PXD101 potentiates hormonal therapy and prevents the onset of castration-resistant phenotype modulating androgen receptor, HSP90, and CRM1 in preclinical models of prostate cancer

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Abstract

Aberrant activation or ‘reactivation’ of androgen receptor (AR) during androgen ablation therapy shows a potential cause for the development of castration-resistant prostate cancer. This study tested the hypothesis that PXD101, a potent pan histone deacetylase (HDAC) inhibitor, may prevent onset of castration-resistant phenotype and potentiate hormonal therapy. A panel of human prostate cancer cells with graded castration-resistant phenotype and in vivo models were used to verify this hypothesis. In this report, we demonstrated that hormonal manipulation favors the onset of castration-resistant phenotype increasing HDAC expression and activity as well as modulating expression and activity of AR, EGFR, HER2, and Akt. Consistent with these observations, the functional knockdown of HDACs by PXD101 prevented the onset of castration-resistant phenotype with a significant downregulation of AR, EGFR, HER2, and Akt expression/activity. The dysregulation of functional cooperation between HDAC6 with hsp90, on the one hand, and between GSK-3β with CRM1, on the other hand, may explain the biological effects of PXD101. In this regard, the HDAC6 silencing or the functional knockdown of hsp90 by 17AAG resulted in the selective downregulation of AR, EGFR, HER2, and Akt expression/activity, while the decreased phosphorylation of GSK-3β mediated by PXD101 increased the nuclear expression of CRM1, which in turn modified the AR and survivin recycling with increased caspase 3 activity. HDAC inhibitors retain the ability to prevent the onset of castration-resistant phenotype and, therefore, merit clinical application.

Key Words
- histone deacetylase
- epigenetic
- PXD101
- hormone refractory prostate cancer
- androgen receptor
Investigation in this setting. However, additional data are needed to develop clinical treatment strategies for this disease stage.

Introduction

PCa is a hormonally regulated malignancy in which the androgen receptor (AR) plays an important role both in disease development and in tumor progression. While most efforts in the clinic are still directed at lowering levels of androgens that activate AR, resistance to androgen deprivation eventually develops, and most PCa deaths are attributable to this castration-resistant prostate cancer (CRPC) form of this disease. Analysis of clinical CRPC revealed that over 90% express elevated levels of AR and androgen-response genes, indicating that the AR remains active and suggesting that AR is inappropriately activated in the absence of or at castration levels of testicular and adrenal androgens (Sharifi 2010, Lonergan & Tindall 2011, Shiota et al. 2011, Wang & Tindall 2011). Four mechanisms have been postulated to account for aberrant AR activation in CRPC: i) activation of AR by non-steroids via deregulated signals, ii) genetic mutations of AR, rendering the receptor hyperactive, iii) amplification or overexpression of AR and its coactivators, which sensitizes cells toward a low level of androgen, and iv) the increase in intracrine androgen. In addition, constitutively active AR variants lacking the ligand-binding domain may promote the development of CRPC (Hörnberg et al. 2011). Recent work has shed light on the importance of epigenetic events including facilitation of AR signaling. Histone modifications are the major driving force for epigenetic gene regulation.

Histone deacetylases (HDACs) are a group of co-repressors of transcriptional activators, including AR (Welsbie et al. 2009). It has also been demonstrated that HDAC1 is overexpressed in 35% of prostate cancer, in metastatic tumors, and in androgen-independent cell lines (Patra et al. 2001, Halkidou et al. 2004a,b). In addition, the acetylation of AR is necessary for co-activator binding to AR as well as reduced co-repressor binding that promotes aberrant cell growth (Korkmaz et al. 2004). HDAC2 had been found to be increased in about 30% of CaP patients (Zhao et al. 2004). HDAC4 was also overexpressed in 23% of prostate cancer specimens (Wang et al. 2009) whereas an altered intracellular localization of HDAC4 in prostate cancers was described in prostate cancer that has become refractory to hormone treatment (Halkidou et al. 2004a,b), suggesting that HDAC4 may be involved in the late events of prostate cancer progression. HDAC5 expression was increased in 57% of prostate cancer specimens (Wang et al. 2009) and this enzyme is able to associate with known AR co-repressors such as SMRT and N-CoR (Fischle et al. 2002), playing a crucial role in prostate cancer tumorigenesis.

HDAC inhibitors (HDACi) are a relatively novel class of relatively specific anticancer drugs that were originally identified by their capacity to reverse the transformed phenotype. As a result of HDAC inhibition, histones become hyper acetylated and DNA is maintained in a relatively open conformation that is conducive to interaction with transcription factors. Consistent with this scenario, HDACi have been shown to alter the transcription of a number of genes and mediate tumor cell differentiation, growth inhibition, and death. A number of HDACi are in clinical trials.

