for the catalysis (serine-, cysteine-, aspartic-, threonine- and metallo-proteases). Most proteases are relatively nonspecific for substrates but some of them show specificity toward a single peptide bond of a particular protein. The complete set of proteases produced by human cells consists of at least 569 proteases and homologues distributed into these five classes.

The action of proteases is tightly controlled by a powerful regulatory system. A crucial role in the regulation of protease activity is exerted by endogenous protease inhibitors. They protect cells and tissues against the proteolytic activity of proteases that are often secreted by or released from normal dying cells or in pathologic situations such as tumor progression. Endogenous inhibitors are always proteins but their number is much lower than that of proteases. One of the systems used to classify these inhibitors is based on the catalytic class of proteases they target.

Another critical component of the protein degradation machinery is the ubiquitin-proteasome system (UPS), which operates both in the nucleus and in the cytosol. UPS consists of a large number of proteins that are dedicated to the identification, targeting and destruction of unneeded or damaged proteins by proteolysis. Proteins are tagged for degradation by a small protein (8.5 kDa) called ubiquitin. This is a multistep process that involves at least three classes of enzymes generally known as ubiquitinating enzymes (UBs): ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin protein ligases (E3). In addition, deubiquitinating enzymes (DUBs) also play a crucial role regulating this process.
proteasome contains a barrel-shaped proteolytic core complex (the proteasome 20S) capped at one or both ends by 19S regulatory complexes, which recognize ubiquitinated proteins. This multicatalytic system includes three different types of proteases with complementary activities: chymotrypsin-like, trypsin-like, and caspase-like activities. Proteases, especially those that degrade extracellular matrix (the ECM), play an important role in tumor development providing access for tumor cells to the vascular and lymphatic systems, thus favoring angiogenesis and invasion. Moreover, certain proteases also promote tumor cell proliferation and resistance to apoptosis showing that proteolytic enzymes can contribute to all stages of tumor progression. Accordingly, proteases have been studied in multiple cancer types, with special attention to the matrix metalloprotease (MMP) family but also, more recently, to the cysteine and serine protease families. Due to the crucial role of proteases in tumor development, the identification and study of their inhibitors and their role in tumor progression are of pressing interest. Remarkably, however, recent studies have provided evidence of intra- and extra-cellular proteases with antitumor properties. These results support an emerging and paradoxical role for some proteases in tumor suppression.

Vitamin D has antitumor effects

Vitamin D is a fat-soluble secosteroid prohormone obtained from diet, dietary supplements and, mainly, from the conversion of 7-dehydrocholesterol to vitamin D₃ in the skin by the action of solar UV-B radiation (280-320 nm). The active form of vitamin D₃, 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, Calcitriol), is obtained after 25-hydroxylation of vitamin D₂ in the liver and subsequent 1α-hydroxylation in the kidney and in certain vitamin D target tissues. 1,25(OH)₂D₃ has pleiotropic effects in the organism: in addition to regulating calcium and phosphate metabolism and bone
biology, it has antiproliferative, anti-invasive, pro-apoptotic and prodifferentiation activity. These novel actions suggest, together with epidemiological data and the results of experimental studies, a protective role of 1,25(OH)\(_2\)D\(_3\) against several neoplasias (reviewed in ref. 12). To avoid the toxic hypercalcemic effect associated with the high-dose 1,25(OH)\(_2\)D\(_3\) treatments required for \textit{in vivo} activity, many derivatives generically termed deltanoids have been synthesized.\(^{13,14}\)

1,25(OH)\(_2\)D\(_3\) and its analogs act by interacting with their high affinity receptor (vitamin D receptor, VDR), through a complex network of genomic and non-genomic mechanisms.\(^{15-17}\) To exert its genomic effects, VDR forms heterodimers with another nuclear receptor (retinoid X receptor, RXR), which interacts with specific DNA sequences known as vitamin D response elements (VDREs) in target genes (reviewed in ref. \(^{18}\)). Global transcriptomic studies have identified a large number of candidate 1,25(OH)\(_2\)D\(_3\) target genes in several types of human tumoral cells.\(^{19-25}\) They include genes coding for proteins with a wide variety of functions that are responsible for the antitumor action of 1,25(OH)\(_2\)D\(_3\). Remarkably, some genes encode both proteases and protease inhibitors.

