Cellular Physiology and Biochemistry

Cell Physiol Biochem 2013;31:815-822
DOI: 10.1159/000350099
Published online: June 04, 2013
Accepted: May 14, 2013

1421-9778/13/0316-0815$38.00/0

Original Paper

1α,25-Dihydroxycholecalciferol (Vitamin D3) Induces NO-Dependent Endothelial Cell Proliferation and Migration in a Three-Dimensional Matrix

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Key Words
1α,25-dihydroxycholecalciferol • Endothelial cells • Cell proliferation • Cell migration • Three-dimensional matrix

Abstract
Background/Aims: The 1α,25-dihydroxycholecalciferol (Vit. D) induces eNOS dependent nitric oxide (NO) production in human umbilical vein endothelial cells (HUVEC). To our knowledge, there are no reports directly relating Vit. D induced NO production to proliferation and/or migration in endothelial cells (EC). The aim of this study was to evaluate whether Vit. D addition to porcine EC could affect their proliferation and/or migration in a three-dimensional matrix via NO production. ***Materials and Methods:*** Porcine aortic endothelial cells (PAE) were used to evaluate Vit. D effects on cell proliferation and migration in a three-dimensional matrix. ***Results:*** Vit. D induced NO production in PAE cells. Moreover, it induced a significant increase in cellular proliferation and migration in a three-dimensional matrix. These effects were NO dependent, as inhibiting eNOS activity by L-NAME PAE migration was abrogated. This effect was strictly related to MMP-2 expression and apparently dependent on Vit. D and NO production. ***Conclusions:*** Vit. D can promote both endothelial cells proliferation and migration in a three-dimensional matrix via NO-dependent mechanisms. These findings cast new light on the role of Vit. D in the angiogenic process, suggesting new applications for Vit. D in such fields as tissue repair and wound healing.

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Introduction

The 1α,25-dihydroxycholecalciferol (Vit. D) is the active form of vitamin D3, a pleiotropic hormone playing a key role in a wide array of physiological events such as calcium and phosphorous homeostasis and bone development and maintenance. Moreover, Vit. D is a potent regulator of the cell growth, differentiation and maturation of various normal and cancer cells [1-4]. Vitamin D and its active form mediate different effects in a large number of tissues, as nearly every tissue displays Vit. D receptors (VDR) [3]. VDR is a 48 kDa zinc finger nuclear expressed receptor activating transcription by binding Vit. D response elements (VDRE) within the promoter of Vit. D responsive genes, either as homodimer or heterodimer with the retinoid acid V receptor-α, retinoic acid receptor or thyroid hormone receptors [5]. Vit. D also regulates growth factor expression and cytokine synthesis, as well as receptor expression, thus modulating cellular growth and differentiation of many cellular populations such as endothelial cells (EC) [5]. EC form a dynamic tissue with spontaneous or injury-dependent cell renewal and express specific cell functions at blood/vessel wall interface [6] and they are known to be an important site of Vit. D biosynthesis expressing the key biosynthetic enzyme 25(OH)D_3-1α-hydroxylase [4, 7]. Moreover, EC also express VDR [6, 8-10], thus suggesting the hypothesis that Vit. D could act as a possible autocrine/intracrine modulator of endothelial function [4, 6]. To date Vit. D effects on endothelial cell growth and morphogenesis is unclear. It has been reported that this hormone decreases or has no effect on endothelium proliferation [6, 11-13], but it can induce nitric oxide (NO) synthesis [4]. NO is endogenously synthesized from the guanidino nitrogen atoms of L-arginine or can be produced from exogenous sources, such as nitrovasodilatators by one of several isoforms of NO synthases (NOS) [14, 15]. In the circulatory system, NO is produced by a constitutively-expressed endothelial NOS isoform (eNOS) and acts as an endogenous nitrovasodilatator [16] playing a pivotal role in EC function [17, 18]. Even though NO affects a wide array of physiological processes, such as cell growth and migration [19], to our knowledge there are no reports directly relating Vit. D induced NO production and EC proliferation and/or migration. The aim of this study was to evaluate whether Vit. D could affect porcine EC proliferation and/or migration in a three-dimensional matrix and whether this activity could be mediated by NO production.

