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Targeting 1α,25-dihydroxyvitamin D_3 antiproliferative insensitivity in breast cancer cells by co-treatment with histone deacetylation inhibitors☆

Claire M. Banwell[a,*], Laura P. O'Neill[a], Milan R. Uskokovic[b], Moray J. Campbell[a]

[a] *Department of Medicine, Division of Medical Sciences, Queen Elizabeth Hospital, University of Birmingham Medical School, Edgbaston, Birmingham B15 2TT, UK*
[b] *Hoffman-La Roche, Nutley, NJ 07110, USA*

Abstract

Proliferation of the non-malignant breast epithelial cell line, MCF-12A, is sensitively and completely inhibited by 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) (ED_{90} = 70 nM), We used real time RT-PCR to demonstrate that the relative resistance to 1α,25$(OH)_2D_3$ of MDA-MB-231 cells (ED_{50} > 100 nM) correlated with significantly reduced Vitamin D receptor (VDR) and increased NCoR1 nuclear receptor co-repressor mRNA (0.1-fold reduction in VDR and 1.7-fold increase in NCoR1 relative to MCF-12A ($P < 0.05$)). This molecular lesion can be targeted by co-treating cells with 1α,25$(OH)_2D_3$ or potent analogs and the histone deacetylation inhibitor trichostatin A (TSA). For example, the co-treatment of 1,25-dihydroxy-16,23,Z-diene-26,27-hexafluoro-19-nor Vitamin D_3 (RO-26-2198) (100 nM) plus TSA results in strong additive antiproliferative effects in MDA-MB-231 cells. This may represent novel chemotherapeutic regime for hormone insensitive breast cancer.

Keywords: Histone deacetylation; NCoR1; Chemotherapy

1. Introduction

In the UK and the USA breast cancer is the second most common cause of cancer mortality amongst women. Surgery and radiation-based therapies combined with estrogen ablation can be highly effective in the short term but in a significant proportion of cases the disease will relapse subsequently, in a resistant and invasive form that is frequently lethal. New, better tolerated treatments are required and chemotherapies that induce differentiation or apoptosis in tumours are promising area of research that may be particularly attractive for these indolent tumours.

1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) regulates differentiation of the normal mammary gland. In explant cultures pro-differentiating hormones such as cortisol, prolactin and insulin up-regulate the Vitamin D_3 receptor (VDR), which is initially present at low levels. Similarly, there is impaired ductal differentiation and branching in the mammary gland of VDR knockout mice compared to wild-type litter mates [1,2].

Breast cancer cell lines display a varied antiproliferative response to 1α,25$(OH)_2D_3$. For example, MCF-7 cells, which retain expression of estrogen-receptor (ER) α and β, displays antiproliferative sensitivity to 1α,25$(OH)_2D_3$ and readily undergoes apoptosis in response. By contrast, more aggressive cell lines with altered ER α and β expression profiles, for example MDA-MB-231, are essentially insensitive to 1α,25$(OH)_2D_3$ anti-proliferative effects [3,4] requiring high concentrations of 1α,25$(OH)_2D_3$ associated in vivo with dose-limiting hypercalcaemic side effects. Consequently, a research focus has aimed at developing analogs of 1α,25$(OH)_2D_3$ which are more potent inhibitors of proliferation, but with reduced induction of hypercalcaemia. This has been partially successful with a number of critical changes to the parental hormone resulting in analogs with enhanced antiproliferative potency without enhancing in vivo calcaemic activity. The mechanism underlying the apparent 1α,25$(OH)_2D_3$ insensitivity remains unresolved as, generally, cancer cell lines, retain wild-type VDR and content does not correlate clearly with activity [5–7].

The transcriptional outcome of nuclear receptors is determined by the ligand dependent changes that are exerted on the dynamic balance of histone acetylation and deacetylation, in concert with other post-translational epigenetic events. VDR as a member of the nuclear receptor

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* Corresponding author. Tel.: +44-121-4721311x3923.
E-mail address: claire_banwell@yahoo.com (C.M. Banwell).

