Altered SMRT levels disrupt vitamin D_3 receptor signalling in prostate cancer cells

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Running title: HDAC dysregulation of VDR signalling

Key words: Histone deacetylation inhibitors, 1α,25 Dihydroxyvitamin D_3, prostate cancer, SMRT, GADD45α,

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[c]Supported in part by the Cancer Research Fund, under Interagency Agreement #97-12013 (University of California, Davis contract #98-00924V) with the Department of Health Services, Cancer Research Section. Mention of trade name, proprietary product or specific equipment does not constitute a guaranty or warranty by the Department of Health Services, nor does it imply approval to the exclusion of other products. The views expressed herein represent those of the authors and do not necessarily represent the position of the State of California, Department of Health Services.

[d]Supported in part by grants from the United Birmingham Hospitals Endowment Fund, Prostate Research Campaign UK and National Cancer Research Institute Prostate Research Cancer Collaborative, UK.

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Abstract

We hypothesized that key antiproliferative target genes for the vitamin D receptor (VDR) were repressed by an epigenetic mechanism in prostate cancer cells resulting in apparent hormonal insensitivity. To explore this possibility we examined nuclear receptor co-repressor expression in a panel of non malignant and malignant cell lines and primary cultures, and found frequently elevated SMRT co-repressor mRNA expression often associated with reduced sensitivity to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$]. For example, PC-3 and DU-145 prostate cancer cell lines had 1.8- and 2- fold increases in SMRT mRNA relative to normal PrEC cells (p<0.05). Similarly 10/15 primary tumour cultures (including 3 matched to normal cells from the same donors) had elevated SMRT mRNA levels; generally NCoR1 and Alien were not as commonly elevated. Co-repressor proteins often have associated histone deacetylases (HDAC) and reflectively the antiproliferative action of 1α,25$(OH)_2D_3$ can be 'restored' by co-treatment with low doses of HDAC inhibitors such as trichostatin A (TSA, 15 nM) to induce apoptosis in prostate cancer cell lines.

To decipher the transcriptional events that lead to these cellular responses we undertook gene expression studies in PC-3 cells after co-treatment of 1α,25$(OH)_2D_3$ plus TSA after 6 hr. Examination of known VDR target genes and cDNA microarray analyses revealed co-treatment of 1α,25$(OH)_2D_3$ plus TSA co-operatively upregulated 8 (out of 1176) genes, including MAPK-APK2 and GADD45α. MRNA and protein time courses and inhibitor studies confirmed these patterns of regulation. Subsequently we knocked-down SMRT levels in PC-3 cells using a small interfering RNA (siRNA) approach and found that GADD45α induction by 1α,25$(OH)_2D_3$ alone became very significantly enhanced. The same distortion of gene responsiveness, with repressed induction of GADD45α was found in primary tumour cultures compared and to matched peripheral zone (normal) cultures from the same donor.

These data demonstrate that elevated SMRT levels are common in prostate cancer cells, resulting in suppression of target genes associated with antiproliferative action and apparent 1α,25$(OH)_2D_3$-insensitivity. This can be targeted therapeutically by combination treatments with HDAC inhibitors.

Introduction

Prostate epithelial cells express multiple members of the nuclear receptor (NR) superfamily. These receptors act as ligand-activated transcription factors to regulate genes essential for regulation of proliferation and differentiation. The vitamin D receptor (VDR) typifies this class of transcription factors by associating with vitamin D response elements (VDRE) in the promoter/enhancer region of target genes as part of multimeric, repressive or activating complexes.

Proliferation and differentiation of normal prostate epithelial cells is acutely regulated *in vitro* and *in vivo* by $1\alpha,25(OH)_2D_3$ (Konety *et al.* 1996; Peehl *et al.* 1994) thereby justifying clinical trials in prostate cancer patients (Beer *et al.* 2003). However prostate cancer cells display a spectrum of sensitivities to the antiproliferative action of $1\alpha,25(OH)_2D_3$ (reviewed in (Chen & Holick 2003; Krishnan *et al.* 2003; Peehl & Feldman 2003). Reflective of an antiproliferative role, epidemiological studies have now linked the incidence of prostate cancer to low serum levels of $25(OH)D_3$ as a result of either diet or environment, and specific VDR polymorphisms have been correlated with cancer susceptibility (Hanchette & Schwartz 1992; Ingles *et al.* 1998). Collectively, such data implicate $1\alpha,25(OH)_2D_3$ with a protective action against uncontrolled prostate growth; that initiation or progression of prostate cancer may relate to reduced dietary intake and/or cellular resistance to the antiproliferative effects of $1\alpha,25(OH)_2D_3$.

The molecular mechanisms for $1\alpha,25(OH)_2D_3$-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 growth inhibition by $1\alpha,25(OH)_2D_3$ (Miller *et al.* 1997; Zhuang *et al.* 1997). Indeed, the PC-3 and DU 145 prostate cancer cell lines are not significantly inhibited by physiologically relevant doses of $1\alpha,25(OH)_2D_3$ and consistent with this response, antiproliferative gene targets are not modulated (Campbell *et al.* 2000; Campbell *et al.* 1997). The lack of an antiproliferative response is not reflected by an overall suppression of the capacity of these cells to perceive $1\alpha,25(OH)_2D_3$. 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 – the $1\alpha,25(OH)_2D_3$ metabolising enzyme] (Miller *et al.* 1995).

To resolve why prostate cancer cells appear to loose sensitivity to the antiproliferative action of $1\alpha,25(OH)_2D_3$ we have considered epigenetic mechanisms which regulate promoter access. Nuclear receptors exist in a complexes that are in a dynamic balance between the *apo*, ligand-free state associated with co-repressor and the *holo*, ligand-bound state associated with co-activator (Germain *et al.* 2002; Hermanson *et al.* 2002). In the absence of $1\alpha,25(OH)_2D_3$ the VDR associates in a gene repression complex which contains co-repressor proteins such as NCoR1, SMRT and Alien, and associated histone deacetylases (HDAC) (Polly *et al.* 2000; Guenther *et al.* 2001; Yu *et al.* 2003). These complexes maintain the histone N-terminal 'tails' in a charged state tightly associated with DNA, thereby maintaining a locally closed chromatin structure and suppressing transcription of target genes (Belandia & Parker 2003 Guenther *et al* (2001)]. Ligand binding induces activation which has been demonstrated for related nuclear receptors to include ordered promoter-specific cyclical rounds of complex assembly, gene transactivation, disassembly and proteosome degradation of receptor (Metivier *et al.* 2003; Reid *et al.* 2003b). Central to this are ligand-induced conformational changes which promotes association between the VDR and co-activator complexes containing proteins such as SRC-1, NCoA-62, GRIP-1 and DRIP factors (Zhang *et al.* 2001; Oda *et al.* 2003; Rachez *et al.* 2000; Belandia & Parker 2003; Rachez & Freedman 2000),. The antagonistic actions of these opposing complexes are controlled temporally and spatially to determine the boundaries between heterochromatin and euchromatin and gene promoter responsiveness.

The cell-specific position of the dynamic balance between *apo* and *holo* states is determined by both ligand availability and the unique mileu of co-activator and co-repressors which combine to yield gene specific responses (Hermanson *et al.* 2002). The equilibrium is targeted for disruption in malignancy by aberrant co-activator and co-repressor function resulting in both gain and loss of nuclear receptor function. For example, increased co-activator expression enhances the transcriptional activity of androgens and estrogens in breast and prostate cancer, respectively (Gnanapragasam *et al.* 2001; Feldman & Feldman 2001; Planas-Silva *et al.* 2001; Kawashima *et al.* 2003; Jiang *et al.* 2003b; Hudelist *et al.* 2003; Kollara *et al.* 2001). Similarly RARα-fusion proteins in acute promyelocytic leukaemia bind co-repressors more aggressively than wild type receptors and thereby suppress transactivation

of antiproliferative target genes by all *trans* retinoic acid (Grignani *et al.* 1998; Lin *et al.* 1998).

Therefore we have proposed that VDR signaling is not abrogated in prostate cancer cells but it is skewed by epigenetic mechanisms to repress key antiproliferative gene targets. Previously we showed that co-treatment of three prostate cancer cell lines (LNCaP, PC-3 and DU 145) with $1\alpha,25(OH)_2D_3$ plus HDAC inhibitors, either trichostin A (TSA) or sodium butyrate, resulted in additive and synergistic inhibition of proliferation associated with apoptosis (Rashid *et al.* 2001a). For the current study we hypothesised that an imbalance in the co-activator/co-repressor balance is selectively distorting promoter responsiveness of VDR antiproliferative target genes resulting in $1\alpha,25(OH)_2D_3$-insensitivity. We have now examined VDR and co-repressor levels in cell lines and primary cultures, 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 and we have also examined the effects of knocking-down SMRT levels using a small interfering RNA approach.

Materials and Methods

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

$1\alpha,25(OH)_2D_3$ (generous gift of Dr. Milan R. Uskokovic, Hoffman La Roche, Nutley, NJ 07110, U.S.A.) and TSA (Sigma, Poole, U.K.) were all stored as 1 mM stock solutions in ethanol at –20°C. SB203580, an inhibitor of p38 activation, (CalBiochem, Nottingham, UK), was prepared fresh as required as a 1 mM stock solution in DMSO.

Cell culture

Normal prostate epithelial cells (PrEC), as a non-transformed epithelial counter-part were purchased and cultured in PrEGM media (Clonetics, Wokingham, UK) according to manufacturer's instructions. These cells had a finite lifespan passage time but during the course of the current study they had a doubling time approximately equal to that of the prostate cancer cell line PC-3 cells (~24 hr), obtained from the American Type Culture Collection (ATCC, Rockville, MD). PC-3 cCells were maintained in RPMI 1640 medium (Gibco-BRL, Paisely, UK), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Gibco-BRL,), 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.

Primary cultures

Tissues dissected from radical prostatectomy specimens were processed for primary culture of prostatic epithelial cells according to previously described methods (Peehl 2004). None of the patients had received prior chemical, hormonal or radiation therapy. Histological assessment was performed as described (Schmid & McNeal 1992). Normal peripheral zone (PZ) and benign prostatic hyperplasia (BPH) were established from tissue with no cancer present in adjacent tissue sections. Cancer cultures (CA) were established from adenocarcinomas of varying Gleason grade listed in Table 1. Each cell strain was ascribed a number and serially passaged and cells in secondary or tertiary passages were used for clonal growth assays or RNA isolation. All studies were undertaken in actively proliferating cultures prior to senescence.

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, U.K.) with previously optimised conditions according to the manufacturer's instructions (Rashid *et al.* 2001a). Briefly, cells were plated in 96- well, white-walled, tissue culture-treated plates (Fisher Scientific Ltd., Loughborough, U.K.) (LNCaP, PC-3 and DU 145 at 2 x 10^3 cells/well; PrEC at 3.5 x 10^3 cells/well). Growth media containing varying concentrations of TSA, 1α,25$(OH)_2D_3$, or SB203580 was added to a final volume of 100 µl/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 minutes 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.