**Vitamin D regulates cysteine proteases and cystatins**

Recently, we have demonstrated in human colon cancer cells that 1,25(OH)\(_2\)D\(_3\) induces the expression of cystatin D, an inhibitor of several cysteine proteases of the cathepsin family, which had not been previously linked to cancer.\(^{26}\) Cystatin D has a narrow inhibitory profile. Unlike other members of the cystatin family, it inhibits only cathepsin S, H, and L, but not cathepsin B, the most widely characterized cathepsin. In addition, cystatin D expression had only been found in parotid and submandibular glands and saliva.\(^{27,28}\) Cystatin D was identified as a candidate 1,25(OH)\(_2\)D\(_3\) target gene in a
transcriptomic analysis performed in human SW480-ADH colon cancer cells.\textsuperscript{22} 1,25(OH)\textsubscript{2}D\textsubscript{3} increases cystatin D RNA and protein expression in these cells due to a direct transcriptional activation that required functional VDR.\textsuperscript{26} Transactivation assays showed that full activation is mediated by a cluster of VDR binding sites located close to the transcription start site at the cystatin D promoter. This is consistent with the proposal that isolated, simple VDREs may be not functional \textit{in vivo} but that, on the contrary, VDR binding site clusters or modules contribute to chromatin decondensation which makes it easier for the transcription machinery to accede to the promoter.\textsuperscript{18}

Importantly, ectopic expression of cystatin D in SW480-ADH cells inhibits proliferation, mimicking the effect of 1,25(OH)\textsubscript{2}D\textsubscript{3}. The expression of certain genes was also similarly altered by exogenous cystatin D and by 1,25(OH)\textsubscript{2}D\textsubscript{3}. Thus, classical target genes of 1,25(OH)\textsubscript{2}D\textsubscript{3} such as \textit{CDH1} encoding the adhesion protein E-cadherin or the c-\textit{MYC} oncogene were induced or repressed, respectively, in cystatin D-expressing cells. To corroborate the hypothesis that cystatin D was an important mediator of 1,25(OH)\textsubscript{2}D\textsubscript{3} actions, we knocked-down cystatin D by stable shRNA expression. We found that the antitumor effects of 1,25(OH)\textsubscript{2}D\textsubscript{3}, such as the acquisition of a highly adhesive epithelial phenotype and the inhibition of proliferation, were partially blocked in these cells. In contrast to the effect of cystatin D overexpression, cystatin D knocked-down cells showed decreased expression of E-cadherin protein and increased expression of c-MYC.\textsuperscript{26} Together, our data indicate that cystatin D has an unexpected crucial role in 1,25(OH)\textsubscript{2}D\textsubscript{3} antitumor activity in human colon cancer cells (Fig.1).

How this protease inhibitor exerts its action is unclear. Based on our results obtained using mutant cystatin D proteins with reduced antiproteolytic activity, it seems that not all the antitumor effects of cystatin D are mediated by the inhibition of cathepsins. Thus, the reduction of cell proliferation and c-MYC downregulation are
independent of cathepsin inhibition (Fig. 1). These results open the door to further studies to identify the mechanism by which this protease inhibitor partly exerts its antitumor effects and contributes to the action of 1,25(OH)₂D₃. The finding that cystatin D was also regulated by the 1,25(OH)₂D₃ analog EB1089 in xenografted mice and the significant correlation found between cystatin D expression and VDR protein levels in human colon cancer biopsies sustain a role for 1,25(OH)₂D₃ in the regulation of cystatin D in vivo.