Materials and Methods

Cell culture

Porcine aortic endothelial (PAE) cells were grown in DMEM medium (Euroclone, Milan, Italy) supplemented with 10% heath inactivated foetal bovine serum (FBS) (Euroclone), penicillin (100 U/ml) (Euroclone), streptomycin (100 mg/ml) (Euroclone) and L-glutamine (2 mM) (Euroclone) in a humidified atmosphere containing 5% CO_2 at 37°C.

NO production detection

PAE cells (1x10^5cells/ml) were plated in 96 well plates and allowed to adhere; then complete cell culture medium was changed with DMEM medium without serum and without phenol red for cell starvation. Cells were then treated with vitamin D3 (1-10-100 nM) both in presence or absence of the synthetic vitamin D receptor antagonist ZK159222 (Bayer Pharma AG, Berlin, Germany) (10 nM). As positive control some samples were stimulated with 10 μM acetylcholine (Sigma Aldrich, St. Louis, MO, USA) or 10 μM forskolin (Calbiochem, Darmstat, Germany). NO production was measured in cell culture supernatants using Griess reagent (Promega, Medison, WI, USA), following manufacturer's instructions. Cell culture supernatants absorbance was read at 490 nm.

Proliferation

In order to evaluate vitamin D3 (Sigma Aldrich, St. Louis, MO, USA) influence on cell proliferation, 2.5x10^5 cells were plated onto Petri dishes and allowed to adhere for 5h. Non adherent cells were then

removed by gentle wash in phosphate buffer (PBS, pH=7.4) and complete cell culture medium was changed with low FBS (1%) medium for 24h. Cells were then treated in 1% FBS medium with vitamin D3 (1-100 nM, dissolved in ethanol), ethanol (maximum concentration 0.1%), or left untreated. After 24 h incubation, cell culture medium was removed and cells were fixed in 3.7% formaldehyde – 3% sucrose solution, stained with 1% toluidine blue solution and samples were photographed at 10X magnification, using an optical microscope (Leica ICC50HD). Cell proliferation was evaluated by counting cells in 10 random fields in three samples for each experimental condition from three different experiments. Results were expressed as cells/ mm^2 ± standard deviation (S.D.).

Three-dimensional matrix migration assay

PAE cells were seeded in 12 wells plates and grown in DMEM complete medium to reach a ~ 70% confluent monolayer. The three-dimensional hydrogel matrix (Epigel B, without added growth factor, Epinova Biotech, Novara, Italy) were lean onto PAE monolayers in 250 µl of complete cell culture medium containing different amounts of vitamin D3 (1-100 nM) and cell migration was monitored daily by optical microscopy. After 3 days, cell culture medium was replaced with fresh medium. After 7 days, hydrogel samples were fixed in 3.7% formaldehyde – 3% sucrose solution, stained with 2 µg/ml of Hoechst 33342 solution (Sigma Aldrich, St. Louis, MO, USA) in order to stain cell nuclei and then transferred onto glass microscope slides before observation under UV light using a Leica DM500 fluorescence microscope. Cell migration was evaluated by counting migrated cells into 3D matrix. For each experimental condition, three samples were analyzed at 10X magnification, selecting 10 random fields and results were expressed as no. cells/HPF (high power microscope field) ± standard deviation (S.D.).

NO synthesis inhibition

To evaluate NO synthesis involvement in PAE proliferation and migration following vitamin D3 treatment, some experiments were performed in the presence of the NOS inhibitor N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma Aldrich, St. Louis, MO, USA). L-NAME was dissolved in serum free medium and used at a final concentration of 10 mM [4].

Zymography

In order to detect gelatinolytic activity, conditioned media from PAE cells migrated into the three-dimensional matrix for 7 days were separated by electrophoresis on SDS-polyacrylamide gels containing 0.2% gelatin. Samples were loaded onto zymograms without denaturation. After running, gels were washed at room temperature for 2 h in 2.5% Triton X-100 solution and incubated overnight at 37°C in 0.5 M Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ buffer. Gels were then fixed in MeOH/Acetic Acid (50:10) solution and stained in 0.5% Coomassie Blue in MeOH/Acetic Acid (40:10) solution. Images of stained gels were acquired after appropriate destaining. Gelatinolytic activity was detected as white bands on a dark blue background and quantified by densitometric analysis using ImageJ software.

