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Epigenetic repression of transcription by the Vitamin D_3 receptor in prostate cancer cells☆,☆☆

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Abstract

Normal prostate epithelial cells are acutely sensitive to the antiproliferative action of 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), whilst prostate cancer cell lines and primary cultures display a range of sensitivities. We hypothesised that key antiproliferative target genes of the Vitamin D receptor (VDR) were repressed by an epigenetic mechanism in 1α,25$(OH)_2D_3$-insensitive cells. Supportively, we found elevated nuclear receptor co-repressor and reduced VDR expression correlated with reduced sensitivity to the antiproliferative action of 1α,25$(OH)_2D_3$. Furthermore, the growth suppressive actions of 1α,25$(OH)_2D_3$ can be restored by co-treatment with low doses of histone deacetylation inhibitors, such as trichostatin A (TSA) to induce apoptosis.

Examination of the regulation of VDR target genes revealed that co-treatment of 1α,25$(OH)_2D_3$ plus TSA co-operatively upregulated GADD45α. Similarly in a primary cancer cell culture, the regulation of appeared GADD45α repressed. These data demonstrate that prostate cancer cells utilise a mechanism involving deacetylation to suppress the responsiveness of VDR target genes and thus ablate the antiproliferative action of 1α,25$(OH)_2D_3$.

Keywords: Histone deacetylation inhibitors; 1α,25-Dihydroxyvitamin D_3; Prostate cancer; Co-repressors

1. Introduction

Prostate epithelial cells express multiple members of the nuclear receptor (NR) superfamily which regulate proliferation and differentiation in the prostate gland. Their action is disrupted in prostate cancer, by both gain and loss of function; for example, the androgen receptor signalling is enhanced through multiple mechanisms, while expression of other receptors is lost, such as retinoic acid receptor (RAR) β [1–3].

Multiple epidemiological studies have now linked the incidence of prostate cancer to low serum levels of the 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) precursor, 25$(OH)D_3$ as a result of deficiency in either diet or environment [4,5]. Furthermore, certain Vitamin D receptor (VDR) polymorphisms are also associated with an elevated incidence of prostate cancer [6]. In vitro and in vivo proliferation of normal prostate epithelial cells is acutely regulated by 1α,25$(OH)_2D_3$ and this antiproliferative response is retained in some primary and established prostate cancer cell lines, justifying clinical trials of 1α,25$(OH)_2D_3$ in prostate cancer patients [7,8]. However, the antiproliferative response is reduced to various degrees in other prostate tumours [9]. Collectively, such data link 1α,25$(OH)_2D_3$ with a protective action against uncontrolled prostate growth, and suggest that reduced exposure, or cellular resistance to the antiproliferative effects, may play roles in the initiation or progression of prostate cancer.

The molecular mechanisms for 1α,25$(OH)_2D_3$-insensitivity in prostate cancer are as yet unclear. We and others have demonstrated that the VDR is neither mutated nor is there a clear relationship between VDR expression and

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growth inhibition by $1\alpha,25(OH)_2D_3$ [10,11]. Indeed, the PC-3 and DU 145 prostate cancer cell lines are relatively $1\alpha,25(OH)_2D_3$-insensitive and yet VDR transactivation is sustained or even enhanced, as measured by induction of the highly $1\alpha,25(OH)_2D_3$-inducible *CYP24* gene (encoding $25(OH)D_3$-24-hydroxylase) [12].

VDR associates with Vitamin D response elements (VDRE) in the promoter/enhancer region of target genes as part of multimeric, repressive or activating complexes. A dynamic balance exists between these divergent complexes which are regulated by ligand. In the absence of $1\alpha,25(OH)_2D_3$, the VDR associates with NCoR1, SMRT and Alien co-repressor complexes, which include histone deacetylases (HDAC). These complexes maintain the histone N-terminal 'tails' in a charged state tightly associated with DNA, thereby maintaining a closed chromatin structure and locally suppressing transcription of target genes [13,14]. Ligand binding induces VDR conformational changes and promotes association with co-activator complexes containing proteins such as SRC-1, NCoA-62, GRIP-1 and DRIP factors. Some of these co-activators conversely have associated histone acetylase (HAT) activity, thereby relaxing DNA–histone associations, opening up the chromatin structure and facilitating signalling to the pre-initiation complex [15–17]. Thus, the promoter-specific, ordered regulation of histone modifications forms a 'histone code' that selectively determines the transcriptional activity of VDR target genes [18].

