DNA demethylation and histone deacetylation inhibition act synergistically to re-express oestrogen receptor beta and induce apoptosis in prostate cancer cell-lines

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Introduction

Epigenetic silencing mechanisms are increasingly thought to play a major role in the development of human cancers, including prostate cancer. The two major epigenetic regulatory mechanisms involve alterations in DNA methylation and histone acetylation status. Promoter CpG island hypermethylation and histone hypoacetylation, catalysed by DNA methyltransferase (DNMT) and histone deacetylase (HDAC) respectively, are associated with transcriptional repression. Evidence in other cancers suggests the two mechanisms are dynamically linked, yet few studies have examined the potential interaction of the two pathways in prostate cancer. Here we report a synergistic, apoptotic effect of treatment with a demethylating agent and a histone deacetylase inhibitor on cultured prostate cancer cells, with associated re-expression of the putative anti-proliferative agent oestrogen receptor beta.

Methods

LNCaP, DU-145 and PC-3 cells were pre-treated with the DNMT inhibitor 5'-aza-2'-deoxycytidine (5-AZAC) for 72 hours followed by 24 hours combined treatment with 5-AZAC and the HDAC inhibitor trichostatin A (TSA). Following treatment, cells were either processed for cell proliferation, cell toxicity, cell viability and apoptosis assays, or harvested for quantitative real-time RT-PCR gene expression analyses. Assessed target genes were oestrogen receptor beta (ERβ), androgen receptor (AR), progesterone receptor (PGR) and prostate specific antigen (PSA).

Results

In all cell-lines co-treatment with 5-AZAC and TSA was associated with significantly reduced cell proliferation when compared with control groups ($p<0.05$); associated reduced cell viability and increased cell death was seen in all cases. Caspase activation was significantly increased in the co-treatment group, indicating that apoptosis was a major mechanism of cell death. Most marked effects were seen in the androgen-dependent, AR-positive LNCaP cell-line. In all cell-lines a marked synergistic re-expression of ERβ was identified in the co-treatment group, a finding which was not seen for either AR or PSA. A similar re-expression of PGR was also identified.

Conclusions

At concentrations associated with gene re-expression, the DNA demethylating agent 5-AZAC and the HDAC inhibitor TSA co-operate in an additive and synergistic way to induce apoptosis in prostate cancer cell-lines. Increased apoptosis in the co-treatment group was associated with marked re-expression of ERβ, which is an intriguing finding, raising the possibility of further targeting of prostate cancer cells with ERβ-selective agents such as phytooestrogens and selective oestrogen receptor modulators (SERMs).

Introduction

The regulation of gene expression in eukaryotes is complex and multilayered. Control may be exerted at the level of initiation of transcription, following transcription, or through epigenetic events. Epigenetic events are defined as alterations in gene expression without changes in the DNA coding sequence that are heritable through cell division (1), and are thought to be crucial in perpetuating tissue specificity. Two mechanisms are integral to epigenetic transcriptional control; DNA methylation and histone modification.

DNA methylation, catalysed by DNA methyltransferase (DNMT), describes the addition of a methyl group to the cytosine residue of a CpG dinucleotide. The distribution of CpG dinucleotides in human genome is conspicuously restricted, except for certain CpG-rich 'islands', commonly located in the promoter region of approximately 60% of genes (2), which are typically unmethylated. Hypermethylation of CpG islands is associated with repression of downstream gene expression (3), possibly due to interference with the binding of transcription factors, or through the action of methylated DNA binding proteins (MBDs). Whilst DNA methylation is an important control mechanism in normal cells, inappropriate DNA methylation is thought to be important in the development of a majority of cancers, including prostate cancer. To date over 30 genes have been shown to be epigenetically silenced by hypermethylation in prostate cancer, including genes controlling cell cycle progression, tumour cell invasion, DNA repair, and hormonal response (4).

ERβ is one such hormonal response gene known to be hypermethylated in prostate cancer. It contains a typical CpG island within its promoter (5), and has been shown to be re-expressed in prostate cancer cell-lines treated with DNMT inhibitors such as 5-aza-2'deoxycytidine (6-8). Furthermore, methylation-specific PCR studies have shown that the promoter region of the ERβ gene is hypermethylated in prostate cancer tissue studies (6, 8, 9). The loss of ERβ in prostate cancer cell-lines and in tissue studies adds to an accumulating body of evidence supporting an antiproliferative role for ERβ in the prostate. For example, ERβ knockout mice (βERKO) develop severe epithelial hyperplasia in the ventral prostate, whereas no such abnormality is present in either ERα knockout mice or ERα/ERβ knockout mice (10, 11). ERβ also binds the cell cycle inhibitory protein, mitosis arrest deficient 2 (MAD2)(12), and antagonises ERα-mediated cyclin D1 expression (13), offering a potential mechanism for the regulation of cellular proliferation. An attenuation of androgen receptor activity has also been described (14). In βERKO mice AR is over-expressed in many tissues which normally express ERβ (15, 16), whereas genistein-mediated reduction in AR-expression in LNCaP cells is dependent upon ERβ (17). ERβ is also thought to confer protective effects on the cell by upregulating expression of anti-oxidative enzymes including quinone reductase and glutathione-S-reductase (14, 18), thereby protecting basal cells

against oxidative damage, known to be a risk factor in neoplastic transformation. As the basal layer is thought to harbour progenitor cell populations responsible for cancer initiation and progression (19), and is also the site of strongest staining for ERβ (20, 21), it is possible ERβ exists to counter the proliferative and pro-oxidant actions of both ERα and AR. ERβ thus represents a potential novel target for therapeutic intervention in prostate cancer.

The second mechanism of epigenetic transcriptional control results from histone modifications. Histones form the core around which double-stranded DNA is coiled to form a nucleosome. They are subject to a number of post-translational protein modifications, including acetylation, methylation, phosphorylation, and ubiquitination (22). Histone acetylation is associated with transcription activation, whereas deacetylation, catalysed by a family of histone deacetylases (HDACs), is associated with transcriptional silencing. Dysregulated epigenetic control of gene expression by histone modification is thought to be important in the development of a number of cancers, including prostate cancer (4). Typical abnormal findings in cancer cells include increased histone deacetylase activity (23) and associated transcriptional silencing of selected constitutive genes, including insulin-like factor growth factor-binding protein 3 (24) tissue inhibitor of matrix metalloproteinase (25), and carboxypeptidase A3 (26). To our knowledge there have been no reports to date documenting a similar effect on ERβ expression in prostate cancer cells.

Although histone modification and DNA methylation are both independent processes, they are also integrally linked (27). For example DNA methyltransferases are known to independently recruit histone deacetylases, leading to histone deacetylation and transcriptional repression (28, 29). In addition, MBDs can independently recruit HDACs to the site of DNA methylation (3, 30). A number of *in vitro* studies have demonstrated that DNA methylation and histone deacetylation may co-operate to repress gene transcription (31-33). In addition, the combination of HDAC and DNMT inhibition has also been shown to induce apoptosis, differentiation and/or cell growth arrest in human lung, breast, thoracic, leukemia and colon cancer cell-lines (34). To our knowledge there have been no reports to date documenting the effects of combination therapy in prostate cancer cell-lines.

Methods

Cell-culture

DU-145, LNCaP, and PC-3 prostate cancer cell-lines were purchased from the American Type Culture Collection (ATCC). DU-145 cells were grown in Dulbecco's Modified Eagle's Medium (Cambrex Bioscience Ltd.), LNCaP cells were grown in RPMI 1640 Medium (Cambrex) and PC-3 cells were grown in Nutrient Mixture F-12 Ham medium (Sigma-Aldrich Company Ltd.). All cells were grown in media supplemented with 10% foetal calf serum (Cambrex) and 2 mM L-glutamine, and were incubated at 37°C in an atmosphere containing 5% CO_2.