Statistical analysis

Unpaired Student's *t*-tests were used for statistical analysis. Probability values of $p<0.05$ were considered statistically significant.

Results

Vitamin D3 induces NO production in PAE cells

It has been reported that PAE cells produce NO following both forskolin (FK) and acetylcholine (Ach) stimulation [20], therefore in our experimental model these two drugs were used as positive control. In fact, 10 µM FK increased NO production of 39.55 ± 5.98% over the basal after 3 min stimulation, while 10 µM acetylcholine stimulation resulted in an increase of 31.55 ± 3.62% (data not shown). As shown in figure 1, PAE cells produced NO also after 3 min vitamin D stimulation. In fact, NO accumulation increased compared to basal values after 1 nM (18.03 ± 5.13%), 10 nM (18.67± 0.83%) and 100 nM (25.11± 4.91%) Vit. D stimulation.

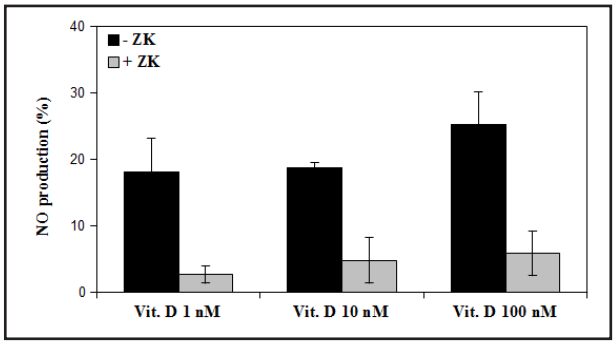

Fig. 1. NO production. Quantification of NO production measured by means of Griess method and expressed as percentage of control values. NO production was evaluated after 3 minutes stimulation in the presence of different concentrations of Vit. D (1-100 nM). Black bars = cells without ZK159222 addition, gray bars = cells + 10 nM ZK159222 ($P<0.05$).

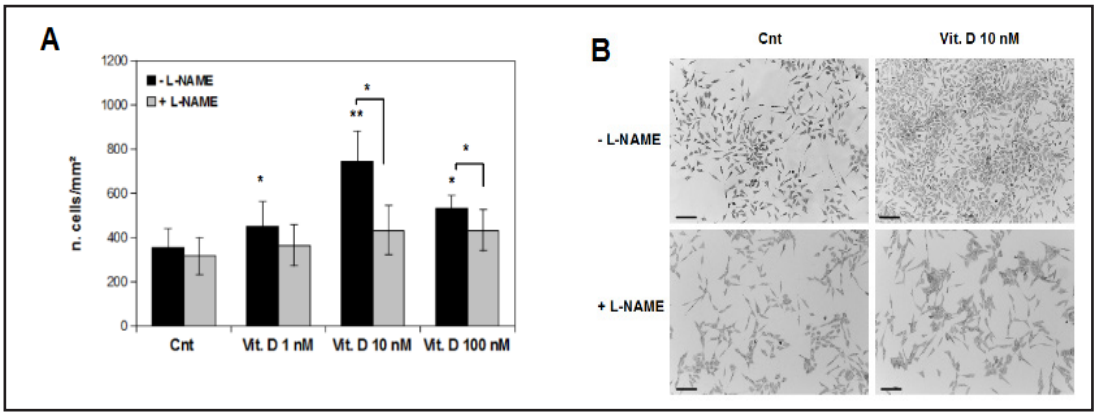

Fig. 2. PAE cells proliferation. A) Determination of PAE proliferation in the presence of different concentrations of Vit. D (1-100 nM) after 24 hours of incubation. Results are expressed as n. cells/mm^2 ± S.D. Black bars = cells without L-NAME addition, gray bars = cells + 10 mM L-NAME. * $p<0.05$; ** $p<0.001$. B) Optical microscopy images of control and Vit. D (10 nM) treated cells both in absence or presence of 10 mM L-NAME after 24 hours of incubation, stained with toluidine blue. Magnification = 10X. Scale bar = 150 µm.

To verify whether Vit. D effect on NO production was mediated by Vit.D receptor, PAE cells were treated with Vit.D in the presence of the specific synthetic antagonist ZK159222 (10 nM). The antagonist concentration used was higher than its IC_{50} value in order to assure that all vitamin D receptors were saturated [21]. As expected ZK159222 presence almost completely inhibited Vit.D-induced NO production (Fig. 1), reducing NO levels to basal.