Guided by these data, we studied a novel hydroxamate, PXD101, that inhibits HDAC activity in cancer cell lysates with IC_{50} (nM) potency (Plumb et al. 2003, Qian et al. 2006, Gravina et al. 2011a,b). Specifically, using castrated-resistant LnCaP cell derivatives (104R, 104S, and C-81) and in 22rv1 bicalutamide-resistant cells generated in our laboratory (Gravina et al. 2011a,b), we performed in vitro and in vivo experiments to investigate the relationship between HDAC expression/activity and PCa progression to androgen-independent phenotype. Here, we demonstrated that PXD101 prevented the onset of castration-resistant phenotype affecting AR signaling and other related pathways such as Akt, EGFR, and Her2. Additionally, the dysregulation of functional cooperation between HDAC6 with hsp90, on the other hand, may explain the biological properties of this HDACi.

Materials and methods

Reagents

All the materials for tissue culture were purchased from Hyclone (Cramlington, NE, USA). Antibodies when not otherwise specified were purchased from Santa Cruz.
Plasticware was obtained from Nunc (Roskilde, Denmark). PXD101 was a trademark of TopoTarget A/S (København Ø, Denmark) and kindly provided by TopoTarget. Bicalutamide was obtained from AstraZeneca. 17-Allylamino-demethoxygeldanamycin (17AAG) was purchased from Sigma–Aldrich. KP7-6 was purchased from Merck. KgaA and Z-IETD-FMK was purchased from Enzo Life Sciences GmbH (Lorrach, Germany).

Cell lines
LnCaP and 22rv1 cell lines were originally obtained from American Tissue Culture Collection (Rockville, MD, USA). The LnCaP sublines (LnCaP-104-S, LnCaP-104-R1, and LnCaP-C-81; Kokontis et al. (1991, 1998) and Karan et al. (2002)) were kindly provided by John M Kokontis (University of Chicago, IL, USA) and Min-Fong Lin (University of Nebraska, Omaha, NB, USA). The LnCaP cell derivative C81 was developed by culturing parental cells (LnCap) for 81 passages in the absence of androgens (Karan et al. 2002). The obtained cell line expressed the same level of LnCap in terms of functional ARs but with different responsiveness to androgen stimulation. This model resembles some biological characteristics of an androgen-independent phenotype seen in prostate cancer patients. C4-2B cells, a metastatic and androgen-insensitive LnCaP subline, derived from orthotopic injection of C4-2 (Lin et al. 2001), were kindly provided by Dr Cecchini from University of Berne. As a further model to study progression from androgen dependence to androgen independence, the androgen-independent human prostate cancer cell line, LnCap 104-R1, derived from androgen-dependent LnCap 104-S cells after long-term androgen deprivation was used. The growth of LnCap 104-R1 cells in vitro does not require androgen but it is instead suppressed by physiological concentrations of androgen. Additional AR protein levels increase during the progression from androgen-dependent LnCap 104-S cells to androgen-independent LnCap 104-R1 cells. BCLT-resistant 22rv1 PCa cell line was generated upon BCLT 5 µM + dihydrotestosterone (DHT) (10−12 M) treatment as already described (Gravina et al. 2011a,b).

Cell proliferation inhibition assay
Cell proliferation was evaluated by [3H]thymidine uptake. To quantify the proliferation rate of tumor cells during the generation of bicalutamide-resistant 22rv1 cells (22rv1-BCLTR) as well as upon different treatments, proliferation studies were conducted by measuring the uptake of [3H]thymidine. In order to exclude that decrease in thymidine uptake could be imputable to apoptotic cells adherent cells were collected and total and viable cells were counted using the NucleoCounter NC-100 (automated cell counter systems; Chemotec, Cydevang, Denmark). Viable cells were titrated at 1×10⁶ cells/ml and 200 μl of suspension, containing 2×10⁴ viable cells, plated in 24-well plates grown for 48 h in medium containing 10% CS-FCS. Next, cultures were exposed to treatments for 72 h and pulsed with [3H]thymidine (1 μCi/well) for 4–6 h, fixed (5% trichloroacetic acid), and solubilized (0.5 M NaOH) before scintillation counting. To determine the effect of the combined drug treatments, any strengthening was estimated by multiplying the percentage of cells remaining (% growth) for each agent.

HDAC activity assay
HDAC activity was evaluated by a colorimetric HDAC activity assay kit (Enzo Life Sciences GmbH) in nuclear extracts of cells treated according to the treatment protocols and according to the manufacturer’s instructions.

siRNA transfection
LnCap (1.5×10⁵ cells/well) and C81 tumor cells were plated in six-well plates and grown in phenol red-free DMEM containing 10% CSS for 2 days. These cells were transfected by HDAC6 and HDAC4 siRNA (h): sc-35544 and sc-35541 respectively (Santa Cruz Biotechnology). Two further HDAC6 and HDAC4 siRNAs were used to minimize the off-target effects: H00010013-R01 and H00009759-R01 respectively. All siRNA duplexes were transfected using Oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions. After HDAC silencing, cells were grown in phenol red-free DMEM containing 10% CS-FCS for 48 h and then treated with DHT (10−10 M) or vehicle for 72 h.