Clonal proliferation assays were used to evaluate responses of primary cultures to 1α,25$(OH)_2D_3$. Each 60-mm tissue culture dish, coated with collagen and containing 5 ml of serum-free medium [Peehl *et al* (2002)] and vehicle or 1α,25$(OH)_2D_3$, was inoculated with 500 cells per dish. After 10 days of incubation, cells were fixed with formalin and stained with crystal violet [Peehl *et al* (2002)]. Total cell growth was measured with an Artek image analyzer (Dynatech, Chantilly, VA, USA).

Extraction of RNA and reverse transcription

PrEC, LNCaP, PC-3 and DU 145 cells were seeded at a density of 2x10^4/cm^2 and allowed to grow for 36 h to ensure that cells were in mid-exponential phase upon treatment. Cells were harvested or dosed by adding cycloheximide (20 µg/ml) (Sigma-Aldrich), 1α,25$(OH)_2D_3$ and/or TSA as indicated with fresh media and incubating for various times. Total RNA was extracted using the GenElute RNA extraction system (Sigma-Aldrich) according to manufacturer's instructions. Primary cultures were serially passaged and grown to 80% confluency in standard serum-free medium. Cells were fed and dosed with 1α,25$(OH)_2D_3$ as

indicated one 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) at 42°C for 60 min in the presence of 100 mM Tris-HCl, pH 9.0, 500 mM KCl, 2 mM $MgCl_2$, 100 pM random hexamers (Pharmacia, Pisacataway, NJ), 2 mM dNTP and 20 U RNAsin (Promega) in a 20 µl reaction volume.

Real-Time Quantitative RT-PCR (Q-RT-PCR)

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 1x 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 *p*moles FAM-labelled TaqMan probe and 22.5 *p*moles primers. All reactions were multiplexed with pre-optimized control primers and VIC- labeled 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 sec 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.

cDNA microarray analyses

PC-3 cells were seeded at a density of $2x10^4/cm^2$ in T75 flasks and allowed to grow for 36 h. Fresh medium was added with the following treatments: $1\alpha25(OH)_2D_3$ (100 nM) and/or TSA (15 nM) or left untreated as control. After 6 h total RNA was isolated as indicated above, DNAse treated and resuspended at a concentration of 2.5 µg/µl. From these RNA stocks 2 µl was used for subsequent generation of radiolabelled probe according to the manufacture's

instructions (Clontech, Wokingham, UK). Clontech Human 1.2 cDNA arrays were incubated overnight with radiolabelled probe. After careful washing the membrane was exposed to film (Kodak, XOMAT) overnight and for 72 h. Images were scanned and the relative difference in the intensity of the signals analysed using the Clontech AtlasImage 1.5 software, which with reference to internal controls, pre-set limits at which differences in signal intensity and/or fold differences (greater than 2-fold) were reported.

Repeat experiments involved stripping the membranes (which were checked to ensure zero signal) and reprobing with radio labelled probes prepared with mRNA isolated from repeat treatments of cells as described above. Tables of differences for each individual and combined treatment were compared to control. For each of the treatments the adjusted difference in the intensity of signals for gene targets that were reported in both experiments were averaged to generate the change in the mean adjusted intensity (ΔMAI). Mean fold differences were only calculated where the basal level of target was detectable. Only genes that were reported as modulated in the same manner in both experiments were included in the results.

Western immunoblot analysis

PC-3 cells were seeded at a density of $2x10^4/cm^2$ in T75 flasks and allowed to grow for 36 h. Fresh medium was added with the following treatments: $1\alpha25(OH)_2D_3$ (100 nM) and/or TSA (15 nM) or left untreated as control. Whole cell lysates were prepared at indicated time points and Western immunoblot analysis performed. Briefly, 30 μg of total protein for each sample was electrophoresed through an sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred onto PVDF membrane (Immobilon-P; Millipore, Bedford, MA) and blocked with TBS-T containing 5% milk powder for 1 h. For detection of GADD45α, a rabbit polyclonal antibody (C-20, Santa Cruz Biotechnology, USA) was diluted 1:500. The secondary antibody, donkey anti-rabbit-HRP conjugate (Amersham, Amersham, UK) was diluted 1:1000. Proteins were detected using ECL (Amersham) and autoradiography. To ensure even loading and transfer of protein, membranes were washed for 15 min with TBS-T and incubated with a 1/10000 dilution of primary mouse monoclonal β-actin antibody (AC-15, Sigma-Aldrich). An anti-mouse-HRP secondary antibody (Binding Site, UK) was used at 1:2000, and signals developed with ECL and autoradiography as described above. To quantify

the relative changes in protein levels, densitometry analysis was performed on the autoradiographs and values normalised to β-actin levels.

Small interfering RNA to target SMRT.

SiRNA fragments (22mers) were generated *in vitro* using a Dicer generation system according to the manufacturer's protocol (Gene Therapy Systems, San Diego, CA). Briefly, a 650 bp region from the human SMRT gene sequence corresponding to 112 to 762 was amplified using the following primers containing T7 promoter sequences;

FS: 5' – gcgtaatacgactcactatagggagacgggctcctggagtaccagc – 3' and

RV: 5' – gcgtaatacgactcactatagggagagctccacctggggccccagg – 3'. This was subsequently cloned into the pGEM T easy vector (Promega), using the multiple cloning sites to allow for sequencing, large scale harvest. Digestion with ECoR1 released a pure concentrated template for *in vitro* translation with primers containing T7 recognition sequences to generate double stranded mRNA. This was subsequently cleaved with recombinant human Dicer enzyme to generate a pool of 22mers which cover the region targeted in the 5'prime region of the SMRT gene. Five hundred ng of purified double stranded RNA 22mers were transfected into each well ($2x10^5$ cells/well in 24-well plates) for 12 hrs. Cells were then left for a further 72 hr to allow gene silencing and subsequently treated with $1\alpha,25(OH)_2D_3$ (100 nM) for 6 hr and total RNA was harvested and Q-RT-PCR for SMRT and target genes was undertaken as above.

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 (Campbell *et al.* 1998). 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.

Results

SMRT mRNA levels are elevated in prostate cancer cell lines and primary cultures.

We reasoned that cancer cells can escape proliferative control from nuclear receptors, such as VDR, by elevating co-repressor expression. We therefore measured the mRNA levels of VDR and the nuclear co-repressors SMRT, NCoR1 and Alien in primary cultures of cancers (E-CA-1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 15, mean age of 55.5), benign prostatic hyperplasia (E-BPH-1, E-BPH-2, mean age 61) compared to the mean of seven normal peripheral zones cultures (E-PZ-1 to 7, mean age of 55) (Figure 1A), three matched tumour and normal pairs from the same donor (E-CA-7, 8, 9 and E-PZ-5, 1, 4 respectively) (Figure 1B), and LNCaP, PC-3 and DU-145 cancer cell lines and PrEC normal prostate epithelial cells (Figure 1C). These data are summarised in and Table 2.

Several non-matched tumour cultures displayed elevated co-repressor levels (>2- fold elevation) compared to the mean level in the 7 normal cultures with SMRT being the most frequently elevated (7/12), with NCoR1 and Alien less frequently elevated (2 and 1 cultures, respectively). Amongst the primary cancer cultures and cell lines there was a trend towards increased SMRT mRNA levels in the least differentiated and/or androgen-independent cells. Thus 2 out 6 cancer cultures with Gleason score 3/3 had elevated SMRT mRNA whilst 3 out of 4 cultures with Gleason score of 4/3 and greater had elevated SMRT. Similarly both androgen-insensitive cell lines had elevated SMRT (Table 1). Despite the relatively large spread of the fold-differences changes in SMRT in all primary cancer cultures (0.4 to 31.6), compared to the normal cultures, the mean δδct for the cancer cultures (where reduced δδct values indicate elevated target gene mRNA transcript) was 9.92+/- 2.004 (SD) compared to the normal which was 11.28 +/- 1.02 (SD), reflecting the reduced variation in levels in the normal peripheral zone cultures (Figure 1A). These data were supported by the matched cancer and peripheral zone normal cultures, each pair from the same donor, in which all cancers displayed elevated SMRT mRNA compared to the normal counterpart elevation (> 2-fold) (Figure 1B). Interestingly the spread of SMRT levels in all cancer cultures samples correlated very strongly with those of VDR ($r = 0.85$, $p<0.0001$). A similar elevation in SMRT mRNA levels was observed in PC-3 and DU 145 cells [(1.8-fold, ($p<0.05$), and 2.2-fold, ($p<0.05$)], respectively compared to PrEC cells (Figure 1C). The levels of SMRT did not

correlate with VDR, as seen in the primary cancer cultures tumours, and all cancer cell lines had a significant reduction in the VDR expression compared to PrEC cells.

We compared these expression patterns with sensitivity to the antiproliferative actions of $1\alpha,25(OH)_2D_3$ in primary cultures and cell lines (Table 1). The two assays measure related aspects of cellular responses to $1\alpha,25(OH)_2D_3$. The ATP proliferation assay measures total cellular ATP after 4 days, whilst the colony assay measures growth of cells after 10 days by measuring the total mass of cells (protein) per dish. In previous studies we have established that both of these readouts are proportional to cell number and thus these assays whilst different, are readily comparable (Peehl *et al.* 1994; Rashid *et al.* 2001a). Reflective of this the ED_{50} of normal cultures in the two assays were comparable, which we attribute to $1\alpha,25(OH)_2D_3$ having a pleiotropic set of actions regulating both cell cycle progression, proliferation and invasion (Polek *et al.* 2003; Blutt *et al.* 2000; Blutt *et al.* 1997; Schwartz *et al.* 1997). Elevated SMRT in PC-3 and DU-145 correlated with their insensitivity to $1\alpha,25(OH)_2D_3$ (ED_{50} > 100 nM). The same diversity of responsiveness was seen in a representative panel of primary cancer cultures compared to normal, and BPH cultures. For example, in colony formation assays the benign culture (E-BPH#1) had ED_{50} = 0.2 nM, which was comparable with the responses of the normal strains we have characterised previously (Peehl *et al.* 1994), whereas the cancer cultures displayed a spectrum of sensitivities. This spectrum is typified by the cancer culture E-CA-7, where the cancer culture which is insensitive to $1\alpha,25(OH)_2D_3$ (ED_{50} > 100 nM), whereas its matched normal counterpart (E-PZ-5) is acutely sensitive (ED_{50} = 5 nM). Taken together these data suggest that resistance of prostate cancer cells to $1\alpha25(OH)_2D_3$ relates to increased co-repressor expression/activity. We subsequently went on to examine if the elevated co-repressor levels could be targeted by co-treatment with HDAC inhibitors.