Vitamin D3 induces PAE cells proliferation through a NO dependent pathway

PAE cells proliferation has been evaluated in low serum conditions, after 24-hour-pre-incubation in these same conditions to synchronize cell culture and to minimize serum induced cell proliferation. As shown in Figure 2, Vit. D induced a significant dose-dependent increase in PAE growth after 24-hour-incubation. The maximal effect was reached stimulating PAE cells with 10 nM Vit. D (Fig. 2A and 2B). The observed cellular density almost doubled compared to control samples (690±210 cells/mm^2 vs 354±84 cells/mm^2, $p<0.0001$). On the other hand, at the highest concentration tested (100 nM), Vit. D effect on cell proliferation was less potent ($p<0.05$). Ethanol used as vehicle for Vit. D administration did not affect cell proliferation (data not shown). In order to evaluate NO involvement in the observed vit. D effects, proliferation assays were performed in the presence of 10 mM L-NAME, an arginine analog inhibiting NO synthesis. Under these conditions, Vit. D was not able to induce cell proliferation. As shown in Figure 2A and 2B L-NAME presence did not alter control PAE proliferation, while completely reverted Vit. D induced cell proliferation.

Vitamin D3 induces PAE cells migration in a 3D matrix through a NO dependent pathway

PAE cells migration has been evaluated in a three-dimensional model. A 3D matrix was lean on 70% confluent PAE monolayers. The 3D matrix used in these experiments was an

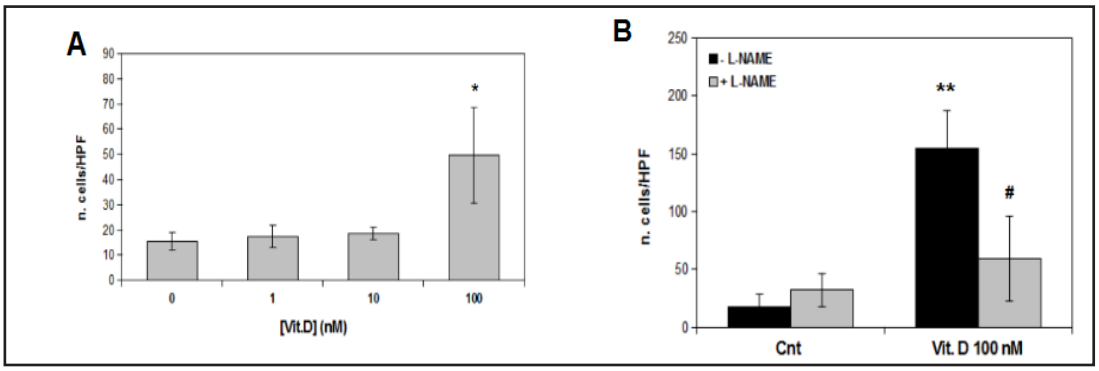

Fig. 3. PAE cells migration into a three-dimensional matrix. A) Determination of PAE migration after 7 days of incubation in the presence of different concentrations of Vit. D (1-100 nM). Results are expressed as n. cells/HPF ± S.D. * $p<0.05$. B) Determination of control and Vit. D (100 nM) treated cells migration both in the presence or absence of 10 mM L-NAME after 7 days of incubation. *$p<0.0001$ compared to control sample; # $p<0.05$ compared to Vit. D 100 nM alone.