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superfamily, acts as multi-meric ligand-dependent transcription factors by binding to specific VDR response elements in the promoter/enhancer regions of primary responding genes. In the absence of ligand the VDR associates with large complexes, containing co-repressors such as either nuclear receptor co-repressor 1 (NCoR1) or silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), and histone deacetylase (HDAC) enzymes [8,9]. This complex stabilises DNA-histone contacts creating a closed chromatin structure that actively represses gene transcription. $1\alpha,25(OH)_2D_3$ causes VDR conformational changes promoting association with co-activator complexes which conversely contain histone acetylases, which destabilise histone–DNA contacts and provide a more transcriptionally permissive environment.

We hypothesised that the dynamic balance of histone modicifications is disrupted in $1\alpha,25(OH)_2D_3$ insensitive breast cancer cells through the altered co-repressors activity and/or expression. We have therefore examined whether altered expression of co-repressors accounts for $1\alpha,25(OH)_2D_3$ resistance in MDA-MB-231 cells and whether sensitivity can be restored by co-treatment with an HDAC inhibitor, trichostatin A.

2. Materials and methods

2.1. Cell culture

The breast cancer cell lines MDA-MB-231 were obtained from Dr. Kay Colston (St. George's Hospital, London, UK) and maintained in 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Gibco-BRL, Paisley) in RPMI media and passaged by trypsinising with trypsin-EDTA (Gibco-BRL). MCF-12A cells were a generous gift of Prof. H. Phillip Koeffler (Cedars-Sinai Medical Ctr/UCLA School of Medicine, Los Angeles, USA). These cells are a non-tumorigenic epithelial cell line, established from tissue taken at mammoplasty reduction from a nulliparous patient with fibrocystic breast disease that contained focal areas of intraductal hyperplasia. They are not tumorigenic in immunosuppressed mice, but do form colonies in semi-solid media. These cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone, 95%; horse serum, 5% [10]. All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Vitamin D_3 compounds and trichostatin A

$1\alpha,25(OH)_2D_3$ and 1,25-dihydroxy-16,23,Z-diene-26,27-hexafluoro-19-nor Vitamin D_3 (RO-26-2198) and trichostatin A (TSA) (Sigma–Aldrich, Poole, UK) were dissolved in ethanol at 1 mM as a stock solution and stored in the dark at −20 °C.

2.3. Proliferation assays

2.3.1. Liquid culture

The action of individual agents alone and in combination was examined using a bioluminescent technique to measure changes in cellular ATP (ViaLight HS, LumiTech, Nottingham, UK) with previously optimised conditions according to the manufacturer's instructions [11]. Briefly, cells were plated in 96 well white-walled tissue culture-treated plates (2×10^3 cells per well) (Fisher Scientific Ltd., Loughborough, UK). Media containing varying concentrations of TSA, $1\alpha,25(OH)_2D_3$, or RO-26-2198 was added to a final volume of 100 μl per well and plates were incubated for 96 h, with re-dosing after 48 h. After the incubation period, 100 μl of nucleotide releasing reagent was added to each well and cells were left for 30 min at room temperature. Liberated ATP was quantitated by adding 20 μl of ATP monitoring reagent (containing luciferin and luciferase) and measuring luminescence with a microplate luminometer (Berthold Detection Systems, Fisher Scientific Ltd.). ATP levels were recorded in relative luciferase units and inhibition of proliferation was expressed as a percentage of control.

2.3.2. Clonal proliferation in soft agar

Trypsinised and washed single-cell suspensions of cells from 80% confluent cultures were enumerated and plated into 24 well flat-bottom plates (Costar, Bucks, UK) using a two-layer soft agar system with a total of 1×10^3 cells per well in a total volume of 600 μl per well. Both layers were prepared with sterile agar (1%) that had been equilibrated previously at 42 °C. TSA (15 nM) and/or $1\alpha,25(OH)_2D_3$ were added to the wells prior to the addition of the feeder layer (20% FCS, 40% 2xRPMI, 40% agar). The cells were mixed into the top layer (20% FCS, 30% 2xRPMI, 30% agar, 18% media containing cells, 1% L-glutamine (100 mg/ml), 1% β-mercaptoethanol (1 mM)) and plated onto the pre-set under layer. After 14 days incubation at 37 °C in a humidified atmosphere of CO_2 in air, the colonies (more than 50 cells) were counted under an inverted microscope. All experiments were performed three times and in triplicate.