Cell-culture treatment with TSA and 5-AZAC

Following optimisation, prostate cancer cells were pre-treated with the DNMT inhibitor 5'-aza-2'-deoxycytidine (5-AZAC), followed by the addition of the HDAC inhibitor trichostatin A (TSA). On day 0, equal numbers of cells were seeded into 16 identical culture vessels (flasks or wells). For 25 cm^2 culture flasks 1 x 10^5 cells were seeded in 10 mls of media; for 96-well plates, 2000 cells were seeded in 100 µl of media per well. Cells were allowed to seed overnight. The following day (day 1) quadruplicates were assigned to control, TSA only, 5-AZAC only, and TSA and 5-AZAC (T&A) treatment groups. 5-AZAC (Sigma-Aldrich) dissolved in Dulbecco's Phosphate Buffered Saline (DPBS, Cambrex) was added to 5-AZAC and T&A assigned flasks at a final working concentration of 8.8 µM. An equivalent volume of DPBS was added to control and TSA flasks. Flasks were left for 72 hours. After 72 hours (day 4) trichostatin A (Sigma-Aldrich) dissolved in ethanol was added to TSA and T&A assigned flasks at a final working concentration of 300 nM. An equal volume of ethanol was added to control and 5-AZAC flasks. After a further 24 hours (day 5) cells were harvested for RNA/protein extraction, or used for cell proliferation/apoptosis assays.

Tritiated thymidine proliferation assay

[^{3}H]-thymidine (Amersham Biosciences UK Ltd.) was added to cells grown in a 96-well plate at a final concentration of 0.037 MBq/ml 16 to 18 hours prior to cell harvesting. Cells were harvested onto a 96-well Unifilter™ GF/C microplate (Perkin Elmer UK Ltd.) and the plate was left to dry for 1 hour at 37 °C. 40 µl of Microscint-O™ scintillin (Perkin Elmer) was added to each well and the plates were counted on a TopCount NXT™ microplate scintillometer.

Cytotoxicity, cell-viability and apoptosis assays

The Toxilight™ cytotoxicity assay (Cambrex) detects adenylate kinase, which leaks from cells in the terminal stages of cell death. The Vialight™ Plus assay (Cambrex) measures abundance of cellular adenosine triphosphate (ATP), which is a marker for cell viability. The Caspase-Glo™ 3/7 apoptosis assay (Promega UK Ltd.) measures levels of caspases 3 and 7, which are key effectors of apoptosis in humans. Cells were grown in white-walled, clear-bottomed 96-well tissue culture plates (Lumitrac™ 200, Jencons Scientific Ltd.) to reduce light contamination between wells. Quadruplicate blank wells were included containing assay reagent, vehicle, and cell-culture medium without cells to control for background luminescence. For the Toxilight™ assay, a further set of control quadruplicates was incorporated to assess luminescence following 100% lysis using the ToxiLight™ 100% Lysis Reagent Set. Following addition of respective Toxilight™ and Vialight™ reagents, bioluminescence was measured after 5 mins on a PlateLumino™ luminometer (Stratec Biomedical Systems). For apoptosis assays Caspase-Glo™ reagents were mixed and added to the microplate, agitated for 30s, wrapped in foil and incubated at room temperature for 90 mins before determination of luminescence. Relative luminescence (RLU) was expressed as average assay luminescence minus average background fluorescence.

RNA isolation and reverse transcription (RT)

An RNeasy™ Mini Kit (Qiagen Ltd.) was used to isolate RNA from cell-lines for real-time RT-PCR. Prior to RNA extraction, cell lysates were homogenised using a QIAshredder™ (Qiagen), centrifuged at 14,000g for 2 mins. The homogenate was added to an RNeasy® spin column and the protocol for animal tissues was followed, including an on-column RNase-free DNase step (Qiagen). Isolated RNA was reverse transcribed as follows: 2 µg of RNA was added to an Eppendorf tube along with 0.5 µg of oligo$(dT)_{15}$ primer (Promega). The tube was heated at 70°C for 5 minutes and immediately placed on ice. 5 µl M-MLV 5x reaction buffer, 1 µl dNTPs (12.5 mM, Bioline Ltd.), 25 units RNasin® ribonuclease inhibitor (Promega), and 200 units M-MLV reverse transcriptase (Promega) was added along with RNase-free water (Sigma-Aldrich) to a final volume of 25 µl. The contents of the tube were mixed gently and incubated at 39ºC for 80 minutes, following which the tube was heated at 95°C for 5 mins to inactivate the reverse transcriptase.

Real-time RT-PCR

Real-time RT-PCR reactions were performed on an Mx4000 Quantitative PCR System (Stratagene Inc.). Prior to thermocycling total cDNA concentration of standards and specimens was determined using a NanoDrop® ND-1000 UV spectrophotometer (Nanodrop Technologies). Thermocycling was done in 0.2 ml optical PCR strip tubes containing 12.5 µl of QuantiTect™ SYBR® green master mix (Qiagen), 2 µl of gene-specific

forward and reverse primers, and 8.5 µl of RNase-free water (Qiagen), to which 2 µl of template cDNA was added. Each specimen assay was performed in duplicate. Common PCR reaction conditions were as follows: 94°C for 5 mins; 95°C for 30 s; *n* °C for 30 s; 72°C for 30 s: cycling through denaturation, annealing and extension for *x* cycles; 72°C for 5mins. Gene-specific primer sequences, annealing temperatures (*n*) and cycle numbers (*x*) are shown in Table 1. All primers were designed for real time RT-PCR, and all except ribosome h18S were intron-spanning. Two housekeeping genes were used; ribosome h18S (h18S) and glucose-6-phosphate dehydrogenase (GAPDH). Mean specimen starting template RNA concentration was determined from constructed calibration curves. Derived target gene expression was normalised by expression relative to individual housekeeping gene expression.

SDS-PAGE and Western Blotting

Protein lysates were obtained using standard techniques. 30 mg of reduced protein sample was loaded into each well of a 10% polyacrylamide gel and submerged in SDS running buffer. Electrophoresis was performed at 100V for approximately 90 mins. Proteins were transferred onto a nitrocellulose membrane over 45 minutes at 13V. After overnight incubation in 5% milk protein/TBS-Tween (TBST), either 1:1000 monoclonal mouse anti-α-tubulin antibody (T6199, Sigma-Aldrich) or 1:1000 polyclonal goat anti-ERβ antibody (sc-6820, Santa Cruz Biotechnologies, Inc.) was added to one of duplicate membranes containing specimen samples and incubated for 3 hours at room temperature. Appropriate secondary antibodies (DakoCytomation Ltd.) were added at a dilution of 1:1000 in 5% milk protein/TBST and incubated for 2 hours at room temperature. Protein expression was determined using the ECL™ Western Blotting Detection Kit (Amersham).

Statistical analyses

All statistical analyses were performed using SPSS statistical software (version 11.5, SPSS Inc.) Where continuous data was normally distributed, means were compared by independent samples t-test. Non-parametric continuous data was analysed using the Mann-Whitney U test. Correlation between two continuously distributed variables was assessed using logistic regression for parametric data and Spearman's correlation coefficient for non-parametric data.

Results

DNA demethylation and HDAC inhibition is associated with reduced proliferation, increased cell death and apoptosis in prostate cancer cell-lines

Proliferation following treatment of DU-145, LNCaP and PC-3 cells with 5-AZAC and TSA was determined by tritiated thymidine proliferation assay. The results are shown in Fig. 1. TSA and 5-AZAC were independently associated with reductions in cell proliferation when compared with the control group in all three prostate cancer cell-lines. Of the two drugs 5-AZAC was more potent in inhibiting cell-proliferation in all cell-lines tested. Co-treatment with TSA and 5-AZAC was associated with values for cell proliferation which were significantly reduced compared with those obtained for TSA treatment or 5-AZAC treatment alone ($p<0.001$ for DU-145 and LNCaP; $p<0.05$ for PC-3) indicating a synergistic effect for co-administration.