Previously, we showed that co-treatment of prostate cancer cell lines (LNCaP, PC-3 and DU 145) with $1\alpha,25(OH)_2D_3$ plus HDAC inhibitors, either trichostatin A (TSA) or sodium butyrate, resulted in additive and synergistic inhibition of proliferation associated with apoptosis [19]. These data support the hypothesis that an imbalance in the co-activator/co-repressor balance alters receptor activity. For example, altered expression/activity of a co-repressor, with associated histone deacetylation activity, thus represses the sensitivity of VDR antiproliferative target genes resulting in $1\alpha,25(OH)_2D_3$-insensitivity. We have now dissected the effects of $1\alpha,25(OH)_2D_3$ plus TSA on gene expression patterns to identify antiproliferative pathways that are suppressed in malignancy. Potentially this opens avenues to utilise combined therapy to treat hormonally insensitive disease with Vitamin D analogs and HDAC inhibitors to deliver sustained therapeutic regimes, which overcome the toxic side-effects and sustain anticancer effects.

2. Materials and methods

2.1. $1\alpha,25(OH)_2D_3$ and HDAC inhibitors

$1\alpha,25(OH)_2D_3$ (generous gift of Dr. Milan R. Uskokovic, Hoffman La Roche, Nutley, NJ 07110, USA) and TSA (Sigma, Poole, UK) were all stored as 1 mM stock solutions in ethanol at −20 °C.

2.2. Cell culture

Normal prostate epithelial cells (PrEC) were cultured in PrEGM media (Clonetics, Wokingham, UK) according to manufacturers instructions. The prostate cancer cell line PC-3 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in RPMI 1640 medium (Gibco-BRL), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Gibco-BRL, Paisley) and passaged by trypsinising with 0.25% trypsin–EDTA (Gibco-BRL). All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.3. Primary cultures

Tissues dissected from radical prostatectomy specimens were processed for primary culture of prostatic epithelial cells according to previously described methods [23]. None of the patients had received prior chemical, hormonal or radiation therapy. Histological assessment was performed as described [20]. The cell strains were generated from either the normal tissue of the peripheral zone (pz) or the adenocarcinoma (ca) from the same individual, each cell strain was serially passaged and cells in secondary or tertiary passages were used for RNA isolation.

2.4. Proliferation assays

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 [19]. Briefly, cells were plated in 96-well white-walled tissue culture-treated plates (Fisher Scientific Ltd., Loughborough, UK; PC-3 at 2×10^3 cells per well and PrEC at 3.5×10^3 cells per well). Growth media containing varying concentrations of TSA, $1\alpha,25(OH)_2D_3$, 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 growth inhibition was expressed as a percentage of control.

2.5. Extraction of RNA and reverse transcription

Cells were allowed to grow for 36 h to ensure that cells were in mid-exponential phase upon treatment. Cells were dosed as indicated and total RNA was extracted

using the GenElute RNA extraction system (Sigma) according to manufacturer's instructions. Primary cultures were serially passaged and grown to 80% confluency in standard serum-free medium. Cells were fed 1 day prior to isolation of total RNA using the Qiagen RNeasy Midi Kit (Qiagen, Palo Alto, CA, USA). For real time reverse transcription-polymerase chain reaction (RT-PCR), cDNA was prepared from 1 μg of total RNA by reverse transcription with Mu-MLV (Promega Southampton, UK).