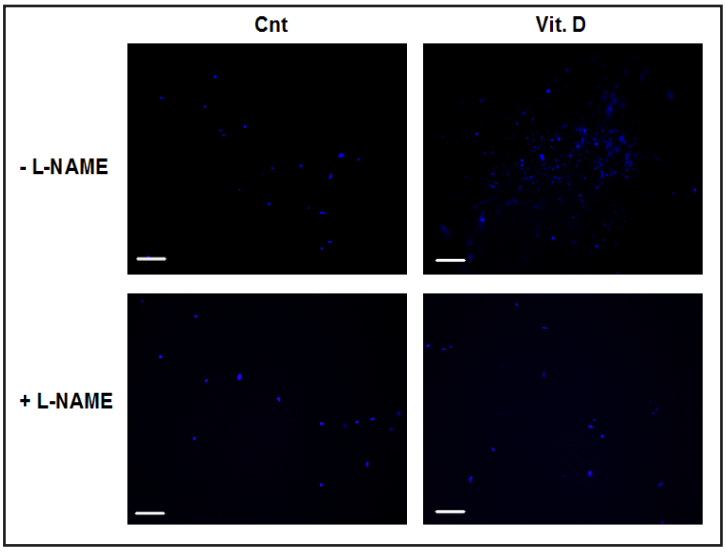

Fig. 4. Optical microscopy images of control and Vit. D (100 nM) treated cells both in the presence or absence of 10 mM L-NAME after 7 days of incubation, stained with Hoechst 33342. Magnification = 10X. Scale bar = 60 µm.

anionic hydrogel made of gelatin and polyglutamic acid, which has previously been described as a good substrate for cell growth [22, 23]. As shown in Figure 3A and 4, PAE migration evaluated counting the cells migrated in the 3D matrix for 7 days increased significantly only in presence of 100 nM Vit.D ($p<0.05$). In order to evaluate NO involvement in the observed phenomenon, under these experimental conditions as well, the experiments were performed in the presence of 10 mM L-NAME. As shown in Figure 3B and 4, L-NAME treatment did not affect control cells migration, while significantly reduced 100 nM Vit. D induced hydrogel invasion ($p<0.05$ compared to Vit.D alone).

Vitamin D induces MMP-2 expression via NO dependent pathway

Extracellular matrix (ECM) degradation is one of the main steps in cell migration and for this reason Vit. D effects on MMP-2 expression in PAE cells migrating into the 3D hydrogel matrix has been evaluated by gelatin zimography after 7 days. As shown in Figure 5A and B, Vit. D addition to cell culture medium increased MMP-2 production in a dose-dependent fashion. The increase in MMP-2 expression appeared to be NO dependent, as L-NAME treatment totally abrogated Vit. D effects on MMP-2 expression (Fig. 5C and D), according to the above described results for cell migration.

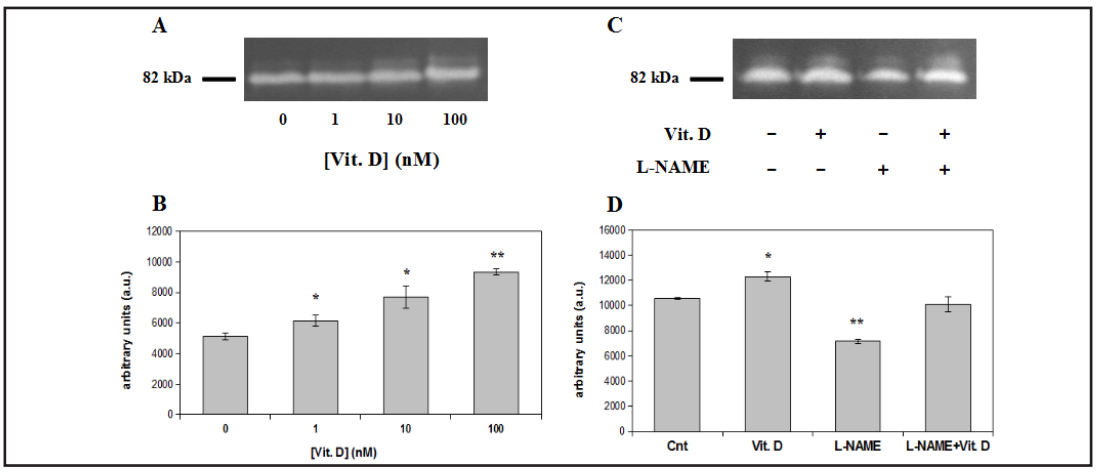

Fig. 5. MMP-2 production. A) Representative zymography of cell growth medium form PAE cells migrated into the three-dimensional matrix for 7 days in the presence of different Vit. D concentrations (1-100 nM). B) Densitometric quantification of MMP-2 expression. C) Representative zymography of cell growth medium from control and Vit. D (100 nM) PAE cells migrated into the three-dimensional matrix for 7 days, both in the presence or absence of 10 mM L-NAME. D) Densitometric quantification of MMP-2 expression.