2.4. Quantitative real time reverse transcription-polymerase chain reaction

Cells were seeded at 5×10^5 cells per T-25 vented tissue culture flask (Costar) and incubated for 36 h at 37 °C in a humidified atmosphere of 5% CO_2 and then total RNA was extracted from approximately 70% confluent cells with Tri Reagent (Sigma) according to manufacturer's instructions. cDNA was prepared from RNA (1 μg) was prepared using standard protocols and expression of specific mRNAs was quantitated using the ABI PRISM 7700 sequence detection system. Each sample was amplified in triplicate wells in 25 μl volumes containing 1× TaqMan Universal PCR Master Mix (3 mM $Mn(OAc)_2$, 200 μM dNTPs, 1.25 U amplitaq gold polymerase, 1.25 U amperase UNG), 3.125 pmol

FAM-labelled TaqMan probe and 22.5 pmol primers. All reactions were multiplexed with pre-optimised control primers and VIC labelled probe for 18S ribosomal RNA (PE Biosystems, Warrington, UK). Primer and probe sequences are given in Table 2. Reactions were cycled as follows: 50 °C for 2 min, 95 °C for 10 min; then 44 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine δCt values (δCt means Ct of the target gene minus Ct of the 18S). The data were transformed through the equation $2^{-\delta Ct}$ to give fold changes in gene expression. To exclude potential bias due to averaging of data, all statistics were performed with δCt values. Measurements were carried out a minimum of three times each in triplicate wells for each condition.

2.5. Statistical analysis

The interactions of two compounds were assessed by measuring the mean effect of either Vitamin D_3 compound or TSA acting alone (±standard error of the mean). The combination of the mean growth inhibition for each compound acting alone was the predicted combined effect. The mean observed combined inhibition was then compared with this value using the Student's *t*-test. Strong additive effects were those with an experimental value significantly greater than the predicted value, and additive was where it did not differ.

3. Results

3.1. MCF-12A and MDA-MB-231 cells show a varying anti-proliferative response to 1α,25(OH)$_2$D$_3$ which correlates with altered expression ratio of VDR to the co-repressor NCoR1

We examined the clonal growth of the MCF-12A and MDA-MB-231 cell lines by dose response assays to 1α,25(OH)$_2$D$_3$ in soft agar. The proliferation of the non-malignant breast epithelial cell line, MCF-12A, is sensitively and completely inhibited by 1α,25(OH)$_2$D$_3$ with an ED_{50} value of 5 nM and an ED_{90} = 70 nM. By contrast, growth of MDA-MB-231 cells was essentially insensitive with an ED_{50} value >100 nM (Fig. 1A). We used real time RT-PCR to demonstrate that the spectrum of sensitivities displayed by MCF-12A and MDA-MB-231 correlated with significantly reduced VDR and increased NCoR1 nuclear