TSA augments the antiproliferative action of $1\alpha,25(OH)_2D_3$

The PrEC cells proliferate at approximately the same rate as the PC-3 cancer cells and yet PrEC cells display a very different and acute response to the antiproliferative action of $1\alpha,25(OH)_2D_3$ (Figure 2A,B). We used PC-3 cells, as a $1\alpha25(OH)_2D_3$-insensitive cell line with increased co-repressor expression, to measure the effect of HDAC activity on the

antiproliferative sensitivity to 1α25(OH)$_2$D$_3$. Control cultures of PC-3 and PrEC cells had comparable dose-response sensitivities to treatment with TSA alone (data not shown) and therefore, we examined the effects on proliferation of a minimally active dose of TSA (15 nM) alone and co-treatment together with escalating doses of 1α25(OH)$_2$D$_3$. The acute inhibition of proliferation of PrEC cells by 1α25(OH)$_2$D$_3$ alone was only affected by TSA when exposed to low doses of 1α,25(OH)$_2$D$_3$ (0.01 nM) (Figure 2A). By contrast, in PC-3 cells the co-treatment resulted in strong additive effects with 100 nM 1α,25(OH)$_2$D$_3$ and additive effects at 10 and 1 nM (Figure 2B); this effectively reinstated the antiproliferative response of PC-3 cells to levels that were comparable to PrEC cells. These data reflect our previous data obtained in prostate cancer cell lines at a single doses of 1α,25(OH)$_2$D$_3$ in both liquid and semi-solid proliferation assays (Rashid *et al.* 2001a).

Regulation of VDR and CYP24 in PC-3 cells co-treated with agents

To examine the transcriptional effects of the co-treatment of agents we examined the induction of VDR and CYP24. The CYP24 gene is among the best-characterized and most profoundly regulated VDR target gene (Dwivedi *et al.* 2000). We and others have shown in prostate and breast cancer and leukaemia cell lines that CYP24 is modulated in a manner that is broadly inversely proportional to the antiproliferative action of 1α25(OH)$_2$D$_3$, and is in part responsible for regulating cellular sensitivity to 1α25(OH)$_2$D$_3$ (Miller *et al.* 1995; Rashid *et al.* 2001b; Ly *et al.* 1999). Similarly in the current study we found exuberant tumour-enhanced CYP24 induction in primary cancer cultures treated with 1α,25(OH)$_2$D$_3$ (50 nM, 6 hr) (Figure 3A). Therefore we examined CYP24 mRNA expression in PC-3 cells over time.

Treatment with 1α,25(OH)$_2$D$_3$ alone resulted in a rapid and very large upregulation of CYP24 mRNA (6.9- fold by 1 h, $p<0.01$), which continued throughout the course of the treatment, resulting in the very significant 6600- fold increase seen at 7 h ($p<0.0001$) (Figure 3B). By contrast, treatment with TSA alone resulted in a relatively modest effect on expression peaking at 7 h (3.7- fold), but in combination it very significantly suppressed the 1α,25(OH)$_2$D$_3$-dependent induction. For example, the mRNA upregulation observed with 1α25(OH)$_2$D$_3$ alone at 7 h was significantly suppressed to a mean fold increase of 1302 ($p<0.001$). At subsequent time points the suppressive action of TSA was diminished.

We reasoned that the significant induction of CYP24 was in part the consequence of direct and indirect responses to treatment. To investigate this further we used the same treatments in the presence and absence of *de-novo* protein synthesis using cycloheximide. A significant reduction in the induction of CYP24 was observed when cycloheximide was added, although this was most pronounced with $1\alpha,25(OH)_2D_3$ alone. Consistent with the time course data, six hours exposure to $1\alpha,25(OH)_2D_3$ alone resulted in a 3342 mean fold increase ($p<0.0001$) in CYP24 mRNA expression (Figure 3C). However this was greatly and significantly suppressed ($p<0.0001$) to an 85-fold increase in cells co-treated with $1\alpha,25(OH)_2D_3$ and cycloheximide. The effect of $1\alpha,25(OH)_2D_3$ was significantly suppressed further by co-treatment of both cycloheximide and TSA resulting in only a 4.4-fold increase.

Taken together, these data suggest a network of transcriptionally regulated co-factors that act in concert to modify the responses to $1\alpha,25(OH)_2D_3$. This does not appear to include the receptor as there were no significant effects on VDR expression with either single or combined treatments over the same 12 hr time course (data not shown).

Identification and regulation of genes in PC-3 cells co-treated with $1\alpha25(OH)_2D_3$ plus TSA.

To characterise more fully the transcriptional effects of the co-treatment of agents we undertook cDNA microarray analyses in PC-3 cells. A number of genes were identified that have been demonstrated to be targets for these agents by other workers. A well known established VDR target gene is the CDKI, $p21^{(waf1/cip1)}$ (Liu *et al.* 1996) and supportively this was modulated in the $1\alpha25(OH)_2D_3$ alone treatment group (2.1- fold), however, this was unaffected by co-treatment with TSA (data not shown). This supports our previous study that indicated that this gene was not co-ordinately modulated by the co-treatment of $1\alpha25(OH)_2D_3$ and TSA (Rashid *et al.* 2001a). Similarly TSA at higher concentrations than used in the current study is a potent inducer of apoptosis (Hirose *et al.* 2003a; Yamashita *et al.* 2003) and in the current study we found Bcl-2 down-regulated 0.48- fold by TSA treatment alone, which was unaffected by the co-treatment (data not shown).

For subsequent studies we focussed on genes that appeared to be co-ordinately regulated by the combined treatments, and have established roles in regulating proliferation, differentiation and apoptosis. We identified 8 genes (out of 1176) that demonstrated co-

operative, additive increases in expression after 6 h exposure to combined TSA and 1α25(OH)$_2$D$_3$ (Table 2). These genes included those that have roles in controlling apoptosis and proliferation [GADD45α (Jin *et al.* 2001), Cyclin K (Mori *et al.* 2002)], and differentiation and/or adhesion [MAPK-APK2 (Alsayed *et al.* 2001; Wang *et al.* 2000), VE-Cadherin (DeFouw & DeFouw 2000)]. For example, expression of GADD45α in 1α25(OH)$_2$D$_3$- or TSA-treated PC-3 cells had a mean adjusted intensity (ΔMAI) of 434 and 381, respectively, whereas co-treatment resulted in a ΔMAI of 6926 (Table 2). From our preliminary validation studies we chose to focus on MAPK-APK2 and GADD45α as these have been shown to be gene targets for nuclear receptor action (Alsayed *et al.* 2001; Wang *et al.* 2000; Akutsu *et al.* 2001).

Real-Time RT-PCR over a 12 hr time course in PC-3 cells confirmed that MAPK-APK2 mRNA was readily detected in control cells, reflecting the relatively strong control signal on the cDNA microarray, and was significantly higher than GADD45α mRNA levels (11.4-fold greater, $p<0.001$). This was reflected by the lack of a basal GADD45α signal detected on the cDNA microarray and hence no fold change could be calculated (Table 2). 1α,25(OH)$_2$D$_3$ alone had limited effect on MAPK-APK2 mRNA expression with a modest 1.3- fold increase after 6 h (Figure 4A). In contrast TSA alone produced an acute induction of 2.3- fold increased mRNA expression at 4 h ($p<0.001$). Co-treatment with 1α,25(OH)$_2$D$_3$ and TSA resulted in a sustained window of MAPK-APK2 upregulation, which was maintained for several hours to approximately 8 hrs post-treatment (Figure 4A). SB203580 is a highly specific inhibitor of p38 that blocks the activation of MAPK-APK2. Importantly SB203580 had no effect on cell growth when used alone or in combination with either agent separately (Figure 4B and data not shown), but abrogated the capacity of TSA to sensitise PC-3 cells to the antiproliferative actions of 1α,25(OH)$_2$D$_3$ (Figure 4B). Taken together, these data suggests that the antiproliferative activity of combined TSA plus 1α,25(OH)$_2$D$_3$ is dependent on the sustained re-expression and activation of MAPK-APK2.

In some respects the pattern of GADD45α mRNA expression in response to 1α,25(OH)$_2$D$_3$ mirrored that of MAPK-APK2. 1α,25(OH)$_2$D$_3$ alone resulted in the steady accumulation of GADD45α mRNA after 3 h, peaking at 2.4- fold increase relative to control. TSA induction occurred between 4 and 6 hr post treatment with a significant mean fold

increase compared to control of 3.1 after 4 h (p<0.0001) (Figure 5A). However, as in the case of MAPK-APK2, the combined treatment sustained a window of increased expression at a level that was higher than the peak responses to either treatment alone (for example, 1α,25$(OH)_2D_3$ 1.0- fold, TSA 1.6- fold and co-treatment 4.3- fold, at 7 h). Consistent with this sustained pattern of mRNA expression, Western immunoblot analysis confirmed the increased expression of GADD45α protein most clearly following 9 h exposure to combined 1α,25$(OH)_2D_3$ and TSA, but not to either agent alone (Figure 5B). Data at earlier time points did not show co-operative changes in protein (data not shown).

Targeted silencing of SMRT with siRNA

To support further the central role of SMRT in distorting transcriptional effects and therefore the antiproliferative actions of 1α,25$(OH)_2D_3$, we used a siRNA approach to reduce SMRT mRNA in PC-3 cells. The Q-RT-PCR primers and probes which detected SMRT mRNA levels were 3' of the region targeted by the SMRT siRNA and therefore we were able to measure the effects of siRNA treatment at the mRNA level. These studies resulted in a mean 0.04 fold reduction in SMRT mRNA levels at 72 hr post-treatment (Figure 6A). Cells were then dosed with 1α,25$(OH)_2D_3$ and the induction of GADD45α measured. Supportively in cells where SMRT levels were reduced, but not mock treated (GFP) cultures, GADD45α became very highly responsive to 1α,25$(OH)_2D_3$ treatment (Figure 6B).

Dysregulation of VDR target genes in matched primary cultures

To examine the significance of the differential gene responsiveness observed in PC-3 cells, we examined CYP24 and GADD45α mRNA induction in response to 1α,25$(OH)_2D_3$ treatment (50 nM, for 6 hr) in three primary tumour cultures (E-CA-7, E-CA-8 and E-CA-14) compare to their matched peripheral zone cultures (E-PZ-5, E-PZ-1, and E-PZ-4 respectively). Initially we characterised the basal expression of the VDR, the three co-repressors and the target genes CYP24 and GADD45α. All three tumour cultures displayed elevated SMRT compared to their matched normal counterpart and in cultures E-CA-7 and E-CA-14 this was matched by elevated VDR levels (Figure 1B). E-CA-7, E-CA-8 and E-CA-14) cultures displayed repressed levels of GADD45α relative to their matched normal controls (0.5-, 0.02- and 0.09- fold decrease, respectively). This was complemented by

elevated basal levels of CYP24 in the E-CA-7 and E-CA-14 cultures of 3.2- and 2.3-fold, respectively. Subsequently we examined the regulation of CYP24 and GADD45α in response to 1α,25$(OH)_2D_3$ (50 nM) (Figure 7 A,B). Upon treatment with 1α,25$(OH)_2D_3$ the tumour cultures displayed distorted responses with simultaneous enhancement of CYP24 and repression of GADD45α induction. CYP24 induction in the E-CA-7 cancer primary culture was approximately twice that seen in the normal counterpart peripheral zone (984- and 567-fold, respectively); enhancement of induction was also seen in the E-CA-8 and E-CA-14. By contrast, GADD45α was not modulated in the tumour culture but was (2.6-fold) in the peripheral zone normal cells (E-PZ-5). Similar dysregulation was seen in the other matched cultures. Interestingly E-CA-7, was essentially insensitive to 1α,25$(OH)_2D_3$ (ED_{50} > 100 nM) whereas its normal counterpart E-PZ-5, was acutely responsive (Table 1). Taken together these data support the concept of elevated SMRT co-repressor levels resulting in promoter-specific distortions of transcriptional responsiveness leading to apparent insensitivity towards 1α,25$(OH)_2D_3$.