Reduced cell proliferation following drug treatment results from either an inhibition of cell growth/division, cell death, or a combination of the two mechanisms. We used the complementary Vialight® and Toxilight® assays to assess the degree of cell viability and cell toxicity respectively in prostate cancer lines treated with TSA and 5-AZAC. The results are shown in Fig. 2. In all cell-lines TSA and 5-AZAC were independently associated with reduced cell viability compared with the control group. In PC-3 cells, 5-AZAC treatment was associated with a significantly greater reduction in cell viability than was seen for TSA treatment ($p<0.001$). There was no significant difference between the drugs in either DU-145 or LNCaP cells. For all cell-lines co-treatment was associated with significantly reduced cell viability compared with the more effective single-agent treatment ($p<0.0001$ for DU-145 and LNCaP cells; $p<0.001$ for PC-3 cells). In terms of magnitude of effect, when compared with the control group, co-treatment was associated with mean reductions in cell viability of 93.3%, 82.3%, and 68.0% for DU-145, LNCaP, and PC-3 cells respectively.

Reduced cell viability usually implies cell death. Toxilight® assays confirmed a broadly reciprocal relationship between cell viability and cell death for all cell-lines. In LNCaP and PC-3 cell-lines co-treatment with TSA and 5-AZAC was associated with significant increases in cell death compared with the more effective single agent ($p<0.001$ each). Interestingly in DU-145 cells co-treatment did not produce any additional cytotoxic effect over TSA treatment alone. The reasons for this are unclear. Cytotoxicity was greatest in AR-positive LNCaP cells. When assessed relative to 100% lysis, co-treatment in LNCaP cells was associated with mean cell death of 84.7%, compared with 33.1% and 47.8% for DU-145 and PC-3 cells respectively. The similar values obtained for reduced cell viability and increased cell death in LNCaP cells suggest that the reduced proliferation seen

with co-treatment is primarily a function of cell-death. In DU-145 and PC-3 cells however the values are less concordant, suggesting that impaired growth *and* cytotoxicity contribute to reduced proliferation.

Visual inspection of cells following drug treatment showed that in addition to reduced numbers, cells changed morphology, particularly following co-administration of both drugs. Typical changes included cell membrane cell shrinkage and membrane blebbing, consistent with apoptosis. Changes were most marked in LNCaP cells (Fig. 3). This initial impression was confirmed by caspase 3/7 apoptosis assay. As shown in Fig. 4, treatment with TSA and 5-AZAC was associated with increases in mean caspase 3/7 activation when compared with control cells in DU-145, LNCaP and PC-3 cells; for TSA treatment the differences were significant in DU-145 and LNCaP cells ($p<0.001$); for 5-AZAC treatment the differences were significant in all three cell-lines ($p<0.001$). As was seen for the cytotoxicity assays, co-treatment did not produce an additional apoptotic effect in DU-145 cells when compared with TSA treatment alone. In LNCaP and PC-3 cells however, co-treatment was associated with significant increases in apoptotic activity compared with the more effective single agent ($p<0.001$ for LNCaP and PC-3). The greatest magnitude of response following co-treatment was again seen in LNCaP cells. When compared with the control group, co-treatment induced an 11.5-fold increase in apoptotic activity in LNCaP cells, compared with 7.5-fold and 1.5-fold increases in DU-145 and PC-3 cells respectively.

<u>DNA demethylation and HDAC inhibition is associated with re-expresssion of ERβ in prostate cancer cell-lines</u>

Ribosome h18S-normalised gene expression for ERβ, AR, ERα, PSA and PGR is shown in Fig. 5. Very similar results were obtained for the GAPDH housekeeping gene (data not shown). This finding, along with the demonstration of strong positive correlations between ERα and ERα-dependent PGR ($p<0.001$), and AR and AR-dependent PSA ($p<0.001$), supports the validity of obtained results.

Co-treatment was associated with ERβ re-expression in all cell-lines tested. When compared with the control group however, results were non-significant in PC-3 and DU-145 cells, although in the latter case the results just failed to achieve statistical significance ($p=0.06$). The results were significant in androgen-responsive LNCaP cells ($p<0.05$). Interestingly, 5-AZAC treatment failed to cause re-expression of ERβ in this cell-line suggesting that chromatin remodelling and not DNA methylation was the primary mechanism of gene silencing. However, co-treatment did increase ERβ expression over TSA treatment alone, suggesting an additional co-stimulatory effect of 5-AZAC. Similar results are seen for ERβ protein expression (Fig 6). Allowing for unavoidable low level non-specific contamination, clearly identifiable in the secondary-only lane, TSA treatment and to a lesser extent 5-AZAC treatment were associated with a re-expression of ERβ protein.

As seen with ERβ gene expression, combination therapy was associated with the most marked re-expression of ERβ protein.

The results obtained for androgen receptor were intriguing. In the androgen-independent AR-negative DU-145 and PC-3 cell-lines, co-treatment with TSA and AZAC caused a marked re-expression of AR which achieved significance in the DU-145 cell-line ($p<0.01$). Very little re-expression of AR was seen following single treatment with either TSA or 5-AZAC. In contrast, TSA treatment induced a *reduction* in AR expression in LNCaP cells lines without any additional additive effect following 5-AZAC administration. Whilst the results for AR did not achieve statistical significance, results for androgen-dependent PSA – which exactly mirrored those of AR – were significant ($p<0.001$). There initially appeared to be a reciprocal relationship in ERβ and AR expression. However, pairwise correlation analyses failed to identify a significant relationship between the genes.

The expression of ERα following co-treatment with TSA and 5-AZAC was very similar to that of ERβ. LNCaP cells, TSA was more effective than 5-AZAC in causing ERα re-expression. Co-treatment with the drugs produced the most marked effect. Levels of ERα-dependent PGR were also re-expressed following co-treatment and closely mirrored those of ERα. Pairwise correlation analyses confirmed a strong positive association between the genes ($p<0.001$).

Discussion

A number of studies have reported reductions in cell proliferation and apoptosis in prostate cancer cell-lines treated with HDAC inhibitors (25, 35-39). Fronsdal et al investigated the response of DU-145 LNCaP and PC3 cells to a number of HDAC inhibitors, including trichostatin A. Treatment with 100 nM TSA resulted in approximate increases cell death of 10% over control cells in DU-145 and LNCaP cells. PC-3 cells however were largely resistant to HDAC treatment. In this study TSA treatment with 300 nM TSA produced a larger increase in cell death in DU-145 cells (~28%), with a similar increase in LNCaP cells (~10%), whilst PC-3 cells were also found to be generally resistant. The reasons for the differences are unclear, but may relate to different cell-culture conditions, treatment regimens, and drug vehicles used. Fronsdal's group also used the relatively inaccurate trypan blue exclusion assay to determine cell death rates, whereas we utilised a highly sensitive bioluminescent method. Whilst a small number of studies have reported inhibited cell proliferation and induction of apoptosis in prostate cancer cell-lines following 5-AZAC treatment (40), the majority report changes in gene expression associated with demethylation without mention of major effects on cell proliferation. The drug dosage used in this study approximates that associated with altered gene expression, yet in this study 5-AZAC administration was associated with significantly reduced proliferation in all cell-lines when compared with controls. Interestingly, relatively modest cytotoxicity (<7.5%) and apoptotic activity was observed in the 5-AZAC treatment group, suggesting that 5-AZAC mediated changes in gene expression reduce cellular proliferation by impairing cell growth and division rather than by initiating apoptosis. This is in contrast to the study by Pulukuri et al who identified activation of p53 and $p21^{WAF1/CIP}$ and subsequent marked upregulation in apoptosis following treatment of LNCaP cells with 5-AZAC.

