Vitamin D receptor as a target for breast cancer therapy

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Abstract

Considerable epidemiological evidence suggests that high levels of circulating vitamin D (VD) are associated with a decreased incidence and increased survival from cancer, i.e., VD may possess anti-cancer properties. The aim of this investigation was therefore to investigate the anti-cancer potential of a low calcaemic vitamin D analogue, i.e., inecalcitol and compare it with the active form of vitamin D, i.e., calcitriol, in a panel of breast cancer cell lines (n = 15). Using the MTT assay, IC50 concentrations for response to calcitriol varied from 0.12 µM to >20 µM, whereas those for inecalcitol were significantly lower, ranging from 2.5 nM to 63 nM (P = 0.001). Sensitivity to calcitriol and inecalcitol was higher in VD receptor (VDR)-positive compared to VDR-negative cell lines (P = 0.0007 and 0.0080, respectively) and in ER-positive compared to ER-negative cell lines (P = 0.043 and 0.005, respectively). Using RNA-seq analysis, substantial but not complete overlap was found between genes differentially regulated by calcitriol and inecalcitol. In particular, significantly enriched gene ontology terms such as cell surface signalling and cell communication were found after treatment with inecalcitol but not with calcitriol. In contrast, ossification and bone morphogenesis were found significantly enriched after treatment with calcitriol but not with inecalcitol. Our preclinical results suggest that calcitriol and inecalcitol can inhibit breast cancer cell line growth, especially in cells expressing ER and VDR. As inecalcitol is significantly more potent than calcitriol and has low calcaemic potential, it should be further investigated for the treatment of breast cancer.

Key Words

- vitamin D
- vitamin D receptor
- calcitriol
- inecalcitol
- breast cancer
- oestrogen receptors
Introduction

The vitamin D receptor (VDR), like the oestrogen receptor (ER), progesterone receptor (PR) and the androgen receptor (AR), is a member of the nuclear family of steroid hormone transcriptional regulators (Leyssens et al. 2013, Narvaez et al. 2014, Feldman et al. 2015). VDR is activated after the binding of the active form of vitamin D known as 1α,25(OH)2-vitamin D3 or calcitriol. Ligand binding results in heterodimerisation with the retinoid X receptor (RXR), which is followed by binding to vitamin D response elements on DNA. After the recruitment of multiple transcriptional regulating proteins such as nuclear receptor co-activators, the calcitriol-bound VDR/RXR complex begins to alter gene expression (Narvaez et al. 2014). VDR has been shown to be both an activator and repressor of transcriptional activity, i.e., its target genes may be upregulated or downregulated (Haussler et al. 2013, Campbell 2014, Christakos et al. 2016).

Unlike ER and AR that have been widely exploited as therapeutic targets in cancer (i.e., ER in breast cancer (EBCTCG et al. 2011) and AR in prostate cancer (Valenca et al. 2015)), less work has been carried out on targeting VDR. There are, however, several preclinical studies suggesting that targeting VDR with calcitriol or its synthetic analogues may have anti-cancer potential (Narvaez et al. 2001, Flanagan et al. 2003, Kasiappan et al. 2014, Lungchukiet et al. 2014, Chen et al. 2015, Murray et al. 2015). In this investigation, using a large panel of breast cancer cell lines that included ER-positive, HER2-positive and triple-negative (TN) cells, we compared the anti-proliferative effects of the low (i.e., negative for ER, PR and not HER2 amplified) cells, ER-positive, HER2-positive and triple-negative (TN) cancer cells (Verlinden et al. 2012, Okamoto et al. 2012, Murthy et al. 2012). These were investigated. These included: Hs578t(i8), MDA-MD-468, MDA-MD-231, HCC1937, HCC1143, BT20, BT549 (all TN); MDA-MD-453, SKBR3, BT474, JIMT-1 (all HER2-positive) and T47D, Cama1, ZR-75-1 and MCF-7 (all ER-positive or luminal). Apart from Hs578t(i8) cells that were supplied by Dr Susan McDonnell, University College, Dublin (Hughes et al. 2008), all other cell lines were purchased from the American Type Culture Collection (ATCC).

Cell lines and reagents

A panel of 15 breast cancer cell lines representing all the main molecular subtypes of breast cancer were investigated. These included: Hs578t(i8), MDA-MD-468, MDA-MD-231, HCC1937, HCC1143, BT20, BT549 (all TN); MDA-MD-453, SKBR3, BT474, JIMT-1 (all HER2-positive) and T47D, Cama1, ZR-75-1 and MCF-7 (all ER-positive or luminal). Apart from Hs578t(i8) cells that were supplied by Dr Susan McDonnell, University College, Dublin (Hughes et al. 2008), all other cell lines were purchased from the American Type Culture Collection (ATCC).