Discussion

The active metabolite of vitamin D, $1\alpha,25(OH)_2D_3$, is readily able to initiate gene transactivation and acutely regulate proliferation of normal prostate, breast and colon epithelial cells and myeloid CD 34 positive precursors (Konety *et al.* 1996; Zinser *et al.* 2002; Tong *et al.* 1998; Ratnam *et al.* 1996; Rashid *et al.* 2001b). By contrast, cancer and leukemic cell lines from these tissues display a spectrum of sensitivities including complete insensitivity to $1\alpha,25(OH)_2D_3$, irrespective of VDR expression (Campbell *et al.* 1997; Palmer *et al.* 2001; Kubota *et al.* 1998; Munker *et al.* 1986). The central hypothesis of the current study is that this apparent hormonal insensitivity is not determined solely by a linear relationship between the levels of $1\alpha,25(OH)_2D_3$ and the VDR, but rather epigenetic events skew the responsiveness to selectively suppress responsiveness of target gene promoters.

We found frequently elevated co-repressor expression, most commonly involving SMRT, in malignant primary cultures and cell lines, with reduced $1\alpha,25(OH)_2D_3$ antiproliferative response, but not normal or BPH cultures, indicating that the ratio of VDR to co-repressor is a more critical indicator of malignant status and $1\alpha,25(OH)_2D_3$ responsiveness. We explored the significance of elevated co-repressor levels in both cancer cell lines and primary cultures. We reasoned that this lesion could be targeted by co-treatment of ligand ($1\alpha,25(OH)_2D_3$) plus HDAC inhibitor (TSA) and supportively demonstrated that the $1\alpha,25(OH)_2D_3$-response of the androgen-independent PC-3 cells was restored to levels indistinguishable from control PrEC cells, by co-treatment with low doses of TSA. This reversal of $1\alpha,25(OH)_2D_3$ insensitivity provided the opportunity to examine patterns of global acetylation and expression of target genes.

In preliminary studies we have examined the global histone acetylation status after co-treatment by extraction of total cellular histone from cells treated by single and combination treatments, and resolution on acid urea triton gels (Sommerville *et al.* 1993). The relatively low dose of TSA used (15 nM) readily increased global histone acetylation acetylation at all lysine residues examined (Histone H4 Lys^{16}, Lys^{8}, Lys^{12}, Lys^{5}, and Histone H3 $Lys^{9\ and\ 18}$, (data not shown)) but only altered expression of approximately 4% of genes on subsequent microarray studies. Similarly there was a lack of $1\alpha,25(OH)_2D_3$-induced acetylation (data not shown) despite its ability to induce a comparable number of genes. The combined treatment

did not alter global acetylation beyond the effect of TSA alone. Collectively these data suggest that differences between global acetylation patterns do not reflect promoter-specific gene transcription.

Microarray studies demonstrated that 1α,25$(OH)_2D_3$ plus TSA uniquely upregulated a group of 'repressed' gene targets associated with the control of proliferation and induction of apoptosis. GADD45α initiates cell cycle arrest, to facilitate DNA repair or apoptosis dependent upon the p53 status (Jin *et al.* 2003; Jin *et al.* 2001); upregulation of GADD45α when p53 function is abrogated induces a predominant apoptotic response (Jin *et al.* 2003). P53 function is abrogated in PC-3 cells and we have reported previously that co-treatment with 1α,25$(OH)_2D_3$ and TSA in these cells results in apoptosis, in the absence of any significant cell cycle changes (Rashid *et al.* 2001a). Recently TSA has also been shown to induce GADD45α mRNA by a combination of stabilisation of transcription factors such as Oct1 on the promoter region (Hirose *et al.* 2003b) and thus in the current study this may well enhance further the activities of the VDR.

Roles for both GADD45α and MAPK-APK2 have been demonstrated in cell lines that retain sensitivity to 1α,25$(OH)_2D_3$ signaling. For example, p38/MAPK-APK2 activation regulates 1α25$(OH)_2D_3$-induced HL-60 myeloid differentiation (Wang *et al.* 2000) and upregulation of GADD45α is a functional part of the antiproliferative action of EB1089 [an analogue of 1α,25$(OH)_2D_3$] in SCC25 squamous carcinoma cells (Akutsu *et al.* 2001) and 1α,25$(OH)_2D_3$ in ovarian cancer cell lines(Jiang *et al.* 2003a). These studies and our own re-expression data highlight these targets as key in mediating the antiproliferative action of 1α,25$(OH)_2D_3$. A siRNA approach demonstrated the significant role that SMRT plays in regulating this response, with its repression resulting in profound enhancement of the induction of GADD45α in response to 1α,25$(OH)_2D_3$. These data support a central role for elevated SMRT levels in suppressing the induction of key target genes resulting in loss of sensitivity to the antiproliferative action of 1α,25$(OH)_2D_3$.

Target gene induction was measured by time course studies using highly sensitive Q-RT-PCR which revealed distinct and relatively acute increases and decreases in mRNA levels. Co-treatment of 1α,25$(OH)_2D_3$ plus TSA generates temporal windows where the equilibrium point of gene transactivation is shifted to favour a more transcriptionally permissive

environment. This is limited by a number of processes including the nuclear receptor proteosomic turn-over (Reid *et al.* 2003a) and the metabolism of both agents (Yoshida *et al.* 1995). The induction of CYP24, which encodes the 1α,25$(OH)_2D_3$-metabolising enzyme 25$(OH)D_3$-24-hydroxylase, is suppressed by the co-treatment and thus one contributory factor to the increased antiproliferative potency of the combination of 1α,25$(OH)_2D_3$ plus TSA treatment may be the greater availability of 1α,25$(OH)_2D_3$. However, CYP24-resistant analogs of 1α,25$(OH)_2D_3$ are not as inhibitory as 1α,25$(OH)_2D_3$ plus TSA, and in turn, we have demonstrated that their potency can be enhanced further by co-treatment with TSA (Rashid *et al.* 2001a). Thus, although ligand availability may be elevated, chromatin remodelling to allow VDR access to other promoters appears more influential.

Suppression of promoter responsiveness is target gene- specific as exuberant induction of CYP24 occurred in PC-3 cells despite a suppression of GADD45α and MAPK-APK2 response. Similarly the microarray analyses also identified a group of 39 genes modulated readily by 1α,25$(OH)_2D_3$ alone, many of which had no clear antiproliferative function, such as Cyclin B1, C-Myc, MCM7 DNA replication licensing factor and PCNA. Furthermore, co-treatment with TSA suppressed the upregulation of CYP24 and many of the 1α,25$(OH)_2D_3$-responsive gene targets. For example, treatment of cells treated with 1α25$(OH)_2D_3$ alone resulted in a ΔMAI with PCNA of 20505 which was suppressed by the addition of TSA to 3954 (data not shown). Intriguingly, co-treatment with cycloheximide suppressed the induction of CYP24; a similar suppression has been observed recently in different cell types (Zierold *et al.* 2002). By contrast upregulation of MAPK-APK2 and GADD45α by TSA and 1α,25$(OH)_2D_3$ was unaffected, supporting a direct role for VDR in their regulation. Others have demonstrated a co-operative interaction between VDR and Ets-1 on the CYP24 promoter resulting in stimulation of gene responsiveness by upstream Ras (Dwivedi *et al.* 2000; Dwivedi *et al.* 2002). Thus, increased oncogenic Ets signalling may explain the paradoxical enhanced 1α,25$(OH)_2D_3$-dependent upregulation of CYP24 observed in cancer cells, and support the concept that high basal or inducible levels of CYP24 are transforming (Albertson *et al.* 2000).

Work in primary prostate cultures has extended beyond cancer cell lines the concept that enhanced co-repressor action selectively suppresses the responsiveness of key

antiproliferative target gene promoters. We found significant and common elevation of SMRT in tumour cultures, and using matched tumour and normal peripheral zone culures we were able to demonstrate the promoter-specific dysregulation of CYP24 and GADD45α observed in the cancer cell lines.

The ability of the VDR target genes to respond differentially reflects both the different arrangement and sequence of VDRE amongst VDR target genes and the number of different *apo* and *holo* complexes which are assembled upon them. It has been proposed that a specific subset of VDRE (IP9) are more commonly associated with genes that mediate growth arrest and apoptosis (Quack & Carlberg 1999; Danielsson *et al.* 1997). Furthermore IP-9- type response elements have been shown to associate with the co-repressors NCoR1 or SMRT (Polly *et al.* 2000). Taken together, these findings and our data support a model whereby elevated SMRT increases the prevalence of repressive complexes such as SMRT-HDAC3, which associate with the promoter/enhancer regions of key antiproliferative target genes thereby sustaining local histone deacetylation, and shifting the equilibrium point of gene activation resulting in reduced sensitivity to ligand.

The primary cultures used in the study were established from patients with organ-confined disease who had not undergone hormonal manipulation and therefore elevation of SMRT probably occurs relatively early in disease progression, and is sustained through disease progression as a similar elevation was observed in cell lines derived from metastatic lesions. Thus this lesion may provide therapeutic opportunities for different stages of disease. Epigenetic repression in prostate cancer cells by elevated SMRT will potentially impact on multiple members of the nuclear receptor superfamily, such as the retinoic acid receptors, peroxisome proliferator-activated receptors and other adopted orphan receptors, resulting in reduced sensitivity to a wide range of dietary-derived macro- and micronutrient ligands. Therefore individual molecular profiling of early stage cancer disease may identify individuals with more aggressive disease, and those whose disease may be controlled by chemoprevention strategies. Current therapeutic strategies for prostate cancer involve a combination of radiotherapy or and radical prostatectomy, and eventually androgen ablation. These therapies are aggressive, with many side-effects and ultimately the cancer cells escape this control and androgen-independent tumours predominate which are invariably lethal. The current study has also highlighted the potential to establish novel chemotherapies centred

around HDAC inhibitors such as butyrate derivatives, TSA, and suberoylanilide hydroxamic acid (SAHA) (Cohen *et al.* 2002; Zhu & Otterson 2003; Wang *et al.* 2004), in combination with potent dietary-derived compounds, to deliver a more focused and sustained 'anticancer' regime for androgen-independent disease.