The combination of HDAC and DNMT inhibition has been shown to induce apoptosis, differentiation and/or cell growth arrest in human lung, breast, thoracic, leukaemia and colon cancer cell-lines (34), but to date no studies have identified a similar relationship in prostate cancer cell-lines. In the data presented herein it is shown for the first time that co-treatment with TSA and 5-AZAC inhibits cell growth and induces apoptosis of prostate cancer cells. In androgen-dependent LNCaP cells a truly *synergistic* effect was identified, particularly for apoptosis, whereas for androgen-independent DU-145 and PC-3 cells the effects were *additive*. The findings suggest that combination treatment may have a role in the treatment of advanced prostate cancer, particularly as recent clinical trials of 5-AZAC (also known as decitabine) in metastatic prostate cancer have been disappointing (41, 42). In a phase II study reported by Thibault et al, only 2 of 12 men with androgen independent metastatic prostate cancer treated with decitabine developed a clinical response leading to delayed progression. The authors concluded that the low-dose decitabine regime was well-tolerated with a modest

clinical activity. Although a number of HDAC inhibitors have shown promising antitumour activity in animal models of prostate cancer (36, 43, 44), as yet there are no published clinical trials in humans. A number of phase I trials have however reported good clinical efficacy and a favourable side-effect profile for HDAC inhibitors in a range of other solid and haematological malignancies (45). It is thus tempting to speculate that combination therapy with HDAC and DNMT inhibitors may improve response rates in prostate cancer over that seen with DNMT inhibitors alone.

In addition to reduced cell proliferation and induction of apoptosis, we have been shown that combination therapy with TSA and 5-AZAC upregulates the expression of ERβ mRNA and protein in prostate cancer cell-lines. In all cell-lines assessed there was a *synergistic* effect of combination therapy, which achieved statistical significance in LNCaP cells. It therefore seems likely that histone acetylation and DNA methylation co-operate to epigenetically silence ERβ in prostate cancer. Although we cannot definitively state this to be true without assessing histone acetylation status and DNA methylation status of the ERβ promoter before and after co-treatment, the observation of similar effects in other studies suggest that this is likely to be the case (34). This finding contributes to the accumulating data identifying layering of epigenetic silencing in prostate cancer. Cameron et al first reported an additive effect for HDAC and DNMT inhibitors in the re-expression of a number of genes silenced in colon cancer, including TIMP3 (31). Since then multiple studies have identified a similar relationship for a variety of genes in breast cancer (46-48), lung cancer (49), lymphosarcoma (50) and prostate cancer (32, 33). Interestingly in the study by Nakayama, co-treatment with TSA and all-trans retinoic acid after treatment with 5-AZAC resulted in re-expression of androgen receptor in DU-145 cells, which corroborates the results presented in this study.

Although ERβ is thought to have anti-proliferative actions in the prostate, the reduced proliferation and apoptosis seen with co-treatment in this study is unlikely to be due to ERβ re-expression alone, given the tendency of HDAC and DNMT inhibitors to preferentially release tumour suppressor and pro-apoptotic genes from epigenetic silencing. The finding does however warrant further investigation. Recent evidence also suggests that upregulation of ERβ may act to inhibit migration of prostate cancer cells. Guerini et al have shown that stimulation of ERβ with the androgen derative 5alpha-androstane-3beta,17beta-diol (3betaAdiol) results in an inhibition of prostate cancer cell migration (51). The mechanism of action is thought to involve upregulation of E-cadherin expression. Thus upregulation of ERβ expression following DNMT and HDAC inhibition may therefore provide a target for subsequent ligand activation with 3beta-Adiol. A number of studies have also identified novel therapeutic agents which target ERβ in prostate cancer and are reported to

induce apoptosis in prostate cancer cell-lines (17, 52). Thus in a similar way upregulation of ERβ following co-treatment may sensitise prostate cancer cells to subsequent treatment with conventional oestrogens and newer agents such as phytooestrogens and selective oestrogen receptor modulators (SERMs).

The results obtained for androgen receptor are intriguing. In androgen receptor negative DU-145 and PC-3 cells, DNMT and HDAC inhibition was associated with a re-expression of androgen receptor, implying epigenetic silencing in these cell-lines. In androgen-receptor positive LNCaP cells however, co-treatment was associated with a significant reduction in androgen-receptor expression. The mechanism of reduced gene expression following HDAC and DNMT inhibition is unclear. Reduced AR expression was predominantly associated with TSA administration, as shown in Fig. 5, yet HDAC inhibitors – including TSA – are reported to maximise AR expression in LNCaP cells (53). The reason for the discrepancy between our findings and those of Korkmaz are unclear, but may to relate to the latter group's pre-treatment of LNCaP cells with the synthetic androgen R1881 prior to HDAC inhibition (53). It remains possible that the reduced expression of AR observed following co-treatment is dependent upon the upregulation of a putative inhibitor of AR-transcription. Although ERβ has been postulated as an inhibitor of AR function in LNCaP cells, (17), pair-wise analyses failed to confirm the presence of a significant negative correlation between the genes.

Traditionally, AR positive, androgen-reponsive LNCaP cells have been used as a model for androgen-dependent prostate cancer, whereas AR negative DU-145 and PC-3 cells have been used as a model for androgen-independent disease. However, it is now known that the majority of metastatic deposits of prostate cancer continue to express androgen receptor (54-56), and current theories of androgen independence have focussed on the presence of a functioning androgen receptor (i.e. AR hypersensitivity, promiscuity, amplification etc.) (57). In addition androgen-independent clones of LNCaP (LNCaP-abl and AI:LNCaP) have been developed which are associated with upregulated AR expression and higher levels of AR reporter gene activity, indicating preserved androgen pathway function (58). For these reasons the validity of AR-negative DU-145 and PC-3 cells as a model for androgen-independent disease has been questioned, and it is believed that of the three-cell-lines used in this study, LNCaP represents the most appropriate model for advanced prostate cancer.

The combination of reduced AR expression and upregulated ERβ expression in LNCaP cells following co-treatment suggests conversion to a more favourable phenotype in prostate cancer. ERβ is associated with antiproliferative and potentially anti-migratory actions in prostate cancer cells. Reduced AR expression induced

by antisense oligonucleotides is also associated with an inhibition of proliferation in LNCaP cells (59). The role of upregulated ERα and PGR expression following co-treatment is less clear. Although ERα is thought to be associated with prostate carcinogenesis the effects of its re-expression in established prostate cancer are unknown. Likewise, although PGR expression is known to be down-regulated in prostate cancer (60, 61), the role of PGR re-expression is similarly undefined. Nevertheless combination therapy with TSA and 5-AZAC induces a spectrum of genotypic changes which are associated with the development of a favourable phenotype, marked reductions in cell proliferation and increased apoptosis in LNCaP cells.