Cell lines were maintained through continued passaging at 37°C with a humidified atmosphere of 5% CO₂. All media was supplemented with 10% foetal bovine serum (FBS) (Invitrogen Life Technologies), 1% penicillin/streptomycin (Invitrogen Life Technologies) and 1% fungizone (Invitrogen Life Technologies). BT549 cells were maintained in RPMI-1640 supplemented with 0.0231U Insulin (Sigma-Aldrich), 10mM Hepes (Sigma-Aldrich), 1.5 g/L sodium bicarbonate (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich). ZR-75-1 cells were maintained in DMEM supplemented with 1 mM oestradiol (Sigma-Aldrich). Cell line identity was confirmed by analysis of short-term repeat loci, and cells were routinely tested for mycoplasma infection.

Cell viability assays

Cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay as previously described (McGowan et al. 2013). To test the effect of calcitriol (Selleckchem, Munich, Germany) or inecalcitol...
(Hybrigenics, Paris, France) on proliferation, cells were plated at a density of $1 \times 10^5$/well in 96-well flat-bottomed plates (Sigma-Aldrich). After overnight incubation, quadruplicate wells were treated with concentrations of compounds ranging from zero to 20 $\mu$M. After 5 days, 0.5 mg/mL MTT was added to each well and incubated at 37°C for 5 h. Media was aspirated, and 200 $\mu$L of DMSO was added to each well, for 5 min. Absorbance was measured at a wavelength of 550 nm on a microplate reader (Multiscan Ascent, Labsystems).

Western blotting

Protein lysates (50 $\mu$g) were separated on a 10% polyacrylamide gel (Bioscience) (200 volts for 60 min). After electrophoresis, protein was transferred to a nitrocellulose membrane (Millipore) and blocked for 2 h at room temperature in 2% low-fat milk (Marvel instant dried skimmed milk) in Tris-buffered saline/0.1% Tween 20 (TBST). The membranes were then probed overnight at 4°C with a mouse monoclonal anti-VDR antibody (clone D-6, Santa Cruz). After 3 washes for 10 min in TBST, membranes were incubated with a horseradish peroxidase-conjugated secondary anti-mouse antibody (Cell Signalling Technology) or anti-rabbit antibody (Cell Signalling Technology) for 2 h at room temperature. Signals were developed using enhanced chemiluminescence (ECL) (Thermo Fisher Scientific) and exposure to X-ray film (Fujifilm). As a control measure for equal loading, blots were re-probed for GADPH. Positivity was defined as a visible band at the appropriate molecular mass location on the blot.

Enzyme-linked immunosorbent assay

Quantification of VDR was carried out using the Human VDR ELISA kit (Cloud Clone Corp., Houston, TX, USA; SEA475Hu), as per manufacturer’s protocol. Absorbance was measured at a wavelength of 450 nm on a microplate reader (Multiscan Ascent, Labsystems).

Measurement of cell migration

Transwell migration assay was performed by seeding cells at a density of $2.5 \times 10^5$/500 $\mu$L in the upper compartment of a cell permeable membrane insert (8 $\mu$m pore size; BioCoat, BD Biosciences). The lower chamber was filled with fibroblast-conditioned medium (supplied by Prof. Ursula Fearon, University College Dublin). After a 5 h incubation period, the cells were treated with 2 $\mu$M calcitriol or 4 nM inecalcitol. After 5 days of incubation, non-migrated cells in the upper chamber were removed with a PBS-soaked cotton swab. This was followed by fixation using 1% glutaraldehyde (Sigma-Aldrich) and staining with 0.1% crystal violet (Pro-Lab Diagnostics, Birkenhead, UK). Cells fixed on the lower face of the Matrigel chambers were counted under a light microscope at a magnification of ×40.

RNA-seq analysis

MCF-7 cells were treated with 2 $\mu$M calcitriol or 4 nM inecalcitol in triplicate for 6 h. DMSO and ethanol acted as controls for calcitriol- and inecalcitol-treated cells, respectively. RNA was isolated using RNeasy Mini kits (Qiagen) according to the manufacturer’s instructions. Quantity and purity were determined using a Bioanalyzer (Agilent). Libraries were prepared using the Illumina TruSeq stranded mRNA library prep kit according to the manufacturer’s instructions (Illumina). Sequencing was performed using an Illumina HiSeq 2500 to produce 51 bp single-end reads, and quality control was conducted using FASTQC. (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were aligned to human genome, version 19, using the sequence aligner subread (Liao et al. 2013) via the bioconductor R package Rsubread (https://bioconductor.org/packages/). The data were scale normalised using the TMM (trimmed mean of M values (TMM)) normalisation method (Robinson & Oshlack 2010). Differential expression was determined using the ebayes function of the R package LIMMA (Smyth 2004).