Figure Legends

Figure 1: Fold elevation of nuclear co-repressor mRNA levels in prostate primary cancer cultures and cell lines. Panel A VDR and co-repressor mRNA levels measured by Q-RT-PCR in primary cultures of cancers (E-CA-1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 15, mean age of 55.5), benign prostatic hyperplasia (E-BPH-1, E-BPH-2, average age 61) compared to the mean of seven normal peripheral zones cultures (E-PZ-1 to 7, mean age of 55). Total mRNA was isolated from cultures in mid-exponential phase, reverse transcribed and the target genes amplified in triplicate according to the Material and Methods. **Panel B.** VDR and co-repressor mRNA levels measured by Q-RT-PCR in three matched cancer (E-CA-7, E-CA-8 and E-CA-14) compared to three normal matched peripheral zone cultures (E-PZ-5, E-PZ-1, and E-PZ-4 respectively) as above. **Panel C**. VDR and SMRT, Alien, and NCoR1 co-repressors were measured in prostate cancer cell lines and compared to PrEC cells. Total mRNA was isolated from triplicate cultures in mid-exponential phase, reverse transcribed and the target genes amplified in triplicate according to the Material and Methods. $*p < 0.05$.

Figure 2: Restoration of $1\alpha,25(OH)_2D_3$ antiproliferative signalling in PC-3 cells by co-treatment with TSA. PrEC cells (**Panel A**) and PC-3 cells (**Panel B**) and were plated into 96 well plates and treated with TSA alone, (15nM) or in combination with a range of concentrations $1\alpha25\text{-}(OH)_2D_3$. After 96 hr, with a re-dose after 48 hr, total ATP was measured according to the Materials and Methods. Additive (Add) and Strong Additive (St. Add) interactions were defined according to the Materials and Methods. Each data point represents the mean of three separate experiments undertaken in triplicate wells+/- S.E.M.

Figure 3: Regulation of CYP24. Panel A. Normal peripheral zone and tumour cultures were treated with 50 nM $1\alpha,25(OH)_2D_3$ for 6 hr, or left untreated (control), and total mRNA isolated, reverse transcribed and CYP24 amplified and the fold increase calculated according to the Material and Methods. **Panel B** Regulation of CYP24 in response to $1\alpha25\text{-}(OH)_2D_3$ (100 nM) and TSA (15 nM) either alone or in combination. $2x10^4/cm^2$ cells plated into 6 well plates and allowed to grow for 36 hours to ensure that cells were in mid-exponential phase.

Total RNA was isolated after the indicated time periods, reverse transcribed and CYP24 amplified according to the Material and Methods. Each data point represents the mean of three separate experiments amplified in triplicate wells. $1\alpha25\text{-}(OH)_2D_3$ treatment was significantly greater that any other at all time points as indicated. Combined treatments that were significantly less than $1\alpha25\text{-}(OH)_2D_3$ treatment are indicated $*p < 0.05$, $**p < 0.001$ $***p<0.0001$, $****p<<0.0001$. **Panel C**. Real Time RT-PCR was used to measure the regulation CYP24 in response to $1\alpha25\text{-}(OH)_2D_3$ (100 nM) and TSA (15 nM) either alone or in combination, in the presence or absence of cyclohexamide (CHX, 20μg/ml). Triplicate sets of $2x10^4/cm^2$ cells were plated into 6 well dishes and allowed to grow for 36 hours. Total RNA was isolated after 6 hr exposure to the indicated treatments, reverse transcribed and the target genes were amplified according to the Material and Methods. Each data point represents the mean of three separate experiments undertaken amplified in triplicate wells. Combined treatments that were significantly greater than either agent alone, and the effect of CHX, are indicated $*p < 0.05$, $**p < 0.001$ $***p<0.0001$.

Figure 4: Regulation and activity of MAPK-APK2 in PC-3 cells. Panel A. Q-RT-PCR was used to measure the regulation of MAPK-APK2 in response to $1\alpha25\text{-}(OH)_2D_3$ (100 nM) and TSA (15 nM) either alone or in combination. $2x10^4/cm^2$ cells were plated in 6 well dishes and allowed to grow for 36 hours to ensure that cells were in mid-exponential phase. Total RNA was isolated after the indicated time periods, reverse transcribed and MAPK-APK2 amplified according to the Material and Methods. 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 $*p < 0.05$. **Panel B.** PC-3 were plated into 96 well plates ($2x10^3$/well) cells and treated with either $1\alpha25\text{-}(OH)_2D_3$ (100 nM), TSA (15 nM) or SB203580 (10 μM) either alone or in combination. After 96 hr, with a re-dose after 48 hr, total ATP was measured using a luciferase-dependent method according to the Materials and Methods and compared to untreated control, as a marker of proliferation. Each data point represents the mean of three separate experiments undertaken in triplicate wells +/- S.E.M. $1\alpha25\text{-}(OH)_2D_3$ plus TSA plus SB203580 is significantly less inhibitory than $1\alpha25\text{-}(OH)_2D_3$ plus TSA ($*p<0.05$)

Figure 5: Regulation of GADD45α mRNA and protein in PC-3 cells. Panel A Q-RT-PCR was used to measure the regulation of GADD45α mRNA in response to 1α25-$(OH)_2D_3$ (100 nM) and TSA (15 nM) either alone or in combination. $2x10^4/cm^2$ cells were plated in 6 well dishes and allowed to grow for 36 hours to ensure that cells were in mid-exponential phase. Total RNA was isolated after the indicated time periods, reverse transcribed and GADD45α amplified according to the Material and Methods. 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 $*p < 0.05$ **Panel B** Parallel T75 flasks seeded at the same density were exposed to 1α25-$(OH)_2D_3$ (100 nM) and TSA (15 nM) either alone or in combination and total protein was isolated after 9 hr. Expression of GADD45α was determined by Western immunoblot analysis. The position of GADD45α is indicated at the left of the panel. Blots were subsequently stripped and re-probed for β-actin

Figure 6: SiRNA targeted towards SMRT results in enhanced induction of GADD45α. 22mer dsRNA targeted towards either SMRT or green fluorescent protein (GFP) as a control was prepared as described in Materials and Methods. PC-3 cells ($2x10^5$) were plated into 24 well plates, transfected and after 72 hr they were subsequently dosed with 1α,25$(OH)_2D_3$ (100 nM) or left untreated (control) for a further 6 hr, and total mRNA was extracted, reverse transcribed. **Panel A** The effects of the above treatments on the basal levels of SMRT were measured on cells untreated with 1α,25$(OH)_2D_3$. **Panel B** The effect of reduced SMRT mRNA was measured on the induction of GADD45α. Each data point represents the mean of two separate experiments amplified in triplicate wells.

Figure 7: Dysregulation of VDR target genes in primary cultures of matched pairs of tumour and peripheral zone. Primary tumour and matched normal peripheral zone cultures form the same donor were cultured and exposed to 1α,25$(OH)_2D_3$ (50 nM) for 6 hr or left untreated (control) and total RNA was extracted, reverse transcribed and the regulation

of CYP24 (**Panel A**) and GADD45α (**Panel B**) was measured according to the Materials and Methods

Cell line or Strain Sample	Age at surgery	AR status and/or Gleason Grade	ED_{50} (nM)	SMRT levels (fold elevation)
PrEC	34	N	10[a]	1
LNCaP	50	AR +ve	100[a]	0.3
PC-3	62	AR –ve	>100[a]	1.8
DU-145	69	AR –ve	>100[a]	2
E-PZ-1	52	N	5[b]	1.0
E-PZ-2	50	N	Nd	1.1
E-PZ-3	59	N	Nd	0.9
E-PZ-4	61	N	Nd	0.9
E-PZ-5	45	N	5[b]	1.2
E-PZ-6	62	N	Nd	1.2
E-PZ-7	56	N	Nd	0.8
E-BPH-1	58	N	0.2[b]	0.8
E-BPH-2	64	N	1[b]	1.1
E-CA-1	50	3 / 3	Nd	0.5
E-CA-2	50	3 / 3	Nd	0.7
E-CA-3	64	3 / 3	Nd	1.5
E-CA-4	54	3 / 3	Nd	2.1
E-CA-5	64	3 / 3	Nd	3.8
E-CA-6	69	3 / 3	Nd	4.4
E-CA-7	45	80% (3), 20% (4)	>100[b]	3.2
E-CA-8	52	70% (3), 30% (4)	Nd	3.3
E-CA-9	68	3 / 4	Nd	0.5
E-CA-10	70	3 / 4	Nd	0.4
E-CA-11	62	3 / 4	Nd	2.4
E-CA-12	62	4 / 3	1[b]	4.3
E-CA-13	54	40% (4), 30% (3), 20% - IDC	Nd	0.4
E-CA-14	31	60% (4), 20% (3)	Nd	17.8
E-CA-15	51	100% (5)	Nd	31.6

Table 2: Characteristics and responsiveness to $1\alpha,25(OH)_2D_3$ of cell lines and primary cultures. N = normal prostate epithelial cells, AR = androgen receptor, ED_{50} = estimated dose of $1\alpha,25(OH)_2D_3$ (nM) required to inhibit cell proliferation as measured

by bioluminescent[a] or colony formation[b] assays according to Material and Methods, Nd = not determined.