References

1. Waddington CH. Gene regulation in higher cells. Science 1969;166(905):639-40.
2. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J Mol Biol 1987;196(2):261-82.
3. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3(6):415-28.
4. Li LC, Carroll PR, Dahiya R. Epigenetic changes in prostate cancer: implication for diagnosis and treatment. J Natl Cancer Inst 2005;97(2):103-15.
5. Li LC, Yeh CC, Nojima D, Dahiya R. Cloning and characterization of human estrogen receptor beta promoter. Biochem Biophys Res Commun 2000;275(2):682-9.
6. Sasaki M, Tanaka Y, Perinchery G, Dharia A, Kotcherguina I, Fujimoto S, et al. Methylation and inactivation of estrogen, progesterone, and androgen receptors in prostate cancer. J Natl Cancer Inst 2002;94(5):384-90.
7. Lau KM, LaSpina M, Long J, Ho SM. Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation. Cancer Res 2000;60(12):3175-82.
8. Zhu X, Leav I, Leung YK, Wu M, Liu Q, Gao Y, et al. Dynamic regulation of estrogen receptor-beta expression by DNA methylation during prostate cancer development and metastasis. Am J Pathol 2004;164(6):2003-12.
9. Nojima D, Li LC, Dharia A, Perinchery G, Ribeiro-Filho L, Yen TS, et al. CpG hypermethylation of the promoter region inactivates the estrogen receptor-beta gene in patients with prostate carcinoma. Cancer 2001;92(8):2076-83.
10. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, et al. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A 1998;95(26):15677-82.
11. Weihua Z, Makela S, Andersson LC, Salmi S, Saji S, Webster JI, et al. A role for estrogen receptor beta in the regulation of growth of the ventral prostate. Proc Natl Acad Sci U S A 2001;98(11):6330-5.
12. Poelzl G, Kasai Y, Mochizuki N, Shaul PW, Brown M, Mendelsohn ME. Specific association of estrogen receptor beta with the cell cycle spindle assembly checkpoint protein, MAD2. Proc Natl Acad Sci U S A 2000;97(6):2836-9.
13. Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, et al. Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 2002;277(27):24353-60.
14. Weihua Z, Warner M, Gustafsson JA. Estrogen receptor beta in the prostate. Mol Cell Endocrinol 2002;193(1-2):1-5.
15. Cheng G, Weihua Z, Makinen S, Makela S, Saji S, Warner M, et al. A role for the androgen receptor in follicular atresia of estrogen receptor beta knockout mouse ovary. Biol Reprod 2002;66(1):77-84.
16. Ogawa S, Chan J, Chester AE, Gustafsson JA, Korach KS, Pfaff DW. Survival of reproductive behaviors in estrogen receptor beta gene-deficient (betaERKO) male and female mice. Proc Natl Acad Sci U S A 1999;96(22):12887-92.
17. Bektic J, Berger AP, Pfeil K, Dobler G, Bartsch G, Klocker H. Androgen receptor regulation by physiological concentrations of the isoflavonoid genistein in androgen-dependent LNCaP cells is mediated by estrogen receptor beta. Eur Urol 2004;45(2):245-51; discussion 251.
18. Montano MM, Jaiswal AK, Katzenellenbogen BS. Transcriptional regulation of the human quinone reductase gene by antiestrogen-liganded estrogen receptor-alpha and estrogen receptor-beta. J Biol Chem 1998;273(39):25443-9.
19. Garraway LA, Lin D, Signoretti S, Waltregny D, Dilks J, Bhattacharya N, et al. Intermediate basal cells of the prostate: in vitro and in vivo characterization. Prostate 2003;55(3):206-18.

20. Leav I, Lau KM, Adams JY, McNeal JE, Taplin ME, Wang J, et al. Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. Am J Pathol 2001;159(1):79-92.
21. Tsurusaki T, Aoki D, Kanetake H, Inoue S, Muramatsu M, Hishikawa Y, et al. Zone-dependent expression of estrogen receptors alpha and beta in human benign prostatic hyperplasia. J Clin Endocrinol Metab 2003;88(3):1333-40.
22. Zhang Y, Reinberg D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev 2001;15(18):2343-60.
23. Halkidou K, Gaughan L, Cook S, Leung HY, Neal DE, Robson CN. Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate 2004;59(2):177-89.
24. Tsubaki J, Hwa V, Twigg SM, Rosenfeld RG. Differential activation of the IGF binding protein-3 promoter by butyrate in prostate cancer cells. Endocrinology 2002;143(5):1778-88.
25. Thelen P, Schweyer S, Hemmerlein B, Wuttke W, Seseke F, Ringert RH. Expressional changes after histone deacetylase inhibition by valproic acid in LNCaP human prostate cancer cells. Int J Oncol 2004;24(1):25-31.
26. Huang H, Reed CP, Zhang JS, Shridhar V, Wang L, Smith DI. Carboxypeptidase A3 (CPA3): a novel gene highly induced by histone deacetylase inhibitors during differentiation of prostate epithelial cancer cells. Cancer Res 1999;59(12):2981-8.
27. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 2002;3(9):662-73.
28. Fuks F, Burgers WA, Godin N, Kasai M, Kouzarides T. Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. Embo J 2001;20(10):2536-44.
29. Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet 2000;25(3):338-42.
30. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 1998;393(6683):386-9.
31. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 1999;21(1):103-7.
32. Chen H, Toyooka S, Gazdar AF, Hsieh JT. Epigenetic regulation of a novel tumor suppressor gene (hDAB2IP) in prostate cancer cell lines. J Biol Chem 2003;278(5):3121-30.
33. Nakayama T, Watanabe M, Suzuki H, Toyota M, Sekita N, Hirokawa Y, et al. Epigenetic regulation of androgen receptor gene expression in human prostate cancers. Lab Invest 2000;80(12):1789-96.
34. Zhu WG, Otterson GA. The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells. Curr Med Chem Anti-Canc Agents 2003;3(3):187-99.
35. Fronsdal K, Saatcioglu F. Histone deacetylase inhibitors differentially mediate apoptosis in prostate cancer cells. Prostate 2005;62(3):299-306.
36. Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C, et al. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. Cancer Res 2000;60(18):5165-70.
37. Gleave ME, Sato N, Sadar M, Yago V, Bruchovsky N, Sullivan L. Butyrate analogue, isobutyramide, inhibits tumor growth and time to androgen-independent progression in the human prostate LNCaP tumor model. J Cell Biochem 1998;69(3):271-81.
38. Maier S, Reich E, Martin R, Bachem M, Altug V, Hautmann RE, et al. Tributyrin induces differentiation, growth arrest and apoptosis in androgen-sensitive and androgen-resistant human prostate cancer cell lines. Int J Cancer 2000;88(2):245-51.

39. Ellerhorst J, Nguyen T, Cooper DN, Estrov Y, Lotan D, Lotan R. Induction of differentiation and apoptosis in the prostate cancer cell line LNCaP by sodium butyrate and galectin-1. Int J Oncol 1999;14(2):225-32.
40. Pulukuri SM, Rao JS. Activation of p53/p21Waf1/Cip1 pathway by 5-aza-2'-deoxycytidine inhibits cell proliferation, induces pro-apoptotic genes and mitogen-activated protein kinases in human prostate cancer cells. Int J Oncol 2005;26(4):863-71.
41. Goffin J, Eisenhauer E. DNA methyltransferase inhibitors-state of the art. Ann Oncol 2002;13(11):1699-716.
42. Thibault A, Figg WD, Bergan RC, Lush RM, Myers CE, Tompkins A, et al. A phase II study of 5-aza-2'deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. Tumori 1998;84(1):87-9.
43. Sasakawa Y, Naoe Y, Inoue T, Sasakawa T, Matsuo M, Manda T, et al. Effects of FK228, a novel histone deacetylase inhibitor, on tumor growth and expression of p21 and c-myc genes in vivo. Cancer Lett 2003;195(2):161-8.
44. Kuefer R, Hofer MD, Altug V, Zorn C, Genze F, Kunzi-Rapp K, et al. Sodium butyrate and tributyrin induce in vivo growth inhibition and apoptosis in human prostate cancer. Br J Cancer 2004;90(2):535-41.
45. Dokmanovic M, Marks PA. Prospects: Histone deacetylase inhibitors. J Cell Biochem 2005;96(2):293-304.
46. Gagnon J, Shaker S, Primeau M, Hurtubise A, Momparler RL. Interaction of 5-aza-2'-deoxycytidine and depsipeptide on antineoplastic activity and activation of 14-3-3sigma, E-cadherin and tissue inhibitor of metalloproteinase 3 expression in human breast carcinoma cells. Anticancer Drugs 2003;14(3):193-202.
47. Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, Davidson NE. Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. Cancer Res 2001;61(19):7025-9.
48. Bovenzi V, Momparler RL. Antineoplastic action of 5-aza-2'-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor beta and estrogen receptor alpha genes in breast carcinoma cells. Cancer Chemother Pharmacol 2001;48(1):71-6.
49. Belinsky SA, Klinge DM, Stidley CA, Issa JP, Herman JG, March TH, et al. Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. Cancer Res 2003;63(21):7089-93.
50. Ghoshal K, Datta J, Majumder S, Bai S, Dong X, Parthun M, et al. Inhibitors of histone deacetylase and DNA methyltransferase synergistically activate the methylated metallothionein I promoter by activating the transcription factor MTF-1 and forming an open chromatin structure. Mol Cell Biol 2002;22(23):8302-19.
51. Guerini V, Sau D, Scaccianoce E, Rusmini P, Ciana P, Maggi A, et al. The androgen derivative 5alpha-androstane-3beta,17beta-diol inhibits prostate cancer cell migration through activation of the estrogen receptor beta subtype. Cancer Res 2005;65(12):5445-53.
52. Kim IY, Seong do H, Kim BC, Lee DK, Remaley AT, Leach F, et al. Raloxifene, a selective estrogen receptor modulator, induces apoptosis in androgen-responsive human prostate cancer cell line LNCaP through an androgen-independent pathway. Cancer Res 2002;62(13):3649-53.
53. Korkmaz CG, Fronsdal K, Zhang Y, Lorenzo PI, Saatcioglu F. Potentiation of androgen receptor transcriptional activity by inhibition of histone deacetylation--rescue of transcriptionally compromised mutants. J Endocrinol 2004;182(3):377-89.
54. Baumann CT, Lim CS, Hager GL. Intracellular localization and trafficking of steroid receptors. Cell Biochem Biophys 1999;31(2):119-27.
55. Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res 1995;55(14):3068-72.