Analysis of apoptosis
Analyses of apoptosis were performed at different times of treatments collecting adherent and suspension cells from cultures and analyzing apoptosis by FACS. After the appropriate treatments, cells (1×10⁶) were fixed for 30 min in 70% ethanol and pelleted by centrifugation (720 ×g; 5 min). After removal of ethanol, cells were incubated and resuspended in 1 ml DNA-staining solution (PBS containing 200 mg/ml RNase A, 20 mg/ml propidium iodide plus 0.1% Triton X-100) and left at room temperature.
temperature for 60 min. Apoptosis was analyzed using Annexin V staining (GenScript, Piscataway, NJ, USA). All cells were then measured on a FACSscan flow cytometer (FACScan; Becton Dickinson, San Jose, CA, USA) with an argon laser at 488 nm for excitation and analyzed using Cell Quest Software (Becton Dickinson, Mountain View, CA, USA). Apoptotic cells were detected by the percentage of Annexin V-stained cells. The results were expressed as the percentage of death by apoptosis induced by a specific treatment. Experiments were performed in triplicate.

Western blot analysis

Cells and frozen tissues were washed with cold PBS and immediately lysed with 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM p-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Lysates were electrophoresed in 7% SDS–PAGE, and separated proteins were transferred to nitrocellulose and probed with the appropriate antibodies using the conditions recommended by the antibody suppliers.

In vivo experiments

Castrated and intact male CD1 nude mice (Charles River, Milan, Italy) were kept in line with University guidelines (University of L’Aquila, Medical School and Science and Technology School Board Regulations, in compliance with Italian government regulation no. 116 of January 27, 1992 on the use of laboratory animals). Before any invasive manipulation, mice were anesthetized with a ketamine (25 mg/ml)/xylazine (5 mg/ml) mixture. All mice received s.c. flank injections of 1×10⁶ in 250 μl of 12 mg/ml Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) of 22Rv1 and C4-2B cells. Treatments were started when a mean tumor volume of about 80 mm³ was reached. Mice were randomly maintained in a group of ten animals assigned to receive i.p. injections of 100 μl PBS or PXD101 (20 and 40 mg/kg bid) for 3 consecutive weeks (5 days/week). Three groups of intact male nude mice received 50 mg/kg per day BCLT by oral gavage for consecutive 21 days alone or in combination with i.p. injections of 100 μl PXD101 (20 and 40 mg/kg bid) for 3 consecutive weeks (5 days/week). PXD101 (20 and 40 mg/kg bid) was also administered in pre-castrated nude mice. The effects on tumor growth of different treatments were evaluated by i) tumor volume measured during and at the end of experiment by biweekly measurement of tumor diameters with a Vernier caliper (length×width), expressed in mm³ according to the formula 4/3πr³ and ii) tumor weight, measured at the end of experiment. Experiments were stopped 30 days after tumor inoculation, mice were killed by carbon dioxide inhalation and tumors were removed surgically. Half of the tumor was directly frozen in liquid nitrogen for protein analysis and the other half was fixed in parafomaldehyde overnight for immunohistochemical analyses including angiogenesis (CD31; Dako, Glostrup, Denmark), proliferation index (Ki67; Dako), and apoptosis (APO-BrdU TUNEL Assay Kit, Invitrogen Ltd.). Indirect immunoperoxidase staining of tumor xenograft samples was performed on paraffin-embedded tissue sections (4 μm). Briefly, sections were incubated with primary antibodies overnight at 4 °C. Next, avidin–biotin assays were performed using the Vectastain Elite kit obtained from Vector Laboratories (Peterborough, UK). Mayer’s hematoxylin was used as a nuclear counterstain. Negative controls were incubated only with universal negative control antibodies under identical conditions, processed, and mounted. Images of the stained blood vessels were taken using a Leitz photo microscope. Positive tumor microvessels were counted at ×400 in five arbitrarily selected fields/tumor and the data are presented as number of CD31+ microvessels per microscopic field at 100× magnification for each group. Ki67 labeling index was determined by counting 500 cells at 100× and determining the percentage of cells staining positively for Ki67.