Primer	Sequence
GADD45α Forward primer	AAGACCGAAAGGATGGATAAGGT
GADD45α Reverse primer	GTGATCGTGCGCTGACTCA
GADD45α Probe	TGCTGAGCACTTCCTCCAGGGCAT
MAPKAPK2 Forward primer	GCCTGCTGATTGTCATGGAA
MAPKAPK2 Reverse primer	TGGTCTCCTCGATCCTGGAT
MAPKAPK2 Probe	TTTGACGGTGGAGAACTCTTTAGCCGT
CYP24 Forward primer	CAAACCGTGGAAGGCCTATC
CYP24 Reverse primer	ACTTCTTCCCCTTCCAGGATCA
CYP24 probe	ACTACCGCAAAGAAGGCTACGGGCTG
Alien Forward primer	CCTCATCCACTGATTATGGGAGT
Alien Reverse primer	CATCATAATTCTTGAAGGCTTCA
Alien Probe	CCCTCAAGTGCATTTTACCACCACATTCTCT
NCoR1Forward primer	TGAAGGTCTTGGCCCAAAAG
NCoR1Reverse primer	TTTGTCTTGATGTTCTCATGGTA
NCoR1Probe	CTGCCACTGTATAACCAGCCATCAGATACCA
SMRT Forward primer	CACCCGGCAGTATCATGAGA
SMRT Reverse primer	CGAGCGTGATTCCTCCTCTT
SMRT Probe	CTTCCGCATCGCCTGGTTTATT
VDR Forward Primer	CTTCAGGCGAAGCATGAAGC
VDR Reverse Primer	CCTTCATCATGCCGATGTCC
VDR Probe	AAGGCACTATTCACCTGCCCCTTCAA

Table 2: Sequences of primers and probes for real time RT-PCR

Treatment		1α,25(OH)$_2$D$_3$		TSA		1α,25(OH)$_2$D$_3$ plus TSA	
Gene	**GenBank accession no.**	**Fold**	**ΔMAI**	**Fold**	**ΔMAI**	**Fold**	**ΔMAI**
DNA-binding protein inhibitor ID-1; Id-1H	D13389	3.8	16038	2.2	16087	6.6	26246
Cyclin K	AF06515	1.2	623	1.5	2756	8.9	15752
MAP kinase-activated protein kinase 2 (MAPK-APK2)	U12779	1.4	7352	1.5	7683	2.7	20143
ras-related C3 botulinum toxin substrate 1; p21-rac1; TC25	M29870; M31467	Nc	6665	Nc	6965	Nc	14965
Zyxin + zyxin-2	X94991; X95735	1.5	2959	0.8	3109	3.5	13898
Growth arrest & DNA-damage-inducible protein (GADD45);	M60974	Nc	434	Nc	382	Nc	6926
beta catenin (CTNNB)	X87838; Z19054	Nc	1231	Nc	1560	Nc	2427
cadherin 5 (CDH5); vascular endothelial cadherin precursor (VE-cadherin)	X79981; X59796	2.4	7160	0.7	221	11.5	20848

Table 3: Co-operative regulation of gene expression patterns by co-treatment with TSA and 1α,25(OH)$_2$D$_3$ Table of fold differences (Fold) and change in the mean adjusted intensity (ΔMAI) for target genes that were co-operatively regulated by the co-treatment of agents, compared to control. From each of the treatments the fold and ΔMAI was calculated from the mean of the two experiments. Only genes that were reported as modulated in the same manner in both experiments were included in the results. Repeat experiments involved stripping the membranes (which were checked to ensure zero signal) and reprobing with radiolabelled probes prepared from repeat treatments of cells.

Nc, fold change not calculated.

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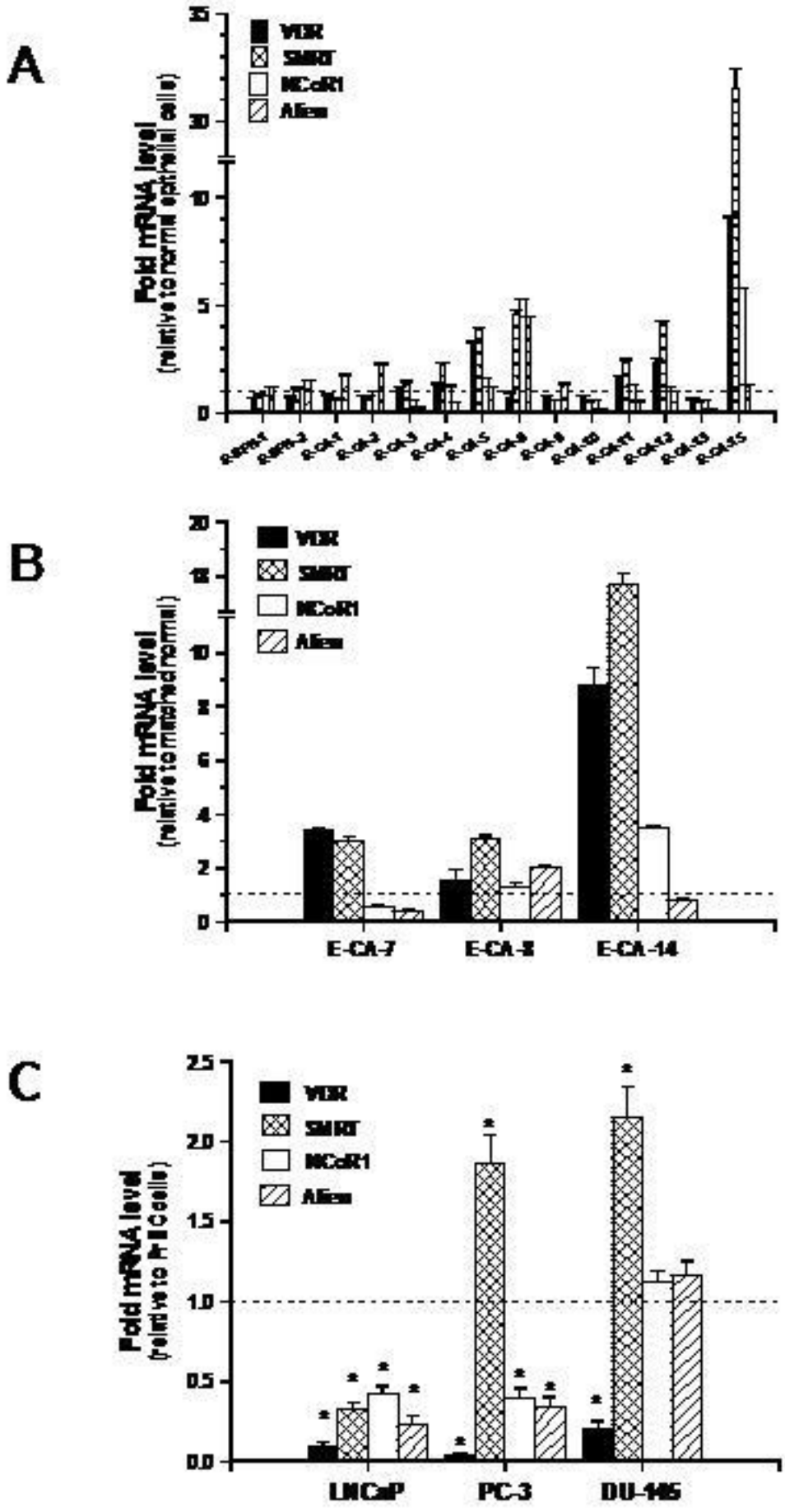
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VDR
SMRT
NCoR1
Alien
Fold mRNA level
(relative to normal epithelial cells)
B
VDR
SMRT
NCoR1
Alien
Fold mRNA level
(relative to matched normal)
E-CA-7
E-CA-8
E-CA-14
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(relative to PrEC cells)
LNCaP
PC-3
DU-145

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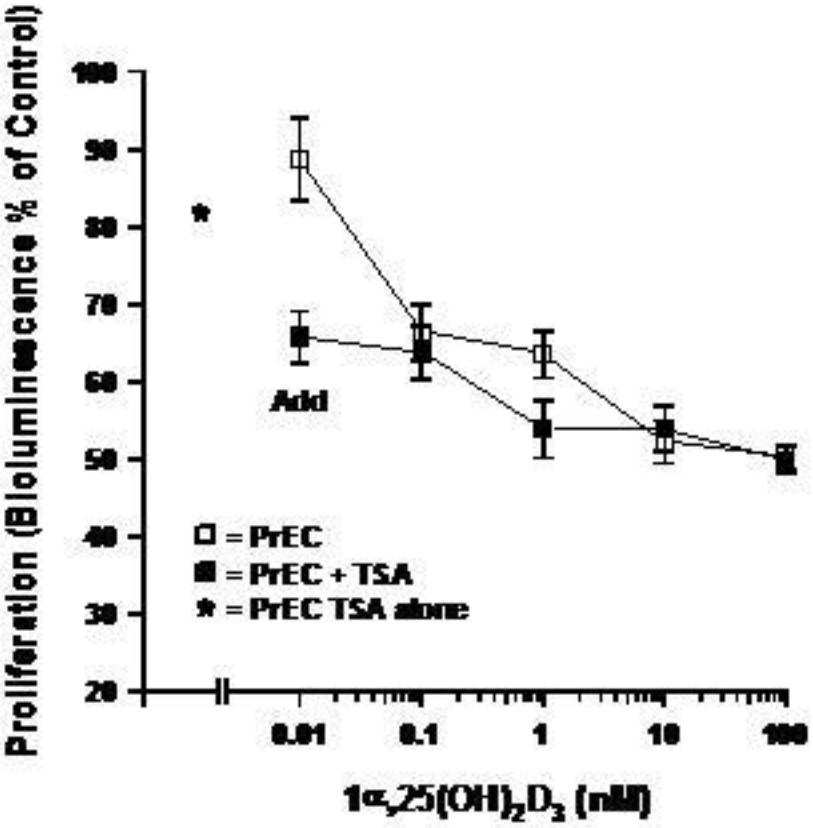
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90
80
70
60
50
40
30
20
Add
□ = PrEC
■ = PrEC + TSA
* = PrEC TSA alone
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0.1
1
10
100
1α,25(OH)2D3 (nM)

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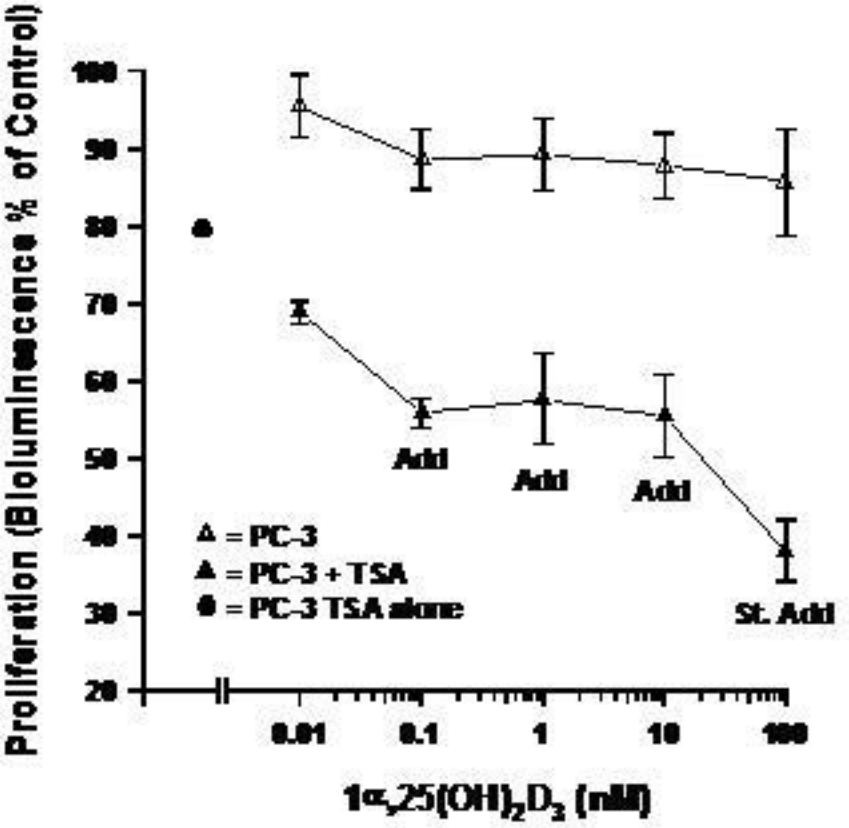
Proliferation (Bioluminescence % of Control)
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90
80
70
60
50
40
30
20
Add
Add
Add
St. Add
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▲ = PC-3 + TSA
● = PC-3 TSA alone
0.01
0.1
1
10
100
1α,25(OH)2D3 (nM)