Cystatin D is not the only cystatin regulated by 1,25(OH)₂D₃. Previously, cystatin E/M had been identified as a target of the 1,25(OH)₂D₃ analog EB1089 in squamous carcinoma cells. The upregulation of cystatin E/M by the hormone was confirmed by Northern blot and immunofluorescence analyses. Interestingly, cystatin E/M is downregulated in cancer, and several authors relate the loss of its expression with breast cancer progression and propose it as a tumor suppressor. However, no functional studies relating cystatin E/M and 1,25(OH)₂D₃ in squamous cell carcinoma have been reported. Similarly, cystatin A is regulated by 1,25(OH)₂D₃ in normal keratinocytes. This protease inhibitor is considered a differentiation marker for this particular cell type. 1,25(OH)₂D₃ increases human cystatin A expression by inhibiting the Raf-1/MEK1/ERK signaling pathway via a non-genomic response.

The view emerging from all these studies is that cystatins are used by 1,25(OH)₂D₃ as mediators of its genomic and non-genomic effects. At least part of the contribution of cystatins to 1,25(OH)₂D₃ action seems to be independent of its capacity to inhibit cathepsin activity.

Although the regulation of cystatins by 1,25(OH)₂D₃ is a relatively recent finding, the induction of cathepsin B by 1,25(OH)₂D₃ in breast cancer cells was first reported in 1996. Cathepsin B is a ubiquitously expressed cysteine protease that has
been described as a general marker of apoptosis and plays an important role in the cell death induced by tumor necrosis factor (TNF)α. Cathepsin B is released from lysosomes during TNFα-induced apoptosis and promotes the release of mitochondrial cytochrome c.\textsuperscript{34-36} In breast cancer cells, 1,25(OH)\textsubscript{2}D\textsubscript{3}-induced apoptosis requires the dissipation of mitochondrial membrane potential leading to the release of cytochrome c, the generation of oxidative stress and the redistribution of BAX from the cytosol to mitochondria.\textsuperscript{37} Therefore, cathepsin B induction by 1,25(OH)\textsubscript{2}D\textsubscript{3} could be attributed to the pro-apoptotic activity of the hormone. In addition, the induction of cathepsin B by 1,25(OH)\textsubscript{2}D\textsubscript{3} in MCF-7 breast cancer cells enhances TNFα-induced apoptosis.\textsuperscript{38} However, the induction of cathepsin B by 1,25(OH)\textsubscript{2}D\textsubscript{3} does not seem to play a central role in the apoptotic pathway but may rather be a downstream event in the induction of cell death.\textsuperscript{37,38}

Another member of the cathepsin family, cathepsin L, has been reported to be a target of 1,25(OH)\textsubscript{2}D\textsubscript{3}. A transcriptomic analysis, later confirmed by qRT-PCR, showed that the expression of cathepsin L is inhibited by 1,25(OH)\textsubscript{2}D\textsubscript{3} in MDA-MB-231 breast cancer cells.\textsuperscript{23} This protease has been related with the promotion of malignancy in multiple cancer types and its inhibition by 1,25(OH)\textsubscript{2}D\textsubscript{3} could be part of the antitumor action of 1,25(OH)\textsubscript{2}D\textsubscript{3} in breast cancer.

**Vitamin D modulates the activity of matrix metalloproteases and serine proteases**

Matrix metalloproteases (MMPs) are a family of zinc-dependent proteases capable of degrading components of the extracellular matrix and basement membrane. MMPs are regulated by the action of specific inhibitors: the tissue inhibitors of metalloproteases (TIMPs). Both MMPs and TIMPs are frequently disrupted in cancer, which promotes
the migration and invasion of cancer cells. A number of studies have shown the
regulation of MMPs and/or TIMPs by 1,25(OH)_{2}D_{3}. Treatment with 1,25(OH)_{2}D_{3} of
breast and prostate cancer cells decreases expression of MMP-9 and increases TIMP-1
levels.\textsuperscript{39, 40} The modulation of the MMP/TIMP balance may be a mechanism by which
1,25(OH)_{2}D_{3} inhibits invasion. In addition, EB1089 inhibits MMP-13 and, surprisingly,
induces MMP-1 and MMP-3 RNA expression in squamous cell carcinoma lines.\textsuperscript{20} The
fact that each MMP has specific, and still not completely understood, function may
eventually explain the differences in their regulation by 1,25(OH)_{2}D_{3}. In fact, MMP-3 is
considered a dual protease in terms of cancer-modulating properties.\textsuperscript{2}