56. van der Kwast TH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CC, Mulder E, Boersma W, et al. Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int J Cancer 1991;48(2):189-93.
57. Navarro D, Luzardo OP, Fernandez L, Chesa N, Diaz-Chico BN. Transition to androgen-independence in prostate cancer. J Steroid Biochem Mol Biol 2002;81(3):191-201.
58. Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines--part 1. J Urol 2005;173(2):342-59.
59. Eder IE, Hoffmann J, Rogatsch H, Schafer G, Zopf D, Bartsch G, et al. Inhibition of LNCaP prostate tumor growth in vivo by an antisense oligonucleotide directed against the human androgen receptor. Cancer Gene Ther 2002;9(2):117-25.
60. Latil A, Bieche I, Vidaud D, Lidereau R, Berthon P, Cussenot O, et al. Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. Cancer Res 2001;61(5):1919-26.
61. Ji Q, Liu PI, Elshimali Y, Stolz A. Frequent loss of estrogen and progesterone receptors in human prostatic tumors determined by quantitative real-time PCR. Mol Cell Endocrinol 2005;229(1-2):103-10.

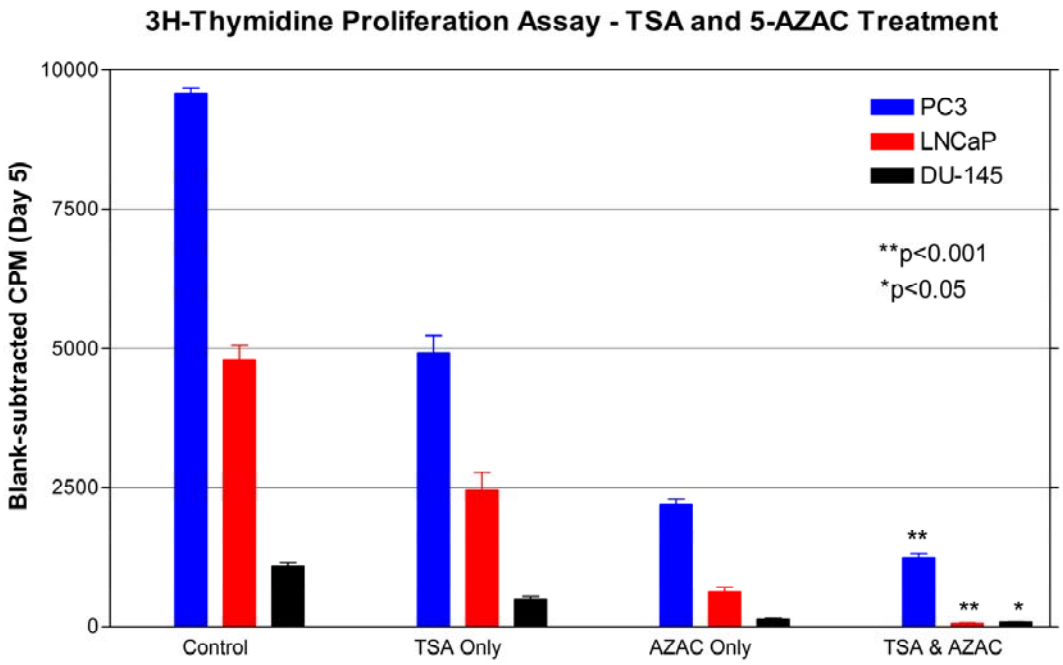

Figure 1 ^{3}[H]-thymidine uptake following treatment with TSA and 5-AZAC in prostate cancer cell-lines
In all cell-lines single-drug treatment was associated with a reduction in cell proliferation, with 5-AZAC displaying a greater magnitude of effect than TSA in all cases. Co-treatment was associated with significant reductions in cell proliferation than single-drug therapy (5-AZAC) for all cell lines ($p<0.001$ for LNCaP and PC-3; $p<0.05$ for DU-145).