An adjusted $P$ value $<0.05$ and a fold-change greater than 1.4-fold was considered significant. The $P$ values were adjusted using the Benjamini and Hochberg method (Benjamini & Hochberg 1995). The package LIMMA was chosen here for differential expression analysis as it has been shown to be particularly robust when dealing with small sample sizes (Seyednasrollah et al. 2015). Gene ontology (GO) analysis was performed using the R package goseq (Young et al. 2010), a package specifically designed for performing GO analysis on RNA-seq data, so as to overcome the inherent bias in RNA-seq data for over-detecting long and highly expressed transcripts. For comparison across public datasets, gene lists as published by the original authors were used. All calculations were carried out in the R statistical environment (https://cran.r-project.org/).
qPCR confirmation of SNAI2 and MYBPH expression

Total RNA isolated using the RNeasy Mini kit (Qiagen) was transcribed into cDNA (0.5 µg of template) using the RT² First Strand kit (Qiagen). RT-PCR analysis of SNAI2 and MYBPH mRNA was performed in triplicate using the RT² qPCR Primer Assay (Qiagen) and GAPDH as housekeeping gene. Cycle threshold (Ct) values were normalised using the housekeeping gene, and the fold-change gene expression was calculated using the ∆∆Ct method.

Evaluation of the effect of VDR expression on prognosis in patients with different molecular subforms of breast cancer

To establish a potential prognostic value for VDR in breast cancer patients, we evaluated a pooled database of 12 publicly available breast cancer datasets (n=2019 patients) containing gene expression data (Madden et al. 2013), as previously described (Caiazza et al. 2016). The median expression level of VDR was used to dichotomise the data, and disease-free survival (DFS) was chosen as the survival endpoint.

Statistical analysis

IC₅₀ data were analysed using Prism, version 5.0b software (GraphPad Software). The correlation coefficients were calculated using the Spearman’s non-parametric test. The IC₅₀ values of calcitriol and inecalcitol were compared using Student’s t-test.

Results

Comparative effects of calcitriol and inecalcitol on cell growth inhibition in a panel of breast cancer cell lines

Table 1 compares the IC₅₀ values for calcitriol and inecalcitol in a panel of 15 breast cancer cell lines. Across the panel, IC₅₀ concentrations for calcitriol ranged from 0.1 µM to >20 µM, whereas the corresponding values for inecalcitol varied from 2.5 nM to 63.5 nM. Overall, IC₅₀ values for inecalcitol were significantly lower for inecalcitol than those for calcitriol (P<0.001) (Fig. 1A), i.e., inecalcitol was significantly more potent in inhibiting proliferation than calcitriol. Irrespective of the cell line, the increased potency for inecalcitol vs calcitriol was >18-fold.

Relationship between response to calcitriol or inecalcitol and cellular VDR level

As calcitriol is believed to mediate its actions via its specific receptor, we related IC₅₀ values for calcitriol...
and inecalcitol to the cell line concentration of VDR. To do this, VDR levels were determined by both Western blotting and ELISA (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article). Overall, a significant correlation was found between VDR levels determined using the 2 methods ($P=0.0009$, $r=0.7634$, $n=15$) (Supplementary Fig. 1C). The BT-549 cell line, however, lacked a visible VDR band after Western blotting but was VDR positive using ELISA. This discrepancy may relate to the specificity of the antibodies used in the different assays. The antibody used in the Western blotting binds to amino acids 344–424, i.e., towards the C-terminal end of the VDR. This region of VDR may be missing in BT-549 cells and if so, no protein would be detected using Western blotting. We are unable to establish the identity of the epitopes recognised by the antibodies in the ELISA, as this information is proprietary. However, if these antibodies bind to a different region in VDR than the antibody used for Western blotting, this might explain our positive finding with ELISA but undetectable levels using Western blotting.

Using Western blotting, VDR was found to be expressed in 10 of the 15 cells lines investigated and absent in 5 cell lines (Supplementary Fig. 1A). As shown in Fig. 1B, $IC_{50}$ values for calcitriol were significantly lower in cell lines expressing VDR than those in cell lines negative for VDR ($P=0.0007$, $n=15$) (Fig. 1B). Indeed, all the VDR-negative cell lines were resistant to calcitriol with $IC_{50}$ values $>$20µM (Table 1). Similarly, response to inecalcitol was significantly greater in VDR-positive vs VDR-negative cell lines ($P=0.008$) (Fig. 1C). However, in contrast to the situation with calcitriol, inecalcitol appeared to be growth inhibitory in VDR-negative cell lines.

**Figure 1**

Effect of calcitriol or inecalcitol on cell line growth and relationship between response to calcitriol or inecalcitol treatment and VDR protein levels. (A) Comparative $IC_{50}$ values for breast cancer cell lines treated with calcitriol or inecalcitol. (B) and (C) scatter plot representing the relationship between $IC_{50}$ values for calcitriol (B) or inecalcitol (C) and VDR status. Data were analysed using Student t test.