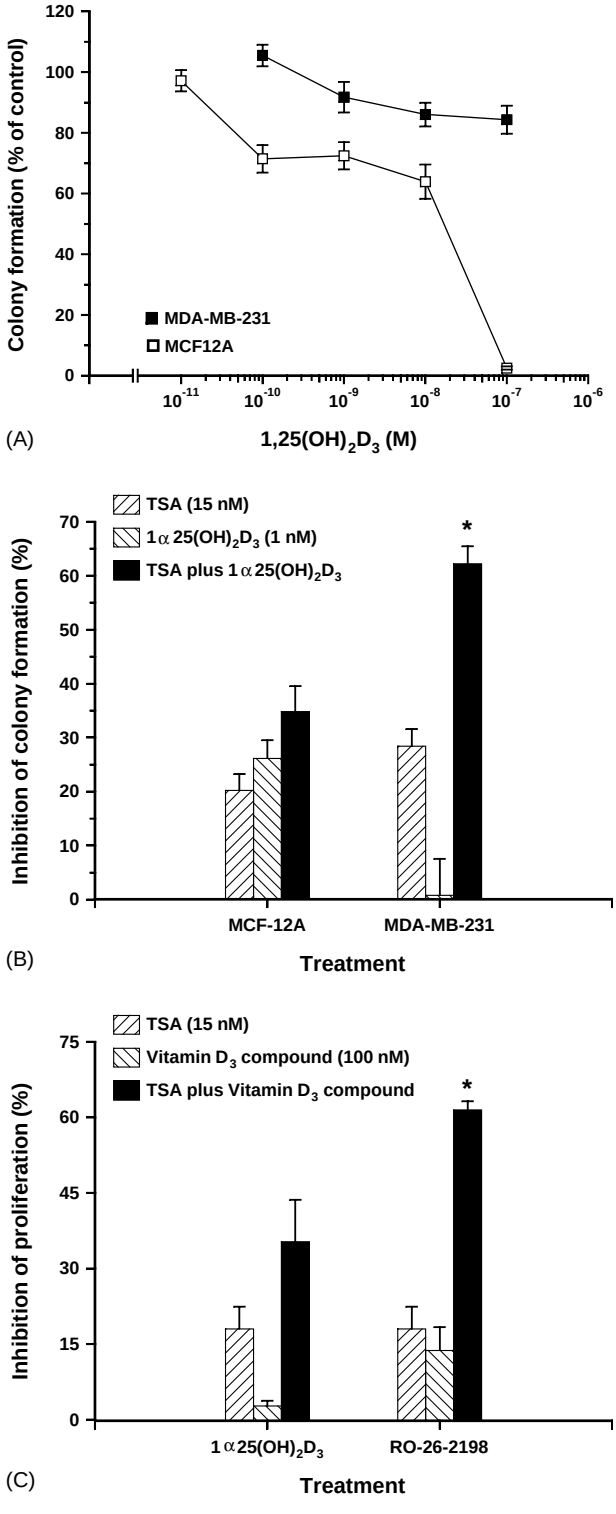

Fig. 1. Insensitivity of MDA-MB-231 cells to inhibition of proliferation in response to 1,25(OH)$_2$D$_3$. Panel A: the dose response of 1,25(OH)$_2$D$_3$ in MCF-12A cells and MDA-MB-231 cells was assessed by inhibition of clonal proliferation in two-layer soft agar assays. The top layer containing 1×10^3 cells per well was plated onto the pre-set feeder layer that contained 1,25(OH)$_2$D$_3$. After 14 days growth, colony number was enumerated and compared to untreated control. Each data point represents the mean of three separate experiments undertaken in triplicate wells. Panel B: MCF-12A and MDA-MB-231 cells were treated with either 1,25(OH)$_2$D$_3$ (1 nM) or TSA (15 nM), alone or in combination and the effects on colony formation were measured as in panel A. Panel C: MDA-MB-231 cells were plated into 96 well plates at a density of 2×10^3 cells per well and treated with either Vitamin D_3 compound alone (100 nM), or 15 nM TSA) or in combination. The cells were incubated for 96 h and then total ATP was measured in each well using a luciferase-dependent method according to the Section 2 and compared to untreated control.

receptor co-repressor mRNA. MDA-MB-231 cells have 0.1-fold change in VDR and 1.7-fold increase in NCoR1 relative to MCF-12A ($P < 0.05$).

3.2. Histone deacetylase inhibitors co-operate with Vitamin D_3 compounds to inhibit cell growth

We reasoned that this molecular lesion can be targeted by co-treating cells with 1α,25$(OH)_2D_3$ and the HDACi, TSA. We therefore went on to examine whether co-treatment with TSA would restore 1α,25$(OH)_2D_3$ antiproliferative signalling by 1α,25$(OH)_2D_3$ or potent analogs. We screened the response of the cell lines to the antiproliferative action of TSA and constructed single agent dose responses (data not shown). Subsequently, we utlised doses of TSA (15 nM), which alone resulted in approximately 25% inhibition of proliferation, in combination with either 1α,25$(OH)_2D_3$ or RO-26-2198.