Statistical analysis

Continuous variables were summarized as mean and s.d. or as median and 95% CI for the median. For continuous variables not normally distributed, statistical comparisons between control and treated groups were established by carrying out the Kruskal–Wallis tests. When Kruskal–Wallis tests revealed a statistical difference, pair-wise comparisons were made by Dwass-Steel-Critchlow-Fligner method, and the probability of each presumed ‘non-difference’ was indicated. For continuous variables normally distributed, statistical comparisons between control and treated groups were established by carrying out the ANOVA test or Student’s t-test for unpaired data (for two comparisons). When ANOVA test revealed a statistical difference, pair-wise comparisons were made by Tukey’s honestly significant difference test and the probability of each presumed ‘non-difference’ was indicated. Dichotomous variables were summarized by absolute and/or relative frequencies. For dichotomous variables, statistical
comparisons between control and treated groups were established by carrying out the exact Fisher's test. For multiple comparisons, the level of significance was corrected by multiplying the P value by the number of comparisons performed (n) according to the Bonferroni correction. P values <0.05 were considered statistically significant. Statistical analysis software package (SPSS) version 10.0 and StatsDirect (version 2.3.3., StatsDirect Ltd, Bonville Chase, Altrincham. Cheshire, UK) were used for statistical analysis and graphic presentation.

Results

Prolonged androgen deprivation and bicalutamide treatment upregulate HDAC activity and expression and correlates with the castration-resistant phenotype

The LnCaP and LnCaP sublines (LnCaP-104-S, -104-R1, -C-81, and -C4-2B) were chosen as well-known cellular models with graded levels of castration-resistant phenotype. Enzymatic and western blot analyses revealed that HDAC activity (Fig. 1A) and HDAC1, HDAC2, HDAC4, and HDAC6 levels (Fig. 1B) were significantly higher in androgen-independent LnCaP-104-R1, -C-81, and -C4-2B cell sublines than in androgen-dependent LnCaP and LnCaP-104-S cells (P<0.05). Thus, the acquisition of an androgen-independent phenotype was accompanied by the upregulation of HDAC activity and expression in androgen-independent LnCaP derivatives. Next, we evaluated the expression levels and the activation status of AR-related pathways such as Akt, EGFR, and Her2 (Fig. 1C) in LnCaP derivatives. These experiments demonstrated that androgen-independent LnCaP cell derivatives showed higher levels of AR, EGFR, p-EGFR, Her2, p-HER2 p-Akt ser473, and thr308 and this paralleled with upregulation of HDAC activity and expression. Next, we observed that, in the androgen-dependent LnCap and LnCap104-S cells, HDAC activity was significantly decreased by DHT treatment in a dose-dependent manner (P<0.05) with respect to the same cells cultured in CS-FCS. Differently, HDAC activity was unchanged in androgen-independent LnCap C-81 and LnCap C4-2B cells (Fig. 1D). BCLT significantly increased HDAC activity in LnCap and LnCap 104-S with respect to the same cells cultured in DHT (P<0.05) and CS-FCS (P<0.05) while its activity was unchanged in androgen-independent LnCap C-81 and LnCap C4-2B cells (Fig. 1E). These findings indicate that in androgen-dependent tumor models, DHT decreases and BCLT or CS-FCS (androgen deprivation condition) increases HDAC activity respectively, although the most evident effects were found upon BCLT treatment. On the contrary, in models that are already androgen independent, no significant modulation of HDAC activity was found and this evidence confirmed the finding that LnCap C-81 and LnCap C4-2B sub-clones are insensitive to DHT and BCLT treatment (Kokontis et al. 1998, Gravina et al. 2011a,b). The correlation between HDAC activity and expression levels with the BCLT-resistant phenotype was then further examined. For this purpose, the AR-positive androgen-independent but BCLT-sensitive 22rv1 cell line was continuously treated with BCLT and/or DHT for 60 weeks as described previously (Gravina et al. 2011a,b). Over the time, BCLT treatment gradually and significantly increased the enzymatic activity (P<0.05, one-way ANOVA for linear trend) of these deacetylases. The modulation of enzymatic activity was associated with a significant increase in HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6 expression (Fig. 2B). Recent reports indicate that alternatively spliced, truncated isoforms may support androgen-independent phenotype. As shown in Fig. 2C, the expression levels of 75 kDa isoform were greatly increased upon BCLT treatment with a concurrent decrease in the levels of 110 kDa isoform. Increasing concentrations of BCLT or the culture of tumor cells in androgen-depleted medium (CS-FCS) significantly decreased 22rv1 proliferation rate (week 0) with respect to cell cultured a medium supplemented with DHT (P<0.05). Interestingly, cell growth in an androgen-depleted medium or continuously treated with BCLT acquired less responsiveness to the growth-inhibiting action of BCLT (Fig. 2D) over the time. This effect started from the 8th week of treatments as attested by the evidence that neither BCLT treatment nor androgen-depleted culture conditions significantly affected the growth rate of tumor cells (Fig. 2D). This effect, in terms of hormonal manipulation, paralleled with the increased expression of HDAC2, HDAC3, HDAC4, and HDAC6, which started from the 4th or 8th week of BCLT treatment (Fig. 2B).