**Effect of molecular subtype of cell line on response to calcitriol and inecalcitol**

Response to both calcitriol and inecalcitol was related to the ER, AR and HER2 status of the cell lines. As shown in Fig. 2, the $IC_{50}$ values for both compounds were significantly lower in ER-positive cell lines than those in ER-negative cell lines, (for calcitriol, $P=0.0430$; for inecalcitol, $P=0.005$) (Fig. 2A and B). In contrast, response to calcitriol and inecalcitol was independent of both the AR (Fig. 2C and D) and HER2 status (Fig. 2E and F). In an attempt to explain why ER-positive cell lines were more sensitive than ER-negative cell lines to both calcitriol and inecalcitol, we compared VDR levels determined by ELISA in the 2 groups of cell lines. Although there was a trend for higher concentrations of VDR in ER-positive compared to ER-negative cell lines, this difference did not reach statistical significance ($P=0.133$). The failure to reach statistical significance may be due to the relatively small number of cell lines investigated.

**Effect of calcitriol and inecalcitol on migration**

In addition to enhanced proliferation, cell migration is essential for cancer progression, especially for invasion and metastasis. We therefore investigated the effects of treatment with calcitriol (2µM) or inecalcitol (4nM) on migration using the Transwell migration assay (Fig. 3A, B, C and D). With MCF-7 cells, calcitriol reduced migration by 41.9% ($P=0.015$) vs 56.3% ($P=0.012$) after treatment with inecalcitol; with BT20 cells, the corresponding reductions in migration were 45.9% ($P=0.045$) and 57.6% ($P=0.007$), whereas with Hs578t(i8), the reductions in migration were 40.3% ($P=0.043$) and 49.3% ($P=0.001$), respectively. Unlike our findings with MCF-7, BT20 and
Figure 2
Relationship between the response to calcitriol or inecalcitol treatment and the molecular subtype of breast cancer cell line. (A, B, C, D, E and F) Scatter plots representing the relationship between response to treatment with calcitriol (A, C and E) or inecalcitol (B, D and F) and the molecular subtype of breast cancer. Scatter plots representing ER+ and ER− cell lines (A and B), AR+ and AR− cell lines (C and D) or HER2+ or HER2− cell lines (E and F). Data were analysed using the Student’s t test. Data points represent the mean of three independent experiments.

Figure 3
Effect of calcitriol or inecalcitol on cell migration. Bar chart representing the percentage cell migration as determined by the Transwell migration, as well as a representative image of Transwell insert (×40) for MCF-7 (A), BT20 (B), Hs578T(i8) (C) or MDA MB 453 cell lines. Cells were treated with either 2 µM calcitriol or 4 nM inecalcitol. Percent migration was determined by quantifying the number of cells on the underside of the insert and normalising to vehicle control. All experiments were carried out in triplicate. Data were analysed using Paired t test. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0463.
Hs578t(i8) cells, calcitriol failed to significantly reduce migration in MDA-MB-453 ($P=0.079$). In contrast to calcitriol, treatment with inecalcitol significantly reduced migration in MDA-MB-453 cells (reduction in migration, 52.9%, $P=0.033$). Overall, across the 4 cell lines, inecalcitol had a significantly greater effect on migration than calcitriol ($P=0.0018$).

**Effect of calcitriol and inecalcitol on gene expression**

In an attempt to establish if calcitriol and inecalcitol had a similar impact on gene expression, we compared the effects of the 2 compounds on MCF-7 cells using RNA-seq. Overall, 106 genes were found to be significantly regulated after treatment with 2 µM calcitriol and 98 after the addition of 4 nM inecalcitol (Supplementary Tables 1 and 2 for full list of altered genes and Table 2 for the top 20 differentially regulated genes). The majority of the genes were upregulated, which is consistent with previous studies (Goeman et al. 2014, Sheng et al. 2015, Simmons et al. 2015). Of the total number of genes significantly differentially regulated by either compound, 77 were altered by both. In addition, there was a strong correlation in the fold changes of these 77 genes between the two treatments ($r=0.9331$, $P=0.0001$) (Supplementary Fig. 2). Indeed, the 2 genes showing the greatest extent of upregulation were the same for both compounds, i.e., cytochrome p450, family 24, subfamily a, polypeptide 1 (CYP24A1) and src homology 2 domain-containing E (SHE). Thirty-two genes, however, were significantly differentially regulated by calcitriol that were not altered by inecalcitol, whereas 24 genes were significantly differentially regulated by inecalcitol but not by calcitriol (Supplementary Table 1).

We specifically investigated if either calcitriol- or inecalcitol-modulated expression of VDR. Using RNA-seq, neither compound was found to alter mRNA levels of VDR in MCF-7 cells. Similarly, using RT-PCR, VDR expression was not found to be modulated by either calcitriol or inecalcitol in this cell line.