Researchers have also analyzed the effect of 1,25(OH)_{2}D_{3} on the expression of
urokinase and tissue plasminogen activators (uPA and tPA, respectively) as well as their
inhibitors (PAIs). The serine proteases uPA and tPA convert the proteolytically inactive
plasminogen into plasmin, a wide-spectrum protease capable of degrading ECM and
basement membrane proteins and activating pro-MMPs. 1,25(OH)_{2}D_{3} decreases the
secretion of uPA and tPA and increases that of PAI-1 in breast cancer cells, leading to a
reduction in total PA activity.\textsuperscript{39} This 1,25(OH)_{2}D_{3}-mediated regulation of the
plasminogen activator system was observed in breast- but not in prostate-cancer cells.\textsuperscript{39, 40}

Quantitative RT-PCR studies recently performed in our laboratory revealed that
1,25(OH)_{2}D_{3} causes a 2.3-fold increase in the expression of PAI-1 in SW480-ADH
human colon cancer cells. Additional work is needed to elucidate the possible
contribution of this inhibitor to the antitumor effects of 1,25(OH)_{2}D_{3} in colon cancer.

1,25(OH)_{2}D_{3} also regulates other members of the serine protease family.
Transcriptomic studies using oligonucleotide microarrays performed by us and others
have identified protease M, also known as neurosin/zyme/kallikrein-6 (KLK-6) as a
target gene of 1,25(OH)_{2}D_{3} in colon cancer and squamous carcinoma cell lines.\textsuperscript{20, 22}
Protease M is a member of the kallikrein (KLK) family, which includes secreted serine proteases with a wide-range of expression that are commonly regulated by steroid hormones in several cancer cell lines. The induction of protease M after 1,25(OH)$_2$D$_3$ treatment was confirmed by Northern blotting and immunofluorescence. We also found that, similarly to protease M, normal epithelial cell-specific 1 (NES1), another member of the kallikrein family (KLK-10), was induced by 1,25(OH)$_2$D$_3$ in SW480-ADH human colon cancer cells. This result was confirmed by Northern blot. Data from our laboratory also revealed that KLK-7 RNA expression is induced 9.5-fold by 1,25(OH)$_2$D$_3$ in these cells. The putative role of these serine proteases in 1,25(OH)$_2$D$_3$ action is unknown since no functional studies have been reported. Paradoxically, recent studies have proposed protease M as a marker of poor prognosis in ovarian and colon cancer, while NES1 has been described as a marker of either favorable or unfavorable prognosis depending on the cancer type (ref. and refs. therein). Other hitherto unknown functions of these proteases could contribute to the antitumor action of 1,25(OH)$_2$D$_3$, as a dual role in tumor progression has been proposed for other proteases.

**Vitamin D and the ubiquitin-proteasome system**

The ubiquitin-proteasome system (UPS) has been implicated in cancer and chemotherapy action. The rationale behind this is the control by the UPS of transcription factors and components of the apoptotic machinery that play important roles in the control of cell phenotype and survival. The clinical use of the proteasome inhibitor Bortezomib (Velcade®) against multiple myeloma is a proof-of-concept. Transcriptomic analyses using human SW480-ADH colon cancer cells performed in our laboratory have revealed numerous UPS genes as candidate targets of 1,25(OH)$_2$D$_3$.
(Table I). These genes include a group encoding ubiquitinating (UBs) and deubiquitinating (DUBs) enzymes and another group corresponding to components of the proteasome particle. The effect observed is an almost general repression of these genes at 48 h of 1,25(OH)2D3 treatment. This could be related to the antitumoral action of the hormone, as cancer cells have higher level of proteasome activity than their normal counterparts.4