2.6. Real-time quantitative RT-PCR

Expression of specific mRNAs was quantitated using the ABI PRISM 7700 sequence detection system (GADD45α forward primer AAGACCGAAAGGATGGATAAGGT, GADD45α reverse primer GTGATCGTGCGCTGACTCA, GADD45α probe TGCTGAGCACTTCCTCCAGGGCAT). 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 units AmpliTaq Gold polymerase, 1.25 units 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). 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 = Ct of the target gene minus Ct of the housekeeping gene). The data was transformed through the equation $2^{-\delta\delta Ct}$ 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 cell lines and once each in triplicate wells for primary material.

2.7. Statistical analysis

The interactions of two compounds were assessed by measuring the mean of either $1\alpha25(OH)_2D_3$ or TSA acting alone or in combination [19]. The mean observed combined effect was compared to the individual effects of the agents added together, using the Student's *t*-test. Classification of the effects were as follows: strong additive effects were those with an experimental value significantly greater than the predicted value, additive effects were those where the experimental value did not significantly differ from the predicted value, sub-additive effects were those where the experimental value was significantly less than the predicated value. All other analyses were compared using the Student's *t*-test.

3. Results

3.1. PrEC and PC-3 cells show a varying anti-proliferative response to $1\alpha,25(OH)_2D_3$ which correlates with an altered expression ratio of VDR to the co-repressor SMRT

We examined cell proliferation in liquid media of PC-3 prostate cancer cells and PrEC cells, as a non-transformed epithelial counterpart. These cells had approximately equal doubling times (22 h) but significantly different proliferative responses to $1\alpha,25(OH)_2D_3$. Fig. 1A demonstrates the differential sensitivities obtained using this relatively insensitive proliferation assay, with PC-3 being essentially insensitive ($ED_{50} > 100$ nM) whereas PrEC cells are significantly sensitive at doses equal to, or greater than, 1 nM ($P < 0.05$). Subsequently, we used real time RT-PCR to demonstrate that the spectrum of sensitivities displayed by these cells correlated with significantly reduced VDR and increased SMRT nuclear receptor co-repressor mRNA. PC-3 cells have 0.1-fold change in VDR and 1.8-fold increase in SMRT relative to PrEC cells ($P < 0.05$) (data not shown).

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

We reasoned that the impact of elevated co-repressor expression on nuclear receptor signalling could be targeted by co-treating cells with $1\alpha,25(OH)_2D_3$ and the HDACi, TSA. We therefore went on to examine whether co-treatment with TSA would restore $1\alpha,25(OH)_2D_3$ antiproliferative signalling by $1\alpha,25(OH)_2D_3$. We screened the response of the cell lines to the antiproliferative action of TSA and constructed single agent dose responses (Fig. 1B). Interestingly at higher doses of TSA, greater than 50 nM, PrEC cells are significantly less sensitive to this agent than PC-3 cells. Subsequently, we utilised doses of TSA (15 nM), which alone resulted in approximately 25% inhibition of proliferation, in combination with $1\alpha,25(OH)_2D_3$.

The combination of $1\alpha,25(OH)_2D_3$ and TSA resulted in a range of strong additive interactions in PC-3 cells. Liquid proliferation demonstrated that doses of $1\alpha,25(OH)_2D_3$ of 100 nM, which were essential inactive alone, were significantly potentiated by the addition of TSA (15 nM) (Fig. 1C). For example, these agents combined to inhibit proliferation by 62 ± 4% (±S.E.M.) compared to control cultures, whereas $1\alpha,25(OH)_2D_3$ alone little effect and TSA inhibited approximately 20% ($P < 0.05$). In PrEC cells, only a sub-additive effect was observed with the combination of agents.