**Gene ontology (GO) terms significantly enriched in differentially regulated genes**

Supplementary Table 3 lists the most significantly enriched GO terms after treatment with either calcitriol or inecalcitol. Of these, 10 were found be significantly enriched after treatment with both compounds. These included pathways involved in muscle cell migration, protein citrullination, negative regulation of cellular processes, negative regulation of locomotion, neuron development and citrulline biosynthetic processes. Some GO terms included in the top 20 enriched list, however, were significantly

### Table 2 Top 20 upregulated genes in MCF-7 cells following treatment with calcitriol or inecalcitol.

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Genes unique to treatment with either calcitriol or inecalcitol are indicated in bold.

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modulated by calcitriol but not by inecalcitol, i.e., bone morphogenesis, ossification and eyelid development. On the other hand, the GO terms, myeloid development, cell development, cell surface receptor signalling pathways and cell communication were amongst the top 20 significantly enriched terms after treatment with inecalcitol but not after treatment with calcitriol.

### Comparison of differentially regulated genes across public transcriptomic studies

Table 3 compares the differentially regulated genes observed in this study with those of previously reported transcriptomic studies involving calcitriol (Goeman et al. 2014, Sheng et al. 2015, Simmons et al. 2015). It is important to state that these different studies

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Values presented are fold-changes. Values in bold represent genes that were altered across all studies. NS, not significant.
for the most part used different cell lines, different concentrations of calcitriol different treatment times and different techniques for investigating gene expression (Supplementary Table 4). Despite these differences, 23, 14 and 27 of the genes whose expression was significantly altered after calcitriol treatment in the present investigation were also significantly modulated in the study of Goeman and coworkers (Goeman et al. 2014), Sheng and coworkers (Sheng et al. 2015) and Simmons and coworkers (Simmons et al. 2015), respectively. Only 4 genes however, were found to be differentially regulated across all studies after treatment with calcitriol, i.e., CYP24A1, transmembrane protease serine 2 (TMPRSS2), calponin-like transmembrane (CLMN) and EFL1 elongation factor-like GTPase 1 (EFTUDE). Interestingly, the same 4 genes were also found to be altered with inecalcitol in this study. Of note, CYP24A1 was the top regulated gene in all studies.

Confirmation of the effect of calcitriol and inecalcitol on gene expression using candidate genes of interest

Although the RNA-seq analysis showed major overlap in the genes regulated by calcitriol and inecalcitol in MCF-7 cells, a small number were specifically regulated by only one of the compounds. Thus, snail family zinc finger 2 (SNAI2) was found to be upregulated by calcitriol only (Supplementary Table 1). On the other hand, expression of myosin-binding protein H (MYBPH) was increased by inecalcitol but not by calcitriol (Supplementary Table 1). We decided to confirm and extend these findings using qPCR. As shown in Fig. 4A, using RT-PCR, the addition of calcitriol increased the expression of SNAI2 not only in MCF-7 cells but also in 3 other cell lines, i.e., in CAMA1 and T47D an MDA-MB-453 cells. Calcitriol, however, did not increase SNAI2 expression in BT20 or Hs578t(i8) cells. Similarly, no increase in SNAI2 was seen in any of the 6 cell lines investigated after the addition of inecalcitol. Indeed increasing the concentration of inecalcitol 4-fold (i.e., up to 16 nM) failed to significantly upregulate SNAI2 expression (data not shown).

In contrast to SNAI2, using RNA-seq, MYBPH was found to be upregulated in MCF-7 cells by inecalcitol but not by calcitriol. Consistent with the RNA-seq data, RT-PCR analysis confirmed that MYBPH was upregulated by inecalcitol in MCF-7 cells (Fig. 4B). Similarly, RT-PCR showed that expression of MYBPH was increased in 5 other cell lines after treatment with inecalcitol. In contrast, calcitriol had no effects in 5 of these cell lines. Although, using RT-PCR, calcitriol was found to upregulate MYBPH expression in MCF-7 cells, the magnitude of this effect was small (Fig. 4B).

Relationship between VDR expression and prognosis in breast cancer

To extend our preclinical findings, we related tumour VDR levels to outcome in a public database of breast cancer survival and gene expression data (Madden et al. 2013). Using the median expression to dichotomise the gene expression data and DFS as the survival endpoint, VDR expression was not significantly associated with survival in breast cancer as a whole (Fig. 5A). Similarly, VDR was not significantly associated with DFS in the
HER2-positive, basal or luminal B molecular subtypes (Fig. 5B, C and D). In contrast, high levels of VDR predicted good outcome in the luminal A patients ($P=0.0056$, HR = 0.74, $n=1035$) (Fig. 5E) and in the luminal A patients known to be treated with adjuvant tamoxifen ($P=0.0003$, HR = 0.27, $n=273$) (Fig. 5F).