Discussion

This study demonstrates for the first time that 1α,25-dihydroxycholecalciferol (Vit. D), the active form of vitamin D, is able to induce PAE cell proliferation and migration into a three-dimensional matrix via NO production.

Most existing studies dealing with the effects of Vit. D on cell growth report an inhibitory effect on cell proliferation coupled with an increased cellular differentiation. However endothelial response to Vit. D stimulation appears to be unclear, producing conflicting results, with studies describing an inhibition of serum induced cell proliferation, and others describing Vit. D as lacking any effect on serum induced proliferation [11]. It is noteworthy that in the above mentioned studies, Vit. D effects were evaluated in presence of serum, whereas in the present study Vit. D stimulation occurred in low serum condition (24 h incubation in low serum medium to syncronize cell culture). Vit. D effect could depend on local cell environment, being stronger when cells are stimulated to proliferate, as in tumors than in situations where only a basal proliferation level is needed [12]. Furthermore, other studies have described that Vit. D effects on cell proliferation were dose dependent. As a matter of fact, *in vitro* studies highlighted that Vit. D displays a biphasic effect on keratinocyte growth, suppressing cell growth at concentrations greater than 10^{-8} M, while promoting it at concentrations below 10^{-9} M [5]. These effects were dependent on cell growth medium composition, in fact cell growth stimulation was detectable only in defined medium without serum, sterol and pituitary extract, whereas in serum containing medium all the tested concentrations suppressed keratinocytes growth [5]. The observed increase in PAE proliferation could be explained by low serum conditions used in this paper, according to what previously observed by Gurlek and coworkers in keratinocytes [5].

Vit. D effects on cell proliferation could also be strongly related to nitric oxide balance, as it is known that at cellular level NO regulates many different processes, such as cell growth, survival, apoptosis, proliferation, and differentiation [24]. In a previous work, Molinari and coworkers highlighted a direct correlation between Vit. D and NO synthesis in HUVEC cells [4]. As NO is known to be a powerful ubiquitous regulator of vascular tone, this correlation appears to be relevant. As described in the results section, this correlation has also been confirmed in PAE cells. Moreover, co-presence of Vit. D and its synthetic antagonist ZK159222 resulted in an almost complete inhibition of Vit. D-mediated NO production. The correlation between Vit. D stimulation and NO production in PAE cells was also confirmed by eNOS

inhibition using the competitive inhibitor N_w-nitro-L-arginine methyl ester (L-NAME) [15], resulting in an effective vitamin D antagonistic effect on cell proliferation and migration.

Nitric oxide is known to be a powerful ubiquitous regulator of vascular tone and its involvement in the angiogenic process has been described [25, 26]. In particular, in the field of angiogenic processes, the role of EC migration is extensively studied. Extracellular matrix (ECM) degradation allows vascular endothelial cells to migrate through their basal membrane and mainly involves matrix metalloproteinases (MMPs) activity. MMPs constitute a tightly regulated family of endogenous zinc dependent endopeptidases, divided into different subfamilies according to their substrate specificity, degrading most of the components of ECM and basal membrane [27, 28]. In particular, the main components of vascular basal lamina (collagen IV, laminin and fibronectin), are degraded mainly by MMP-2 and MMP-9, also known as gelatinases [29]. In particular, MMP-2 has received growing attention as it is the main MMP involved in angiogenesis [30].

In this study EC migration resulted strictly related to MMP-2 expression, as highlighted by gelatin zymography analysis. Moreover, MMP-2 expression resulted dependent on Vit. D and NO stimulation. MMP-2 involvement in PAE cells migration well correlated with previous studies reporting a constitutive MMP-2 expression in endothelium [27, 29], and its upregulation during endothelial cells migration and tube formation in a 3D matrix [30].

In conclusion, the results described herein highlight that Vit. D (1-100 nM) stimulated PAE cells proliferation and migration in a three-dimensional matrix and that these phenomena depend on NO production. The discussed results could be relevant in the light of the use of Vit. D supplementation in very promising fields such as tissue repair and wound healing.

Conflict of Interests

The authors declare they have no conflict of interests.

Acknowledgements

The authors thank Bayer Pharma AG (Berlin, Germany) for the generous gift of vitamin D3 synthetic antagonist ZK159222.

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