3.3. Regulation of GADD45α in prostate cancer cells

GADD45α has emerged from parallel studies by us and others as a strong candidate VDR target gene in

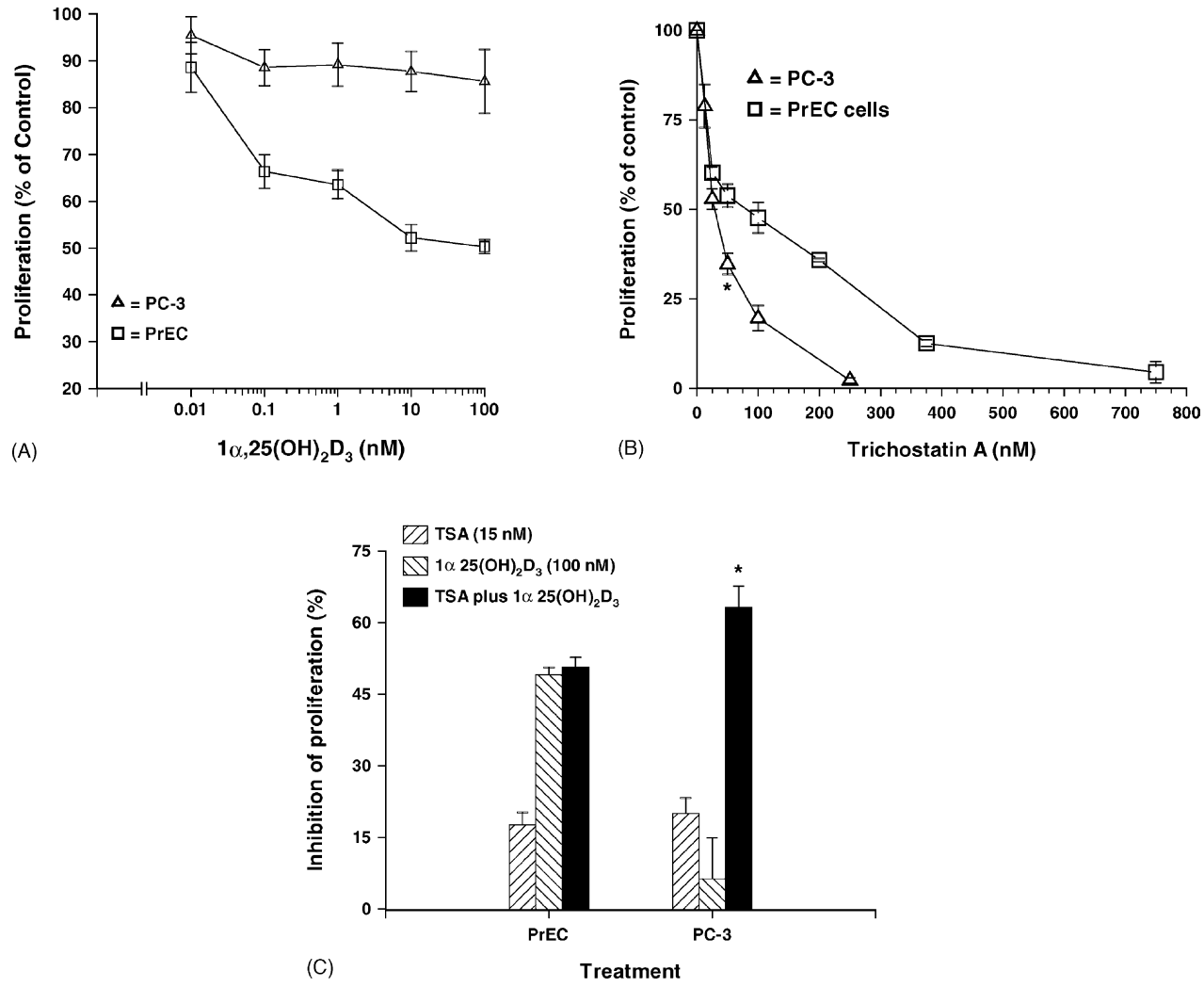

Fig. 1. Sensitivity to inhibition by $1\alpha,25(OH)_2D_3$ can be restored by co-treatment with the HDACi TSA. (A) The effect of $1\alpha,25(OH)_2D_3$ on the proliferation of PrEC and PC-3 cells was assessed by measurement of intercellular ATP by a bioluminescent assay. Cells were plated into 96-well plates and treated with $1\alpha,25(OH)_2D_3$ as indicated. After 96 h, with a re-dose after 48 h, total ATP was measured according to Section 2 and compared to untreated control. Each data point represents the mean of three separate experiments undertaken in triplicate wells (±S.E.M.). (B) The effect of TSA on the proliferation of PrEC and PC-3 cells was measured as in (A). Each point represents the mean of triplicate dishes (±S.E.M.). (C) PC-3 and PrEC cells were plated into 96-well plates and treated with TSA alone (15 nM TSA) or in combination with $1\alpha,25(OH)_2D_3$ (100 nM). After 96 h, with a re-dose after 48 h, total ATP was measured as (A). Strong Additive interactions were defined according to Section 2. Each data point represents the mean of three separate experiments undertaken in triplicate wells (±S.E.M.).