The development of castration-resistant phenotype was prevented by PXD101 in 22rv1 prostate cancer cell line in vitro

The effects of sub-cytotoxic concentration (0.2 μM) of PXD101 were studied in terms of development of castration-resistant phenotype. 22rv1 tumor cells were cultured for 60 weeks in medium supplemented with i) 10% CS-FCS, ii) BCLT (5 μM)+DHT (10−12 M), iii) PXD101 (0.2 μM), or iv) BCLT (5 μM)+DHT.
Figure 1

(A) HDAC activity in LnCap derivatives; (B) western blot of HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6 levels in LnCap derivatives; (C) increased HDAC activity and expression was coupled with the activation of AR, AKT, EGFR, and HER2 signaling pathways; HDAC activity in LnCap derivatives upon increasing concentrations of DHT ($10^{-12}$ to $10^{-10}$ M) (D) or BCLT (5 μM) in the presence of DHT ($10^{-10}$ M) (E). For each experiment, 40 μg total proteins were loaded and analyzed by western blot. Bars represent means of five replicates and are representative of three independent experiments.
The literature indicates that at different time points during the generation of 22rv1-decreased both AR isoform levels in 22rv1 cells examined (Fig. 3D). Under these culture conditions, PXD101 mainly and in 22rv1 cells treated for 2, 8, and 22 weeks with BCLT dependent (LnCaP) and -independent (LnCaP sub-clones) decreased AR expression both in AR-positive/androgen- 

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The acquisition of a more aggressive phenotype of 22rv1 cells in vitro upon BCLT 5 µM + DHT (10\textsuperscript{−12} M) treatment paralleled with (A) increased HDAC activity, (B) increased HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6 expression levels, as well as (C) increased AR expression levels. (D) Tumor cells cultured in androgen-depleted medium or continuously treated with BCLT acquired less responsiveness to the growth-inhibiting action of BCLT (10\textsuperscript{−12} M) DHT (10–12 M) treatment paralleled with (A) increased HDAC activity, (B) increased HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6 expression levels, as well as (C) increased AR expression levels. (D) Tumor cells cultured in androgen-depleted medium or continuously treated with BCLT acquired less responsiveness to the growth-inhibiting action of BCLT (10\textsuperscript{−12} M) + PXD101 (0.2 µM). As shown in Fig. 3A, the co-administration of PXD101 with BCLT prevented the onset of the castration-resistant phenotype with a significant decrease in the tumor cell growth rate with respect to BCLT treatment (P < 0.001). This effect was more sustained starting from the 8th week of culture. A similar trend was observed in terms of number of apoptotic events over the time as attested by [3H]thymidine uptake. Each western blot lane was loaded with 40 µg and the values of fold increases over the controls, arbitrarily set at 1, were obtained by densitometric analysis. Densitometric analyses are representative of three independent experiments. Bars represent means of five replicates and are representative of three independent experiments.

Figure 2

The acquisition of a more aggressive phenotype of 22rv1 cells in vitro upon BCLT 5 µM + DHT (10\textsuperscript{−12} M) treatment paralleled with (A) increased HDAC activity, (B) increased HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6 expression levels, as well as (C) increased AR expression levels. (D) Tumor cells cultured in androgen-depleted medium or continuously treated with BCLT acquired less responsiveness to the growth-inhibiting action of BCLT (10\textsuperscript{−12} M) + PXD101 (0.2 µM). As shown in Fig. 3A, the co-administration of PXD101 with BCLT prevented the onset of the castration-resistant phenotype with a significant decrease in the tumor cell growth rate with respect to BCLT treatment (P < 0.001). This effect was more sustained starting from the 8th week of culture. A similar trend was observed in terms of number of apoptotic events (Fig. 3B). As shown in Fig. 3C, PXD101 dose dependently decreased AR expression both in AR-positive/androgen-dependent (LnCaP) and -independent (LnCaP sub-clones) and in 22rv1 cells treated for 2, 8, and 22 weeks with BCLT (Fig. 3D). Under these culture conditions, PXD101 mainly decreased both AR isofrom levels in 22rv1 cells examined at different time periods during the generation of 22rv1-BCLTR cells (Fig. 3D). The literature indicates that i) HDAC6 is able to modulate AR expression by posttranslational modifications in hsp90 activity (Bali et al. 2005, Kovacs et al. 2005) and that ii) AR transcriptional activity may be regulated by HDAC4 (Halkidou et al. 2004a,b). Therefore, in order to more deeply investigate whether AR pathway is modulated by HDAC6 and/or HDAC4, the selective knockdown of these deacetylases was performed in androgen-dependent (LnCaP) and -independent (C81) cells. In these experiments, HDAC6 silencing reduced AR, HER2, EGFR, and AKT expression levels both in LnCaP and C81 cells. Differently, HDAC4 silencing did not result in AR downregulation in both tumor models. The silencing of HDAC4 and 6 was coupled with a decrease in the expression levels of PSA, suggesting that these two epigenetic mediators have a role in inhibiting AR signaling (Fig. 4A and B). We further investigated whether HDAC6/HDAC4 silencing also affected androgen-responsive cell growth. For this purpose, HDAC6 and HDAC4 silencing was followed by 10\textsuperscript{−10} M DHT in a steroid-reduced condition. This protocol treatment was used to determine whether HDAC6 and HDAC4 silencing pre-treatment can increase the common androgen responsiveness of cell growth. In the presence of DHT, the growth of HDAC4/HDAC6-silenced C-81 cells increased significantly (column 6 vs 5, 14 vs 13, and 16 vs 15 Fig. 4C,
C81 cells, *P < 0.05) compared with no significant effect of controls and scramble. This body of evidence suggests that HDAC6 and 4 may not have a significant role in increasing androgen sensitivity in androgen-dependent models, although these deacetylases may mediate the antitumor effects of PXD101.