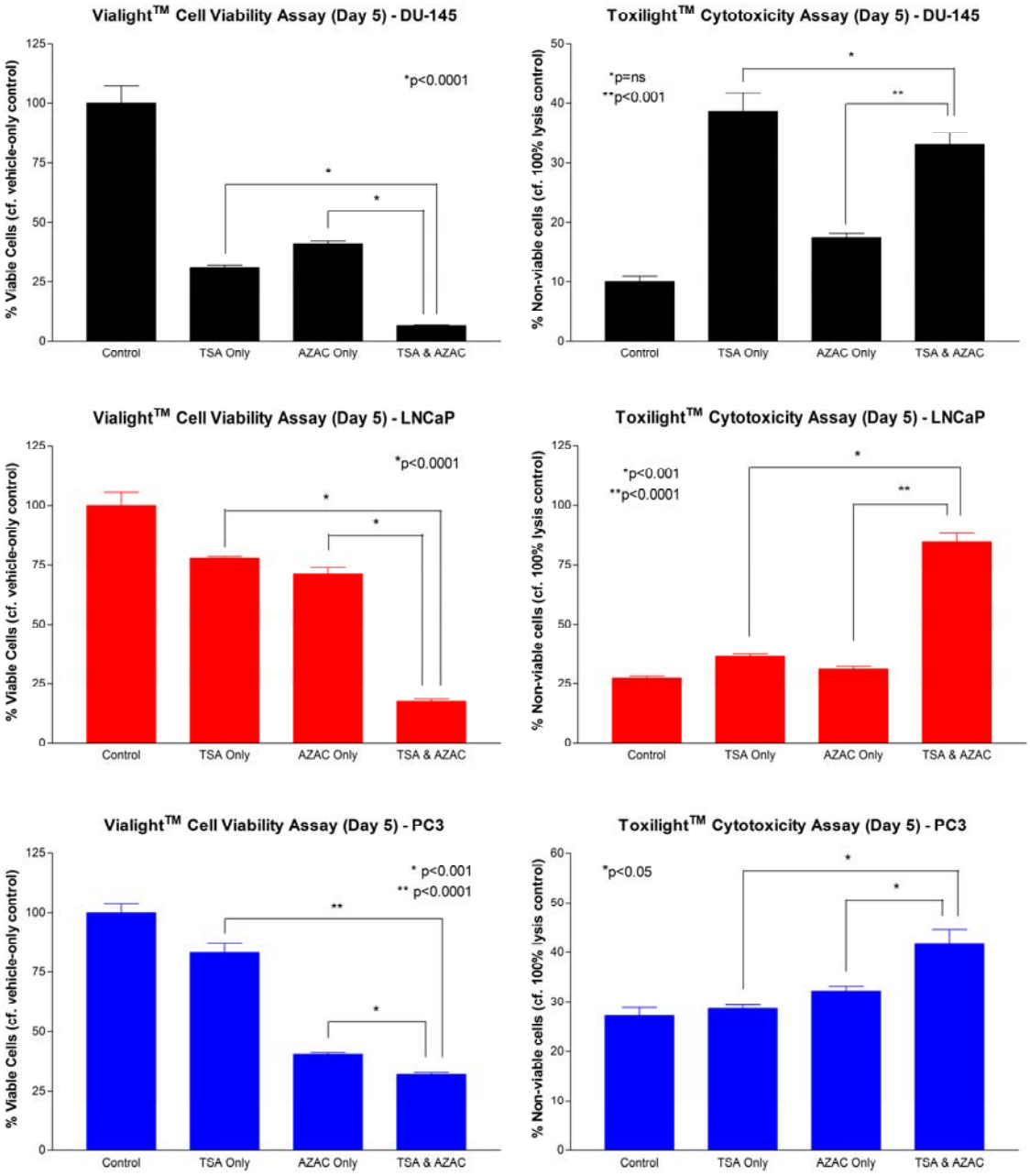

Figure 2 Cell viability and cytotoxicity following treatment with TSA and 5-AZAC in prostate cancer cell-lines
In all cell-lines single-drug treatment was associated with reduced cell viability. When TSA and 5-AZAC were compared, TSA induced a greater reduction in cell viability in DU-145 cells, whereas 5-AZAC was more potent in LNCaP and PC-3 cells. In all three cell-lines co-treatment was associated with significant falls in cell viability compared with the more effective single-drug treatment ($p<0.0001$ for DU-145 and LNCaP cells; $p<0.001$ for PC-3 cells). Co-treatment was also associated with significantly greater cytotoxicity in LNCaP and PC-3 cells when compared with the more effective single agent ($p<0.001$ and $p<0.05$ respectively). For DU-145 cells however TSA treatment appeared to be the main agent responsible for cell death

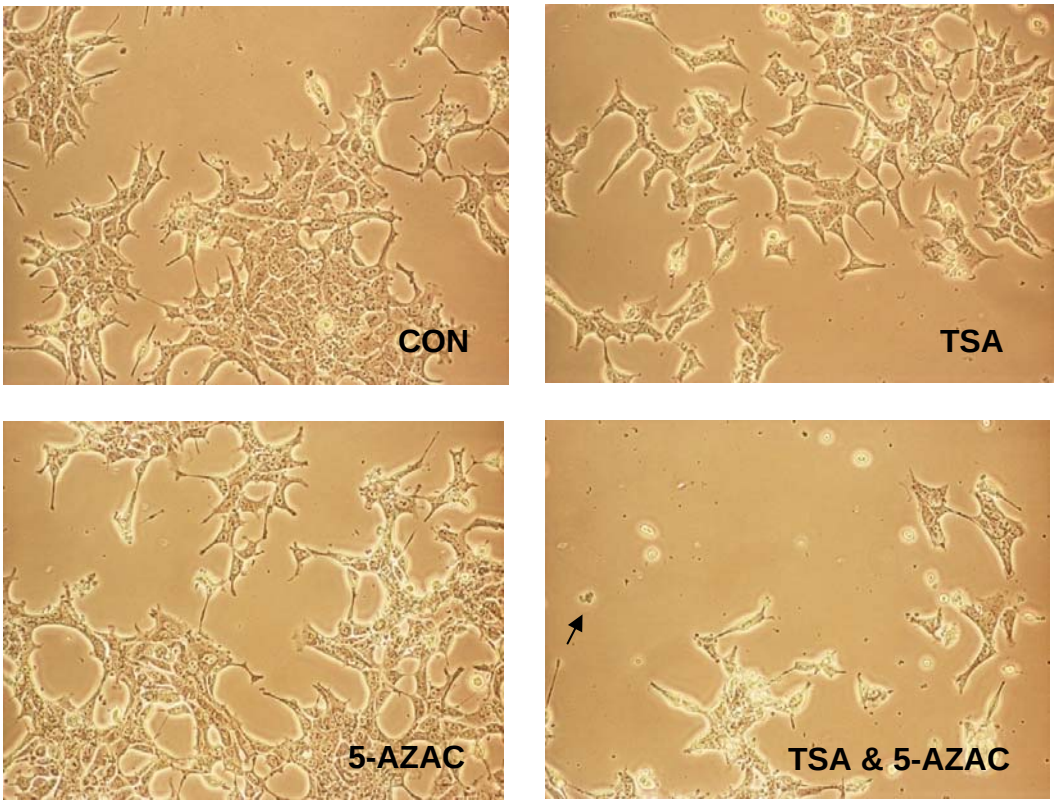

Figure 3 Visual appearance of LNCaP cells following treatment with TSA and 5-AZAC (Day 5)
In the control group there were high numbers of small confluent cells with few dead cells. Following TSA treatment, cell proliferation was reduced with a higher proportion of dead cells; cells were also larger and less confluent. Similar but less striking appearances were seen following 5-AZAC treatment. Combination treatment resulted in a further reduction in cell proliferation with a corresponding increase in dead cells. Commonly, the remaining adherent cells showed characteristic features of a apoptosis including cell shrinkage and membrane blebbing (arrowed).

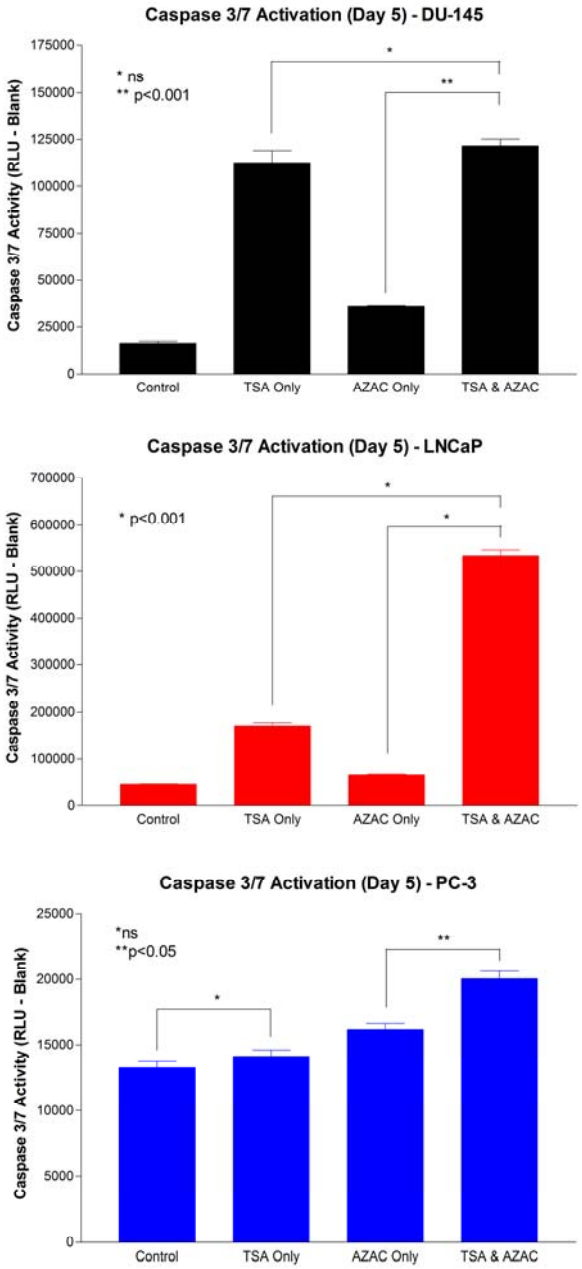

Figure 4 Apoptosis following treatment with TSA and 5-AZAC in prostate cancer cell-lines (day 5)
In all cell lines single-drug treatment was associated with increased apoptotic activity compared with the control group; both drugs achieved statistical significance in DU-145 and LNCaP cells ($p<0.001$), whereas only 5-AZAC treatment achieved significance in PC-3 cells. In DU-145 and LNCaP cells co-treatment was associated with significantly increased apoptotic activity when compared with the most effective single-drug treatment ($p<0.001$). In DU-145 cells however, there was no additional benefit of co-treatment over that of TSA alone.