$1\alpha,25(OH)_2D_3$-sensitive cell lines [21] (data not shown). We therefore investigated its regulation in PC-3 and primary cultures. Real Time RT PCR over a time course in PC-3 cells confirmed that $1\alpha,25(OH)_2D_3$ alone had limited effect GADD45α mRNA at early time points. However, by 7 h post treatment $1\alpha,25(OH)_2D_3$ alone did not result in elevated levels. Similarly, whilst TSA induced gene expression at earlier time points it had little effect at this time point. By contrast the combination promoted a sustained and strong induction of gene ($1\alpha,25(OH)_2D_3$ 1.0-fold, TSA 1.6-fold and co-treatment 4.3-fold, at 7 h).

Supportively, we examined the response of primary cultures of normal and malignant cells from the same individual. Parallel studies have demonstrated that the cancer cells (E–CA–7) have significantly reduced sensitivity to $1\alpha,25(OH)_2D_3$ alone compared to the normal peripheral zone cells (E–PZ–5) which have an ED_{50} of ∼10 nM. Interestingly it is E–PZ–5 cells that modulate GADD45α in response to $1\alpha,25(OH)_2D_3$ (10 nM) alone at 6 h exposure, whereas the E–CA–7 cells do not modulate the gene (Fig. 2B). These data support the hypothesis that cancer cells utilise an epigenetic event, mediated by elevated co-repressor levels, to reduce sensitivity to $1\alpha,25(OH)_2D_3$ by silencing the transcriptional response of key antiproliferative genes such as GADD45α. This can be overcome by co-treatment with HDACi such as TSA.