As our data indicate that HDAC6 silencing decreases AR expression and as the literature indicates that i) heat-shock protein 90 (hsp90) mediates AR protein stabilization (Gibbs et al. 2009) and ii) HDAC6 knockdown inhibits chaperone function of hsp90 (Bali et al. 2005), the modulation of hsp90 client proteins was studied upon 17AAG treatment, an agent able to inducing proteasomal degradation of hsp90 client proteins (Saporita et al. 2007, Chandarlapaty et al. 2008). In Fig. 4D, we demonstrated that AR, Her2, EGFR, and Akt expression was reduced in the LnCaP and LnCaP-C81 cells in a time-dependent manner after 72 h of treatment with 17AAG treatment (10 μM, panel D). Hsp90 inhibition potentiated the PXD101 (0.5 μM) effects in terms of AR, HER2, EGFR, and Akt expression levels.

To verify whether PXD101 was able to modify the sensitivity vs hormonal manipulation, this HDACi was administered alone or in combination with DHT (Fig. 5A) and/or BCLT (Fig. 5B) to androgen-independent LnCaP (C81 and C4-2B) cell derivatives. Single-agent BCLT had minimal antitumor effects on prostate cancer cells with no significant with respect to CS-FCS. The co-administration of PXD101 with BCLT and/or DHT restored the sensitivity vs these two treatments (Fig. 5A and B). Additionally, co-treatment with different concentrations of PXD101 (0.2–1.0 μM) and BCLT (5 μM) induced in LnCaP-C81 cells: i) an increment of p27 and p16 expression with a concomitant p21 decrease was
Two different HDAC6 and HDAC4 siRNA-specific preparations were used as described in the Materials and methods section in order to minimize the impact of off-target effects. LnCap and LnCap-C81 cells were incubated in phenol red-free DMEM containing 10% CSS for 48 h and then treated with 17AAG (0.5 μM) or vehicle for 72 h. AR, Her2, EGFR, and Akt expression levels upon PXD101 (0.5 μM) or PXD101 (0.5 μM) and/or BCLT treatments were studied in LnCaP and LnCaP-C81 cell lines by western blot. Each western blot lane was loaded with 40 μg protein for LnCap and LnCap-C81 cells by western blot (A and B). After silencing, LnCap and C81 cells were cultured for 72 h in a medium supplemented with i) 10% CS-FCS for 48 h and then treated with 17AAG (10 μM) and/or PXD101 (0.5 μM) or vehicle for 72 h. AR, Her2, EGFR, and Akt expression levels upon PXD101 treatment were measured over time (C). The involvement of hsp90 in AR down-modulation, HDAC6 mediated, was studied in LnCaP and LnCaP-C81 cells using a specific inhibitor, the 17AAG (D). Tumor cells were grown in DMEM containing 10% CS-FCS for 48 h and then treated with 17AAG (10 μM) or vehicle for 72 h. AR, Her2, EGFR, and Akt expression levels upon 17AAG and/or PXD101 treatments were studied in LnCaP and LnCaP-C81 cells by western blot. Each western blot lane was loaded with 40 μg total proteins. These experiments are representative of three individual experiments. Bars represent means of five replicates and are representative of three independent experiments.