**Discussion**

In this investigation, we showed that both calcitriol and the low calcemic-inducing analogue, inecalcitol inhibited breast cancer cell growth in a cell line-dependent manner. Although previous studies also showed that calcitriol as well as some of its specific analogues blocked the growth of breast cancer cells, almost all of these reports used only one or a small number of cell lines (Vink-van Wijngaarden et al. 1994, Welsh 1994, Simboli-Campbell et al. 1996, Swami et al. 2000, 2012, Verlinden et al. 2000, Narvaez et al. 2001, Flanagan et al. 2003, Lundqvist et al. 2014). Furthermore, most of these earlier studies failed to measure the IC$_{50}$ values for growth inhibition by calcitriol or its analogues.

In contrast to the limited number of cell lines used in most of the previous preclinical studies evaluating the cell growth inhibitory potential of calcitriol, our investigation used 15 cell lines representing all the different molecular subtypes of breast cancer, i.e., ER positive, HER2 positive and TN. Furthermore, unlike most previous studies, we measured IC$_{50}$ values for all the cell lines investigated, and we related response to calcitriol and inecalcitol to...
the endogenous concentration of the VDR. Although not unexpected, we found that IC\textsubscript{50} values for both compounds were significantly lower in VDR-positive cells vs VDR-negative cells.

However, the growth of some cell lines that had undetectable levels of VDR by both Western blotting and ELISA was inhibited by inecalcitol. A possible explanation for this finding is that inecalcitol may mediate some of its effects independent of VDR. Across the full panel of 15 cell lines, however, IC\textsubscript{50} values for inecalcitol were lower in the VDR-positive cell lines compared to those in the VDR-negative cell lines, suggesting that the predominant anti-proliferative effects of inecalcitol occurs via VDR.

The finding that VDR-positive cell lines were more sensitive than VDR-negative cell lines to both calcitriol and inecalcitol suggests that VDR levels may be a predictive biomarker for response to these compounds. Although this result might be expected, we are not aware of a previous study that related VDR levels with sensitivity to calcitriol or any of its analogues in a relatively large panel of cancer cell lines.

The extent of growth inhibition, however, depended not only on the endogenous VDR level but also on the molecular subtype of the cell line, i.e., ER-positive cell lines were more sensitive to both compounds than ER-negative cell lines. This enhanced response in ER-positive cell lines, may relate, at least in part, to the trend towards higher levels of VDR in these cell lines compared to ER-negative cells. In contrast to the enhanced response in ER-positive vs ER-negative cell lines, response to both compounds was independent of HER2 and AR status.

Although multiple studies have previously reported that calcitriol has cancer cell growth inhibitory properties (Narvaez et al. 2001, Flanagan et al. 2003, Swami et al. 2012, Kasiappan et al. 2014, Lungchukiet et al. 2014, Chen et al. 2015, Murray et al. 2015) and thus theoretically might be used to treat cancer, it is unlikely to be used clinically due to its hypercalcaemia-inducing activity at the concentrations necessary to inhibit cancer cell growth. In an attempt to circumvent the induction of hypercalcaemia, several low calcemic-inducing vitamin D analogues have been synthesised (for review, see Leyssens et al. 2013). Of the synthetic low calcemic vitamin D analogues, inecalcitol is the most clinically advanced for cancer treatment. This compound was previously reported to inhibit the growth of MCF-7 cells both \textit{in vitro} and in an animal model (Verlinden et al. 2000, Swami et al. 2012). As mentioned in the Introduction section, previous studies reported that inecalcitol was at least 10-fold more potent than calcitriol in blocking cancer cell growth and furthermore was less calcemic in the animal models investigated (Verlinden et al. 2000, Swami et al. 2012).

Here, we not only confirm this greater potency of inecalcitol vs calcitriol in MCF-7 cells but extend the finding across a panel of 15 breast cancer cell lines. Indeed, our IC\textsubscript{50} values for inecalcitol were at least 18-fold lower than those found with calcitriol. Inecalcitol has already undergone a phase I clinical trial in combination with docetaxel in patients with advanced prostate cancer (Medioni et al. 2014). Of the 54 patients treated in this early phase trial, 50 were assessable for response based on PSA measurement. In these assessable patients, PSA levels decreased by ≥30% within 3 months of initiation of treatment in 85% of cases. Overall, the treatment was well tolerated except when the highest concentration of inecalcitol was administered (i.e., 4000μg bid). Furthermore, inecalcitol has recently received orphan drug designation status for the treatment of acute myeloid leukaemia and chronic lymphocytic leukaemia in both Europe and the USA (http://www.ema.europa.eu/docs/en_GB/document_library/Orphan_designation/2014/03/WC500163572.pdf, https://www.accessdata.fda.gov/scripts/opdlisting/oopd/detailedIndex.cfm?cfgridkey=432914, http://www.ema.europa.eu/docs/en_GB/document_library/Orphan_designation/2015/08/WC500192011.pdf, https://www.accessdata.fda.gov/scripts/opdlisting/oopd/detailedIndex.cfm?cfgridkey=479715).