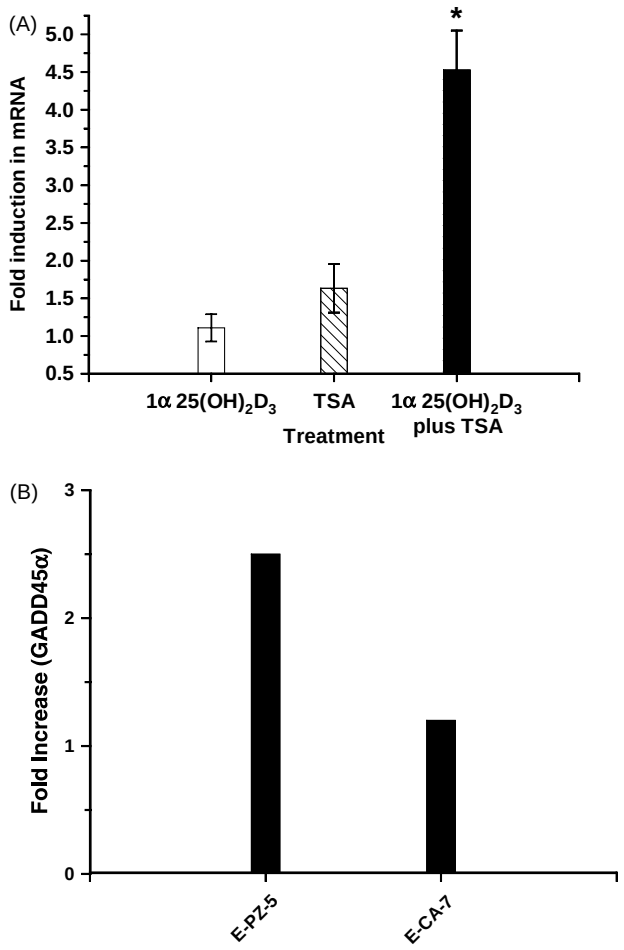

Fig. 2. Regulation of GADD45α mRNA in PC-3 cells and primary prostate cultures. (A) Real-time RT-PCR was used to measure the regulation of GADD45α mRNA in response to 1α,25(OH)$_2$D$_3$ (100 nM) and TSA (15 nM) either alone or in combination. 2×10^4 cm^{-2} cells were plated in six-well dishes and allowed to grow for 36 h to ensure that cells were in mid-exponential phase. Total RNA was isolated after 7 h treatment, reverse transcribed and GADD45α amplified according to Section 2. Each data point represents the mean of three separate experiments amplified in triplicate wells. Combined treatments that were significantly greater than either agent alone are indicated ($^*P < 0.05$). (B) Cultures of the primary normal (E–PZ–5) and cancer (E–CA–7) were grown and treated with 1α,25(OH)$_2$D$_3$ (10 nM) according to Section 2 and total RNA was isolated after 6 h treatment, reverse transcribed and GADD45α amplified according to Section 2.

4. Discussion

The central hypothesis of the current study is that increased co-repressor expression/activity results in epigenetic suppression of antiproliferative target genes. In support of this model we showed that PC-3 cells with reduced 1α,25(OH)$_2$D$_3$ antiproliferative response, but not normal PrEC cells, have significantly elevated SMRT co-repressor levels. Interestingly the 1α,25(OH)$_2$D$_3$ sensitivity of PC-3 cells can be 'restored' to levels that are comparable to PrEC cells by co-treatment with the HDACi TSA.

These data support a model whereby the co-treatment of agents re-activates an epigenetically repressed VDR target gene. Thus, we examined regulation of GADD45α which initiates cell cycle arrest, to facilitate DNA repair or apoptosis. Furthermore upregulation of GADD45α is part of the antiproliferative action of EB1089 (an analogue of 1α,25(OH)$_2$D$_3$) in SCC25 squamous carcinoma cells [21]. However, we saw little or no evidence of these effects in PC-3 cells treated with 1α,25(OH)$_2$D$_3$ alone, but rather could detect them readily when cells were co-treated with 1α,25(OH)$_2$D$_3$ plus TSA. Together these data underscore the concept that inappropriate HDAC activity is suppressing the activity of promoters for key VDR-antiproliferative target genes. The heterogeneity in arrangement and sequence of VDRE amongst VDR target genes may explain their how antiproliferative target genes could be selectively silenced.

Current therapeutic strategies for prostate cancer involve a combination of radiotherapy and radical prostatectomy, and eventually androgen ablation. These therapies are aggressive, with many side-effects. Ultimately the cancer cells escape this control and androgen-independent tumours predominate. HDAC inhibitors such as butyrate derivatives, TSA and more recently suberoylanilide hydroxamic acid (SAHA) are being investigated for a potential role in chemotherapy [22]. Major issues concerning therapy with HDAC inhibitors are the possible toxicity of these compounds, as they target such fundamental processes, and the short half-life in vivo. This study highlighted the combination of 1α,25(OH)$_2$D$_3$ plus TSA to have enhanced antiproliferative activity and allows each agent to be used at lower doses. Interestingly, there is a suggestion that the cancer cells are more potently inhibited by TSA than the normal cells. This cancer cell selectivity has been reported for other cell types and suggests that the therapeutic window for such agents will be greater in cancer cells. Therefore, the combination of selective HDAC inhibitors in combination with potent Vitamin D$_3$ analogs may represent an attractive, more focused and sustained 'anticancer' regime, representing a new avenue in the treatment of aggressive androgen independent prostate cancer tumours.

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