AR expression and androgen sensitivity upon Pxd101 treatment were mediated by GSK-3β and CRM1 activity

PXD101 and BCLT co-treatment significantly reduced the apoptotic effects of the co-treatment between PXD101 with BCLT in LnCaP-C81 (P <0.05; treatments 12 vs 10) and 14 vs 10) (Fig. 6B) cell lines. In addition, the partial role of death receptor pathways upon BCLT and PXD101 co-treatment was also demonstrated using a caspase 8 inhibitor Z-IETD-FMK (treatment 14 in Fig. 6B). As for FasL blocking antibody (Nok-1) or Fas inhibitor Kp7-6, the addition of caspase 8 inhibitor significantly reduced the apoptotic events induced by co-treatment between BCLT and PXD101 (P <0.05; treatment 16 vs 10) (Fig. 6B).
phosphorylation status suggesting that PXD101 may reduce the activation of mediators responsible for the sensitivity to BCLT. In Fig. 6C, we show the effects of combined treatments on AKT signaling pathway on LnCaP, C81, and 22r1-BCLTR cellular models. The phosphorylation of GSK-3β was greatly reduced in LnCaP-C81 and 22r1-BCLTR after co-treatment between PXD101 and BCLT (Fig. 6D). The reduced phosphorylation of this kinase was also associated with an increased activity of GSK-3β, which, in turn, determined an increased nuclear expression of CRM1, a protein involved in nuclear export of AR and different proteins involved in cell cycle progression (cyclin D1 and cyclin inhibitors such as p21 and p27), and apoptosis (survivin, Foxo family members). Specifically, PXD01 resulted in loss of AR cytoplasmic recycling (Fig. 6D) and this was responsible for increased anti-proliferative effects of BCLT. In addition, the reduction of CRM1 expression/activity determined an increased nuclear localization of survivin.

**In vivo effects of PXD101 in combination with castration or bicalutamide treatment**

It has been demonstrated that PXD101 possessed anti-tumor effects in vivo in prostate cancer cell models (Qian et al. 2008). In this report, we studied the in vivo effects of PXD101 under castration condition or bicalutamide treatment. As described in Fig. 7A, androgen-independent 22r1 and C4-2B cell lines were subcutaneously injected in male nude mice. Bi-daily administration of PXD101 (20 or 40 mg/kg) in intact nude male mice resulted in a statistically significant tumor volume decrement with respect to mice treated with vehicle alone was found at day 30 (P < 0.001) in both xenograft models (Fig. 7B and D).
However, the antitumor effect of PXD101 was more evident when it was given at the dose of 40 mg/kg PXD101 with a difference statistically significant with respect to the lower dose (20 mg/kg) ($P<0.001$). When PXD101 was administered to bicalutamide- or castrated-treated mice, its effect, on tumor volume at day 30, was more evident as the dose was increased with the best effect found in combination with BCLT ($P<0.001$; Fig. 7B and D). PXD101 also determined a dose-dependent increment in tumor growth delay (TGD) and a reduction of proliferation index and vessel count in both models. Castration had minimal effects on tumor growth of 22rv1 xenografts whereas a significant reduction of tumor volume (36.3%) was observed in association with BCLT (50 mg/kg daily).

Figure 6
(A) The LnCap-C81 androgen-independent cell line was cultured in phenol red-free DMEM containing 10% CS-FCS for 2 days. After this time point, (A) DHT (10$^{-10}$ M) and/or PXD101 (1.0 $\mu$M) and/or BCLT (5 $\mu$M) were added to medium for 72 h. FasL, FAS, p22, p15, c-FLIP$_L$, c-FLIP$_S$, and survivin expression levels were measured. (B) The C81 androgen-independent cell line was cultured in phenol red-free DMEM containing 10% CS-FCS for 2 days. After this time point, DHT (10$^{-10}$ M) or BCLT (5 $\mu$M) was added to the medium for 72 h. Nok-1 (10 $\mu$g/ml), Kp7-6 (1 $\mu$g/ml), Z-IETD-FMK (10 $\mu$M), and PXD101 (1.0 $\mu$M) were added to the medium in the presence of DHT (10$^{-10}$ M) or BCLT (5 $\mu$M) for 48 h. The percentage of Annexin V-positive/propidium-stained cells (apoptotic cells) was measured in C81 cell line. (C) The expression levels of p-AKT (Thr 308), p-AKT (Ser 473), total AKT, p-EGFR, EGFR, p-HER2, and HER2 were studied in LnCaP, C81, and C4-2B cells by western blot. Each western blot lane was loaded with 40 mg total proteins. (D) p-GSK3$\beta$, GSK3$\beta$, cytoplasmic AR, nuclear AR, CRM1, and survivin were studied in LnCaP, C81, and C4-2B cells by western blot. Each western blot lane was loaded with 100 $\mu$g proteins and normalized vs actin or vs SP1 (nuclear extracts). These experiments are representative of three individual experiments. Bars represent means of five replicates and are representative of three independent experiments.
The proliferation index was significantly lower (33.0%) when compared with those observed (47.4%) in intact nude mice \( (P<0.001) \). Apoptosis was low in 22rv1 tumors grown both in intact and in castrated nude mice whereas 11% of apoptotic cells were present in mice receiving BCLT. Moreover, combined treatments between PXD101 and castration or PXD101 and BCLT were associated with TGD increments, reduction in the proliferation indices, significant increases in the number of apoptotic cells, and reduction in vessel counts. Upon castration or BCLT treatment, no complete response was found whereas by adding PXD101 determined a dose-dependent increment in the number of tumor-free mice \( (P<0.05) \). When we considered C4-2B cells xenografted in intact mice, we find that PXD101 alone induced more than 40% reduction of tumor mass with respect to controls without castration or bicalutamide \( (P>0.001) \) whereas the reduction in the presence of bicalutamide was about 70% \( (P<0.0001; \text{Fig. 7C, Tables 1 and 2}) \). In addition, tumor-free mice were 0 out of 12 in intact nude mice, two out of ten in PXD101-treated (40 mg/kg) mice, and five out of ten in intact nude mice receiving a combination of bicalutamide and PXD101.