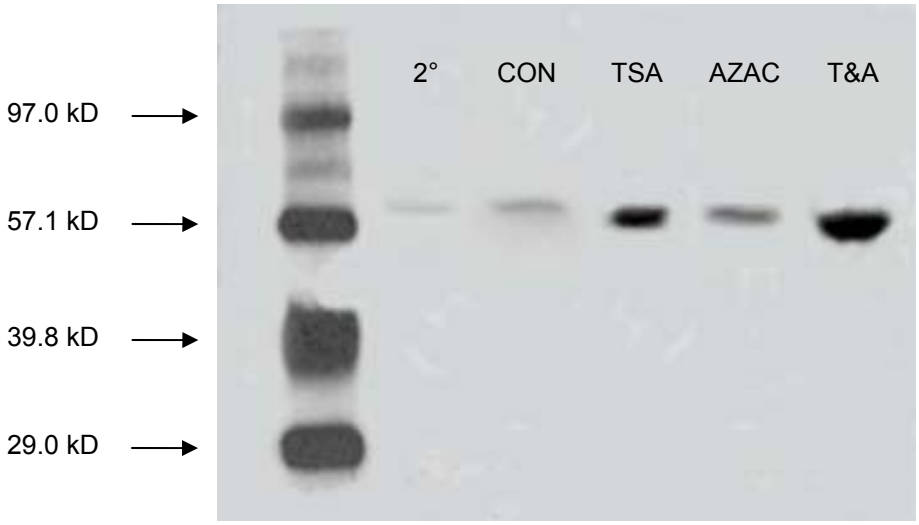

Figure 6 ERβ protein expression in LNCaP prostate cancer cells following 5-AZAC and TSA treatment
This figure shows expression of ERβ protein following treatment of LNCaP cells with TSA and 5-AZAC. Normalisation to total protein load and α-tubulin expression was performed, and a secondary-only control lane was included (2°). Low-level non-specific binding of the secondary-only lane was demonstrated, which we were unable to exclude. However, even accounting for this, ERβ expression was upregulated following single-drug treatment. The effect was more marked with TSA than with 5-AZAC. Co-treatment was associated with a marked increase in ERβ expression, which was greater than that seen with TSA treatment alone, mirroring results obtained for ERβ gene expression.

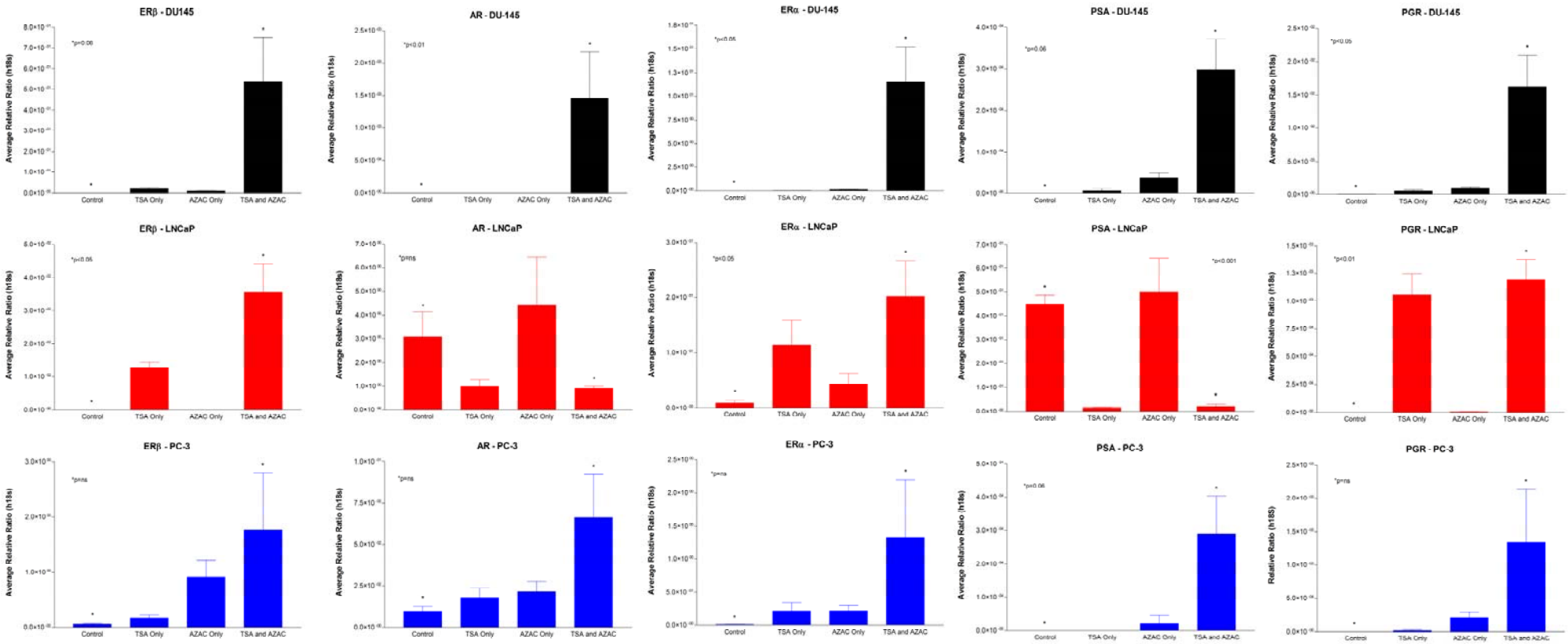

Figure 5 Selected ribosome h18S-normalised gene expression following co-treatment with TSA and 5-AZAC in prostate cancer cell-lines (Day 5)

Expression levels of ERβ increased in all three cell-lines following drug treatment, although when control and co-treatment groups were compared differences were only significant in LNCaP cells ($p<0.05$). A similar increase in DU-145 cells just failed to achieve statistical significance ($p=0.06$). Results obtained for AR and PSA were divergent for androgen-dependent AR-positive LNCaP cells, and androgen-independent AR-negative DU-145 and PC-3 cells. In the latter case, co-treatment was associated with increased AR expression, which achieved significance in DU-145 cells ($p<0.01$). In LNCaP cells co-treatment was associated with a reduction in AR expression which failed to reach significance. A significant reduction was demonstrated for AR-dependent PSA expression ($p<0.001$) however, which showed strong positive correlation with AR expression ($P<0.001$). In all cell-lines ERα expression increased with co-treatment, achieving significance in DU-145 and LNCaP cells ($p<0.05$) when compared with the control group. In LNCaP cells TSA treatment was associated with greater ERα re-expression than was seen following 5-AZAC treatment. Results obtained for ERα and PGR were closely related for all cell-lines, as confirmed by a significant positive relationship following pairwise correlation analysis ($p<0.001$).