These combinations resulted in a range of strong additive interactions in MDA-MB-231 cells. Clonal proliferation in soft agar demonstrated that doses of 1α,25$(OH)_2D_3$ as low as 1 nM were significantly potentiated by the addition of TSA (15 nM). In MCF-12A only a sub-additive effect was observed with the combination of agents. (Fig. 1B). For example in MDA-MB-231 cells these agents combined to inhibited clonal proliferation by 63 ± 4% (±S.E.M.) whereas 1α,25$(OH)_2D_3$ alone had no effect on colony formation and TSA inhibited colony formation by (26% of control) ($P < 0.05$). These effects could be augmented further by using analogs of 1α,25$(OH)_2D_3$ that were resistant to CYP24 metabolism (Fig. 1C). For example, RO-26-2198 (100 nM) and TSA (15 nM) alone each inhibited MDA-MB-231 cells 13% (±4%) and 18% (±4%), respectively, whereas, the combination inhibited proliferation by 61% (±2%) ($P < 0.05$) (Fig. 1C).

4. Discussion

Breast cancer cell lines display a spectrum of antiproliferative responses to 1α,25$(OH)_2D_3$ which include insensitivity not only to the parental hormone but also to 1α,25$(OH)_2D_3$ analogs. In the current study we have examined the basis of insensitivity in one such cell line MDA-MB-231. This cell line is representative of an aggressive hormonally-insensitive stage of disease being insensitive to estrogen withdrawal and having altered expression profiles of ER α and β, and is highly invasive in vivo.

The 1α,25$(OH)_2D_3$-insensitivity in MDA-MB-231 cells appears not to be determined by the VDR content per se, but rather reflects an altered ratio of VDR to co-repressor expression with reduced VDR and elevated co-repressor content. For example, MDA MB 231 cells are at least 500 times less sensitive to the anti-proliferative effects of 1α,25$(OH)_2D_3$ compared to non-malignant MCF-12A cells and comparatively have lower VDR levels and elevated NCoR1 levels. We hypothesised that elevated co-repressor levels combined with reduced VDR content suppresses transactivation of key 1α,25$(OH)_2D_3$-antiproliferative target genes, even when 1α,25$(OH)_2D_3$ is present in the cancer cells. Consistent with this model, the addition of a HDAC inhibitor enhanced the anti-proliferative action of 1α,25$(OH)_2D_3$ in the MDA-MB-231 breast cancer cells.

A major limitation of 1α,25$(OH)_2D_3$ is its rapid metabolism by the cytochrome P450 enzyme 1α,25$(OH)_2D_3$-24-hydroxylase. Therefore, using analogs of 1α,25$(OH)_2D_3$ that are resistant to this action, have been investigated extensively by us and others previously [12,13]. Therefore, in the current study we have demonstrated that the anti-proliferative effects of co-treatment with TSA were potentiated significantly further by combining with CYP24-resistant analogs. Intriguingly, although the hexafluoride analogs have enhanced potency over 1α,25$(OH)_2D_3$, their action can be enhanced further still by co-treating with TSA, suggesting that enhanced metabolism of 1α,25$(OH)_2D_3$ is not the sole mechanism of 1α,25$(OH)_2D_3$ insensitivity.

Resistance to other nuclear receptor ligands has been associated with nuclear receptors that aberrantly bind co-repressor and shift the dynamic equilibrium of histone modifications to maintain the deacetylated state, thereby repressing transcription. For example, the aberrant binding of NCoR1 and SMRT to chimeric retinoic acid receptors (PML-RAR and PLZF-RAR) suppresses retinoic acid signalling, resulting in acute promyelocytic leukaemia [14].

Taken together these data support a model whereby elevated co-repressor levels lead to epigenetic silencing of the transcriptional responsiveness of key antiproliferative target genes and insensitivity towards 1α,25$(OH)_2D_3$. Intriguingly VDR expression is neither lost completely nor mutated, but rather is reduced in expression. This would suggest that some level of expression of wild-type VDR is actually of benefit to malignant cells, possible to promote survival, anti-apoptotic responses. However, the suppression of antiproliferative responsiveness can be targeted by co-treatment with HDACi and Vitamin D_3 compounds. This model is supported by our results in prostate cancer cells and may represent a general mechanism to repress nuclear receptor activity in epithelial tumours [11,15]. Furthermore, the combination of agents may represent an attractive, well tolerated chemotherapy for individuals with hormone insensitive and/or invasive breast cancer.

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