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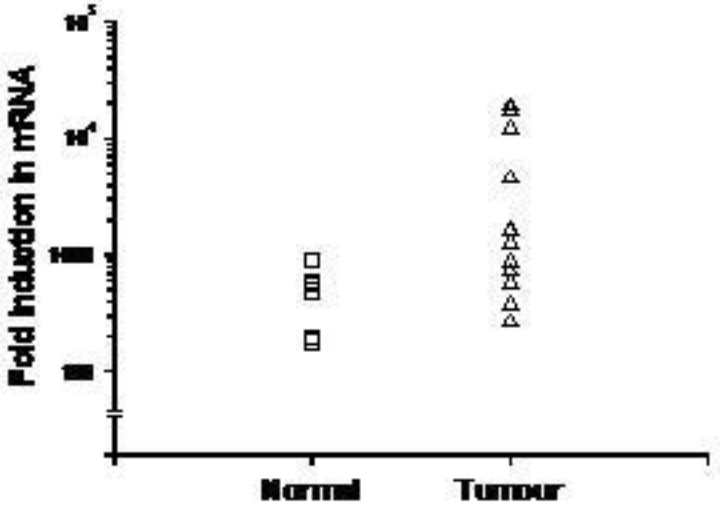
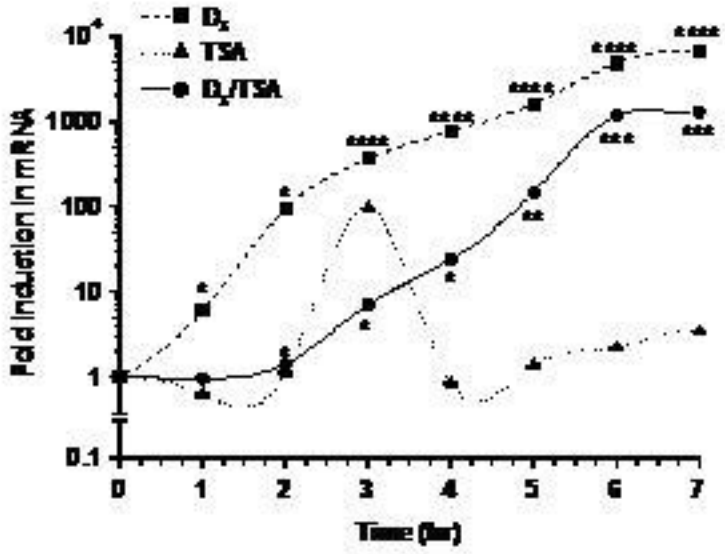
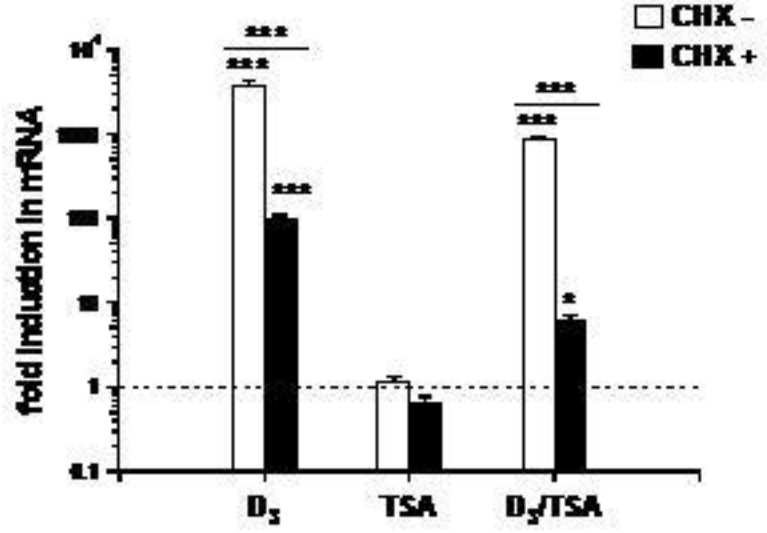
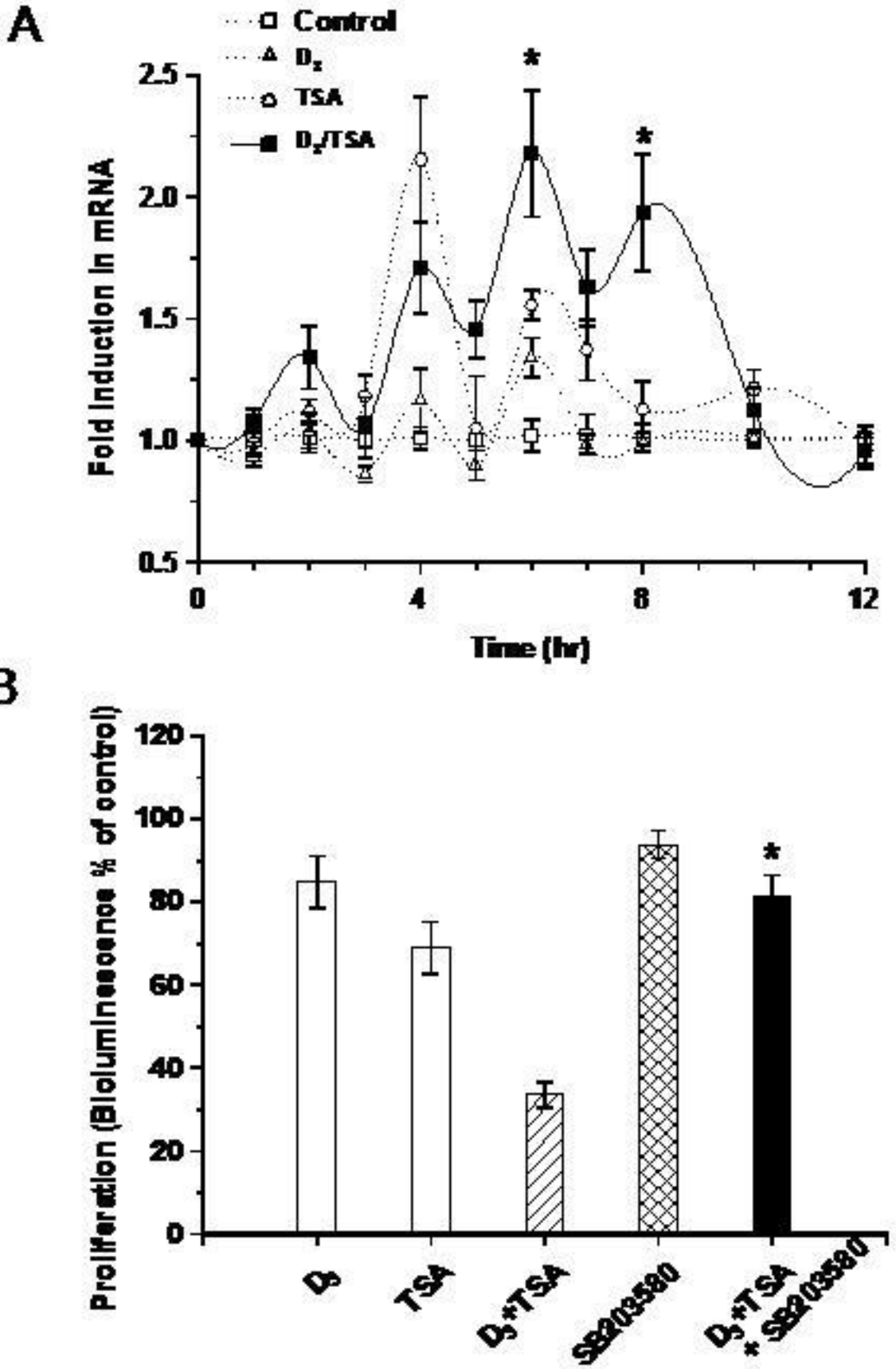

Fold Induction in mRNA
Normal
Tumour

B


D3
TSA
D3/TSA
Fold Induction in mRNA
Time (hr)

C


CHX -
CHX +
fold Induction in mRNA
D3
TSA
D3/TSA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D3
TSA
D3/TSA
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2.0
1.5
1.0
0.5
0
4
8
12
Time (hr)
*
*
B
Proliferation (Bioluminescence % of control)
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100
80
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+ SB203580
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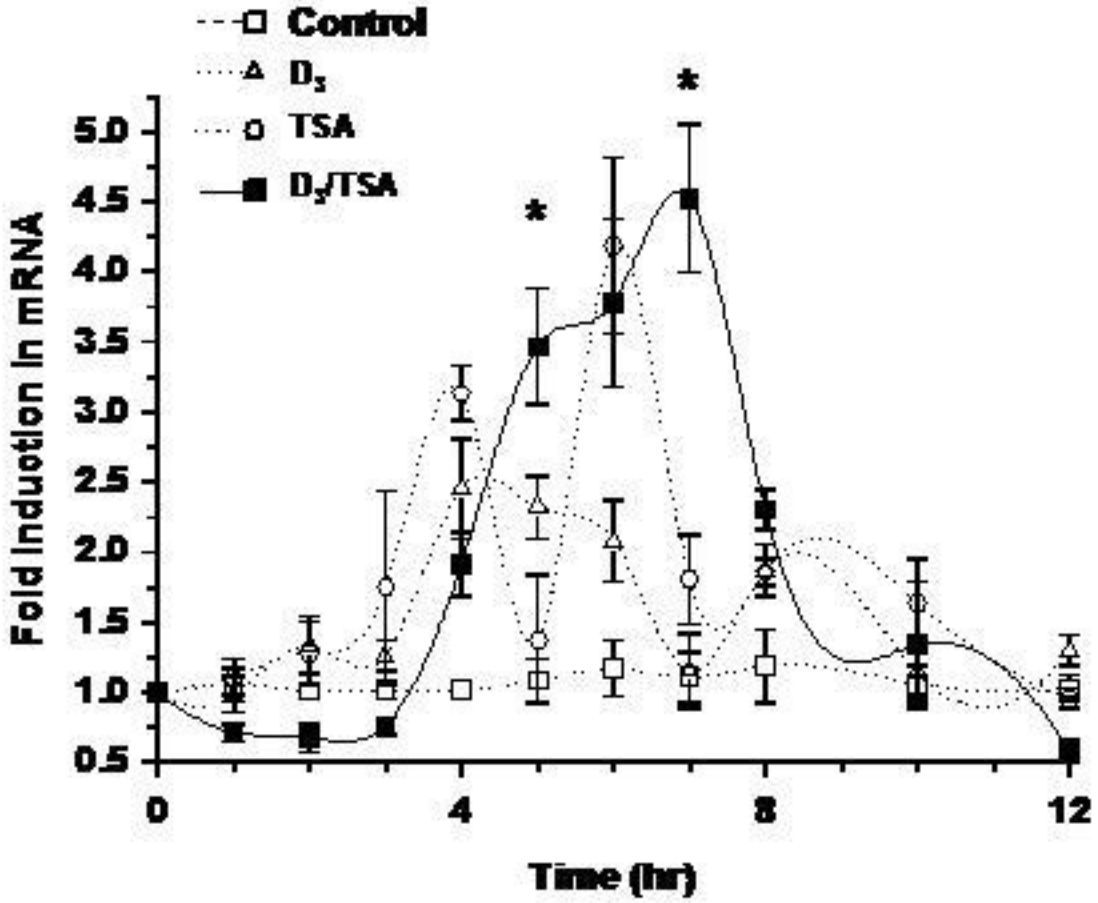
A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TSA
D3/TSA
Fold Induction In mRNA
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4.0
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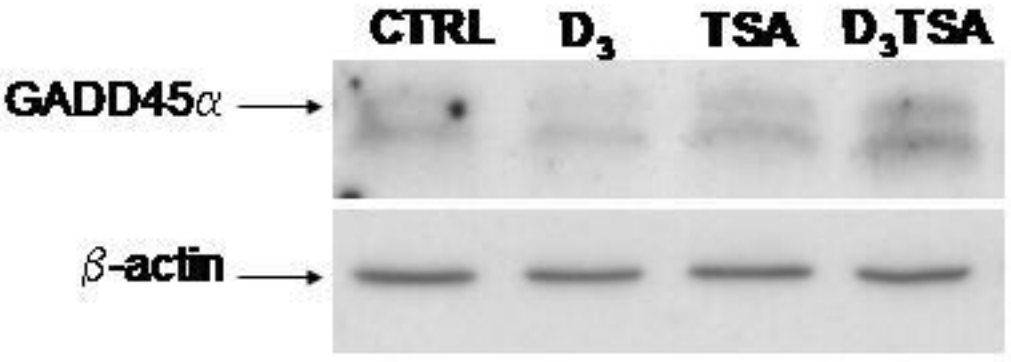
1.0
0.5
0
4
8
12
Time (hr)
*
*