To our knowledge, this is the first study to compare changes in global gene expression after treatment with calcitriol and one of its low calcemic synthetic analogues by RNA-seq analysis. Using the breast cancer cell line, MCF-7, major overlap was found in the genes differentially regulated by calcitriol and inecalcitol. This finding suggests that the 2 compounds act largely in a similar manner. However, a minority of genes were exclusively regulated by each of the compounds, suggesting that they may not act identically.

In contrast to our study showing uniquely regulated genes by calcitriol and its analogue, inecalcitol, Vanoirbeek and coworkers (Vanoirbeek et al. 2009) using microarray previously reported that calcitriol and the analogue, WY1112 regulated the same set of genes. In contrast to our study that used RNA-seq analysis to identify differential gene expression, this previously published report used microarray. It is generally believed that the newer technique of RNA-seq is considerably more sensitive than microarray in detecting gene expression. Thus, our use of the more sensitive technique may have detected more differentially expressed genes than the microarray used.
previously. A further reason why calcitriol and inecalcitol may induce different genes, in contrast with calcitriol and WY1112, is the possibility that not all vitamin D analogues act similarly. Thus, although calcitriol and WY1112 may regulate the same genes, calcitriol and inecalcitol may not necessarily do so.

Using GO term analysis, genes involved in functions such as bone morphogenesis and ossification were specifically enriched by calcitriol, whereas cell surface signalling and cell communication were specifically enriched after treatment with inecalcitol.

A surprising finding from the GO term analysis, however, was that the term, cellular response to vitamin D, was not enriched after treatment with inecalcitol. The likely explanation for this is that this particular GO category is sparse, containing only 6 genes (TRIM24, BAZ1B, SNAI2, KANK2, VDR and CYP24A1). The calcitriol-regulated gene list contained SNAI2 and CYP24A1, and therefore, reached significance (2/6 vs background). In contrast, the inecalcitol-regulated genes only contained CYP24A1 that failed to reach significance (1/6 vs background). This is a quirk of the GOseq approach (Young et al. 2010) and the small size of this particular GO category.

A key question is whether the induction of any of the unique genes can explain the enhanced cell growth inhibitory potential of inecalcitol compared with calcitriol (Verlinden et al. 2000, Swami et al. 2012). In this context, the SNAI2 gene that was upregulated by calcitriol but not by inecalcitol after RNA-seq analysis was previously reported to suppress VDR expression and decrease calcitriol-mediated cancer cell growth inhibition (Pálmer et al. 2004). Using RT-PCR, we confirmed that SNAI2 was upregulated not only in MCF-7 cells but also in MDA-MB-453, CAMA1 and T47D cells. Unlike our findings with calcitriol, SNAI2 expression was not found to be altered by inecalcitol in any of the 6 cell lines tested. As SNAI2 was previously reported to inhibit calcitriol-mediated cell growth inhibition (Pálmer et al. 2004), this observation of different regulation of SNAI2 by the 2 compounds might at least partly explain the enhanced cell growth suppression mediated by inecalcitol vs calcitriol.

In contrast to SNAI2, MYBPH was found to be upregulated by inecalcitol in all of the 6 cell lines investigated. Calcitriol failed to increase MYBPH in 5 of the six cell lines. As MYBPH has previously been implicated in reducing cancer cell migration and metastasis (Hosono et al. 2012), this finding may provide an explanation for the greater potency of inecalcitol compared to calcitriol in reducing migration (Fig. 4).

Several studies have previously investigated the effects of calcitriol on global gene expression in cell lines (Goeman et al. 2014, Sheng et al. 2015, Simmons et al. 2015). The key conclusion from these reports was that only a small number of genes were found to be consistently regulated in the different studies. Thus, Simmons and coworkers (Simmons et al. 2015) identified only 11 genes that were simultaneously altered in 3 different breast cancer cell lines and only 5 overlapping genes in 4 different datasets, i.e., CYP24A1, CLMN, SERPINB1, IL1R1 and EFTUDE. Of these 5 overlapping genes, 3 were found to be modulated by both calcitriol and inecalcitol in the present study, i.e., CYP24A1, CLMN and EFTUDE.

Likely reasons for the different profile of genes found to be regulated in the different reports include the use of different cell lines, different concentration of calcitriol/analogue used, different lengths of treatment, different sensitivity in technique used to measure gene expression, different cut-off points for defining fold-difference in gene expression and different statistical tools used for the analysis of gene expression.