Ubiquitin-mediated events are critical for cell function and proliferation, and the association of DUBs with cancer is increasingly recognized.44 The list of transcription factors regulated by the UPS includes both oncogenes and tumor suppressor genes.4 Moreover, the stability and function of nuclear factor kappa B are tightly regulated by the proteasome. The extremely wide function of the UPS and our limited knowledge of the control of the stability of many of the proteins that are critical in cancer make very difficult to extrapolate or even speculate about the role and consequences of the regulation by 1,25(OH)2D3 of the large series of UPS genes. The first step undoubtedly must be to validate their regulation and then to analyze its effects at the cell level, a task that is hampered by the complexity of the UPS.

Conclusions

1,25(OH)2D3 has a wide range of antitumor activity that has only partially been characterized. Numerous studies have shown that it is a major transcriptional regulator of gene expression. However, the large number of genes regulated by 1,25(OH)2D3 that are related with the protein degradation machinery (proteases, protease inhibitors and components of the UPS) suggests a new role of 1,25(OH)2D3 as a post-translational regulator. Thus, the antitumor activity of 1,25(OH)2D3 may be exerted by a dual transcriptional and post-translational regulation of its target genes. Clearly, validation of
candidate 1,25(OH)$_2$D$_3$ target genes of UPS as well as functional studies with other proteases and protease inhibitors known to be regulated by 1,25(OH)$_2$D$_3$ are necessary to corroborate this view.
References
Table I.

Candidate 1,25(OH)$_2$D$_3$ target genes of the ubiquitin-proteasome system in SW480-ADH human colon cancer cells. RNA levels (fold-change) at 4 or 48 h treatment with 100 nM 1,25(OH)$_2$D$_3$ as compared to vehicle-treated cells obtained using oligonucleotide microarrays.

<table>
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<th>GENE</th>
<th>FOLD CHANGE 1,25(OH)$_2$D$_3$ (h)</th>
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<th>48</th>
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<td><strong>Ubiquitin-conjugating enzymes (E2)</strong></td>
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<td></td>
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<tr>
<td>CDC34 Ubiquitin-conjugating enzyme CDC34</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>UBE2M Ubiquitin-conjugating enzyme E2M</td>
<td>2.30</td>
<td>-3.03</td>
<td></td>
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<tr>
<td>UBE2E3 Ubiquitin-conjugating enzyme E2E 3</td>
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<td></td>
<td></td>
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<tr>
<td>UBE2L3 Ubiquitin-conjugating enzyme E2L 3</td>
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<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td><strong>Ubiquitin protein ligases (E3)</strong></td>
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<td>WWP1 WW domain containing E3 ubiquitin protein ligase 1</td>
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<td><strong>Deubiquitinating enzymes (DUB)</strong></td>
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<td>USP5 Ubiquitin specific peptidase 5</td>
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<td>USP9X Ubiquitin specific peptidase 9, X-linked</td>
<td>2.30</td>
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<tr>
<td>USP13 Ubiquitin specific peptidase 13</td>
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<tr>
<td><strong>Other ubiquitin-related genes</strong></td>
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<tr>
<td>UBE4B Ubiquitination factor E4B</td>
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<tr>
<td>UBC Ubiquitin C</td>
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<td><strong>Proteasome 20S</strong></td>
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<td>PSMB2 Proteasome subunit, beta type, 2</td>
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<tr>
<td>PSMF1 Proteasome inhibitor subunit 1</td>
<td>2.64</td>
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<tr>
<td>PSME2 Proteasome activator subunit 2</td>
<td>-2.30</td>
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Figure legend

Figure 1.

The protease inhibitor cystatin D is induced by 1,25(OH)$_2$D$_3$ and mediates, at least partially, its antitumor activity in colon cancer by cathepsin inhibition-dependent and -independent mechanisms (see ref. 26 for a detailed description).