B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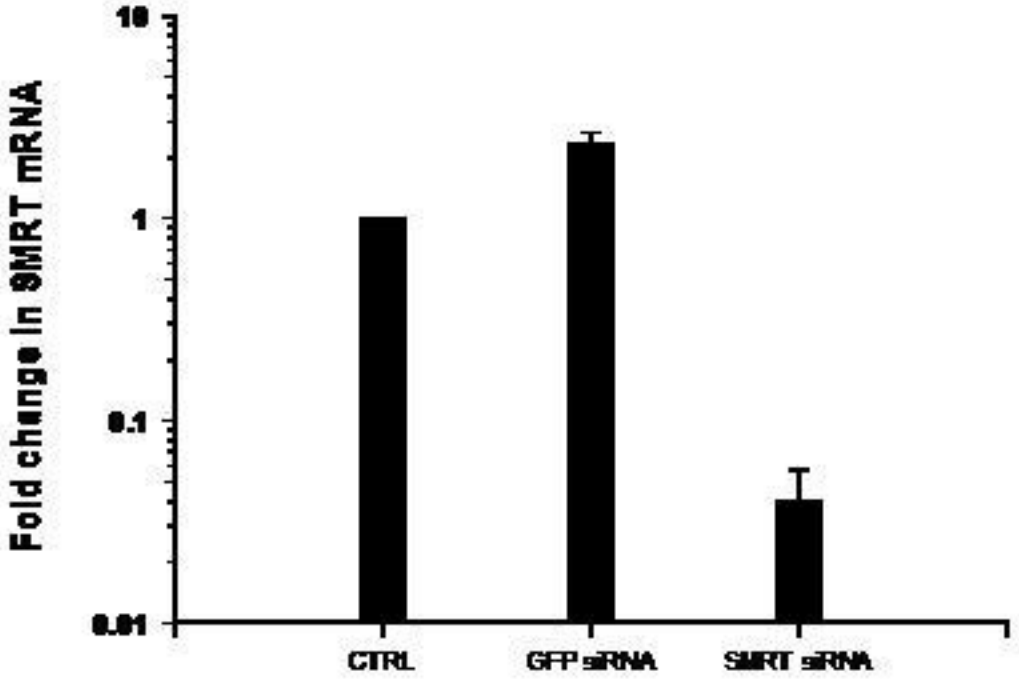
GADD45α
β-actin

A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GFP siRNA
SMRT siRNA

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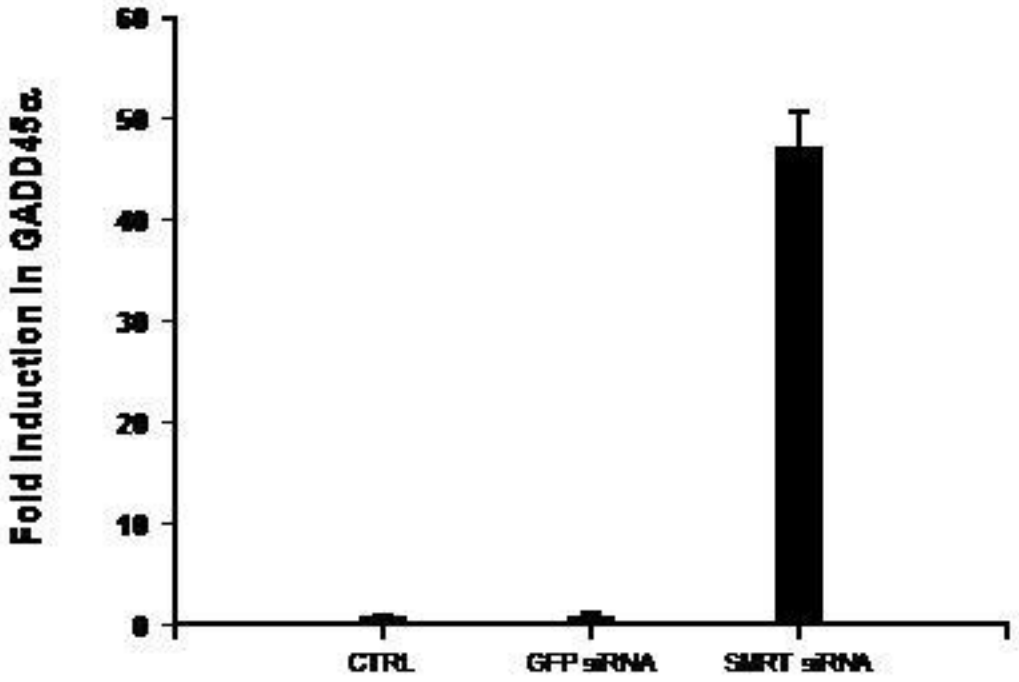
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GFP siRNA
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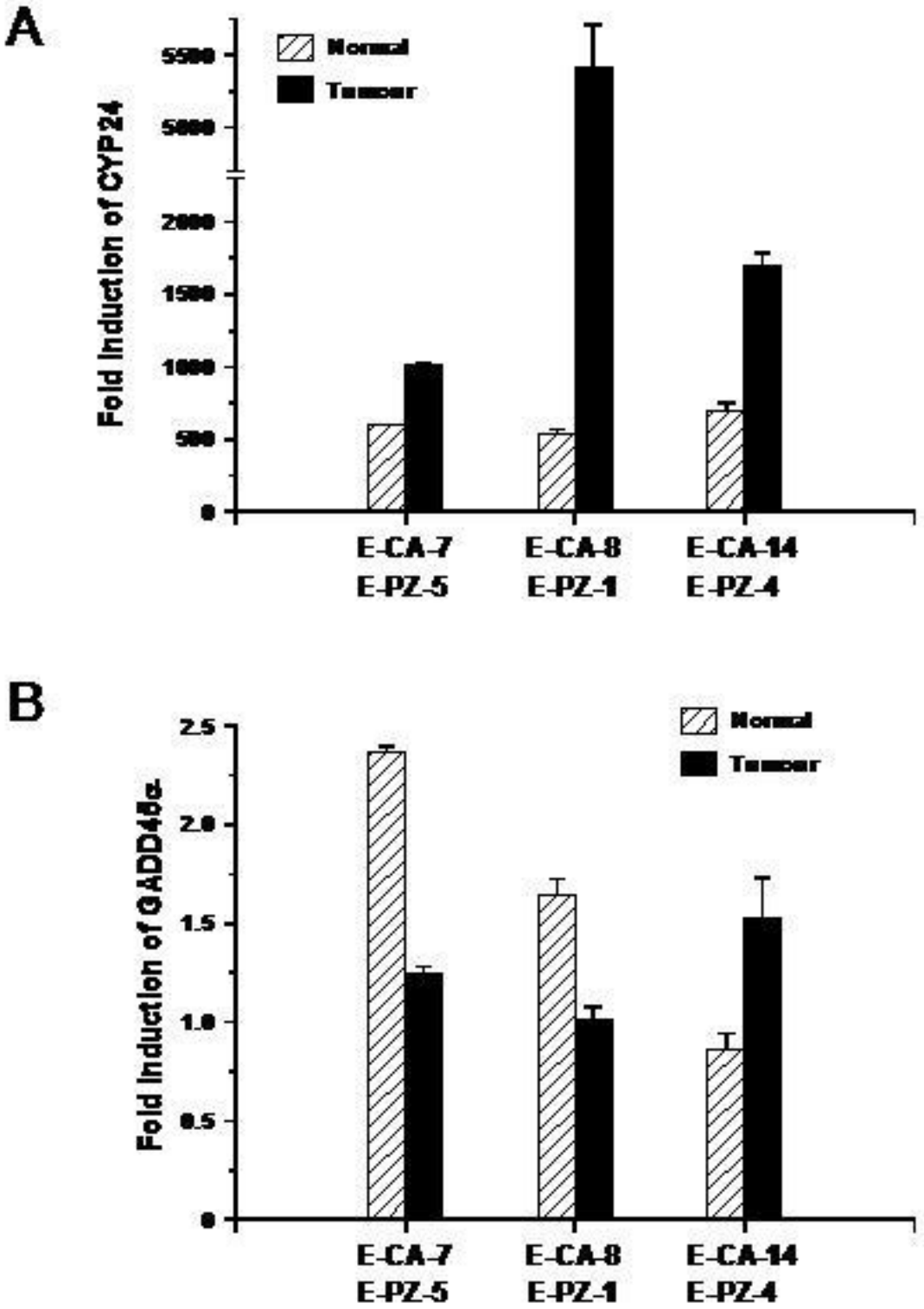
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2000
1500
1000
500
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E-PZ-5
E-CA-8
E-PZ-1
E-CA-14
E-PZ-4
B
Normal
Tumor
Fold Induction of GADD45α
2.5
2.0
1.5
1.0
0.5
0
E-CA-7
E-PZ-5
E-CA-8
E-PZ-1
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