Although the identity of the calcitriol-regulated genes is variable from report to report, a consistent finding is that the top upregulated gene across all studies is CYP24A1. Indeed, in our study, CYP24A1 was also the top upregulated gene by inecalcitol. However, in our study, the upregulation of CYP24A1 by inecalcitol was approximately 3-fold higher than that by calcitriol, although the concentration of the synthetic analogue used was 500-fold lower than that of calcitriol. CYP24A1 is a member of the cytochrome class 1 P450 family of proteins and is believed to be the key enzyme involved in the inactivation of calcitriol. One of its functions thus appears to be preventing the accumulation of potentially toxic concentrations of calcitriol (Luo et al. 2016). In contrast to the greater potency of inecalcitol vis-à-vis calcitriol in inducing CYP24A1, the extent of the fold-change in the differential regulation of most of the other genes was similar for both compounds.

To identify the likely pathways in which the calcitriol-/inecalcitol-regulated genes were involved, we performed GO analysis. Using the R package known a goseq (Christakos et al. 2016), the top GO term associated with treatment with both inecalcitol and calcitriol was muscle migration. Although muscle has not traditionally been regarded as a classical target tissue for vitamin D, several reports suggest that vitamin D plays a role in both skeletal and smooth muscle cell migration (Rebsamen et al. 2002, Tukaj et al. 2010, Owens et al. 2015). In one of these reports, calcitriol was shown...
to promote smooth cell migration by upregulating PI3K signalling (Rebsamen et al. 2002). In another, the compound appeared to enhance migration by inducing cytoskeletal reorganisation (Tukaj et al. 2010).

The second most significantly enriched GO term involving both calcitriol and inecalcitol was protein citrullination. Protein citrullination is a post-translational modification of proteins in which arginine residues are converted to citrulline. After a review of the published literature, we were unable to identify any previous report that linked vitamin D with protein citrullination. However, as histone citrullination has previously been associated with gene regulation (Fuhrmann & Thompson 2015, Clancy et al. 2016), vitamin D-mediated citrullination maybe one of the mechanism by which the compound modulates gene expression. To our knowledge, vitamin D has not previously been implicated in protein citrullination.

Similar to the situation in breast cancer cell lines, VDR is expressed in a subset of breast cancers (Berger et al. 1991, Friedrich et al. 2002, Ditsch et al. 2012, Al-Azhri et al. 2017). Published findings, however, are mixed as regards its potential prognostic value in this malignancy (Berger et al. 1991, Friedrich et al. 2002, Ditsch et al. 2012, Al-Azhri et al. 2017). Here, we show that high VDR expression predicted favourable outcome only in patients with luminal A disease, especially if these patients were treated with adjuvant tamoxifen. In contrast, VDR was unrelated to outcome in the total population of patients or in those with luminal B, HER2 or TN disease. Previously, Berger and coworkers (Berger et al. 1991) reported that high VDR levels correlated with a favourable disease-free interval but not with overall survival. Ditsch and coworkers (Ditsch et al. 2012) however, found that high VDR levels predicted good overall survival. On the other hand, Friedrich and coworkers (Friedrich et al. 2002) and Al-Azhri and coworkers (Al-Azhri et al. 2017) concluded that VDR was not prognostic in breast cancer. Unlike our study, those previously reported investigations failed to separate breast cancer patients into the different molecular subgroups. Our finding of VDR being associated with good overall survival, only in patients with luminal A disease may explain the discrepant finding previously reported. A possible reason why patients with high VDR expression have better survival than those with low levels when treated with tamoxifen is that this subgroup of patients has higher levels of ER than those with low levels of VDR (Al-Azhri et al. 2017). Patients with higher ER levels are well known to derive more benefit from tamoxifen than those with low levels of ER.

In conclusion, we show that the low calcaemic-inducing vitamin D analogue, inecalcitol was substantially more potent in blocking cell growth than calcitriol. Inhibition of cell growth was most pronounced in ER-positive cells lines and was dependent on the concentration of cell line VDR levels. Our RNA-seq analysis suggests that there is substantial overlap in the genes differentially regulated by calcitriol and its analogue, inecalcitol. This finding suggests that both compounds act in a largely similar manner. However, as there were some altered genes and GO terms unique to the treatment with each compound, their mode of action is unlikely to be identical. A challenge for future research will be to establish whether any of the uniquely regulated genes can explain the enhanced cell growth inhibitory potency of inecalcitol compared to calcitriol. As inecalcitol has previously been shown to be growth inhibitory in a mouse model of breast cancer (Verlinden et al. 2000) and to have undergone investigations in an early phase clinical trial (Medioni et al. 2014), it should now be considered for evaluation in a clinical trial in breast cancer, especially in ER-positive patients.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-16-0463.

Declaration of interest
The authors wish to confirm that there are no known conflicts of interest associated with this publication for all co-authors, and there has been no significant financial support for this work that could have influenced its outcome.

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Author contribution statement
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Endocrine-Related Cancer

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