Discussion

Understanding the mechanisms that transform PCa into a castration-resistant state has enabled investigators to explore critical pathways involved in such process allowing for rational therapeutic design. Because functional AR signaling is necessary for the development of prostate cancer, deprivation of androgens remains the mainstay therapy. Unfortunately, resistance to androgen ablation therapy inevitably occurs. Many approaches have been proposed to improve the treatment outcome of this disease, but advanced prostate cancer patients still have a dismal prognosis. Herein, we suggest that PCa may acquire androgen independence upon hormonal therapy through HDAC expression/activity up-modulation as well as by AR overexpression. In this report, we also showed that alternative truncated AR isoforms may be one of the means to diversify its signaling and confer androgen-independent phenotype. We show that there is a time-dependent increase in the expression levels of full and truncated AR upon long-term BCLT treatment. It is possible that the tumor cells use the truncated AR isoforms to escape from hormonal therapy and the aberrant expression of the constitutively active truncated AR isoforms may contribute to ablation-independent growth \( (\text{Hörnberg et al. 2011}) \). Increased histone deacylation highlights a potential role for HDAC as potential molecular targets in prostate cancer therapy \( (\text{Perry et al. 2010}) \) as it has been demonstrated a strengthening of AR transcriptional activity as well as reduction of AR expression by inhibition of histone deacylation \( (\text{Frigo & McDonnell 2008}) \). In LNCaP sub-clones selected for increased androgen independence in androgen-depleted medium, HDAC expression, particularly of HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6, increased in proportion to androgen independence. The relationship between HDACs and BCLT treatment was studied \textit{in vivo} using a 22rv1-BCLT-R cell line selected for resistance to BCLT. This model offered the opportunity to study the interaction between HDACs and anti-androgen response in a tumor environment closer to that of clinical patients. Cell lines that had lost the anti-androgen response were more aggressive than the parent cell line and more rapidly gave rise to increased tumor mass upon BCLT treatment \( (\text{Gravina et al. 2011a,b}) \). Increased vascularity, decreased tumor suppressor expression \( (\text{PTEN}) \), and activation of survival signal transduction pathways \( (\text{AKT and ERK}) \) were found in xenografts from anti-androgen unresponsive cell lines \( (\text{Gravina et al. 2011a,b}) \). Collectively, these results, which are comparable to those obtained in the cultured cell lines, support the findings on the role of BCLT in prostate cancer progression. Similarly, upon long-term treatment with BCLT, 22rv1 was induced to increase the levels of HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6. Although the reduction of AR levels is often associated with increased expression of neuroendocrine markers, HDAC inhibition using different classes of HDACi \( (\text{TSA, SAHA, and valproic acid}) \) have significant therapeutic effects both in androgen-sensitive and -insensitive prostate cancer cells \( (\text{Frønsdal et al. 2005, Welsbie et al. 2009, Chou et al. 2011}) \). Consistent with the idea that hormonal therapy might affect HDAC expression/activity in prostate cancer cells, treatment with PXD101 recovered the anti-proliferative responses of BCLT-chronically treated cell lines. PXD101 was, indeed, able to reduce the insurgence of bicalutamide resistance in 22rv1 chronically cultured with this drug. The results from PCa cells demonstrated that BCLT-mediated changes in protein acetylation status may play a major role in preventing the action of anti-androgen therapy. The possible benefit of PXD101 treatment in androgen-independent prostate cancer cell lines is demonstrated by their increased apoptosis when cultured in the presence of this drug. However, it is possible that the efficacy of PXD101, in recovering the antitumor response under BCLT treatment or upon androgen deprivation (culture in CS-FCS), may
be rather different irrespective of the fact that these two treatments, *per se*, may effectively increase HDAC activity and expression and so result in an androgen-independent phenotype. The reason why we found a discrepancy in terms of anti-proliferative and pro-apoptotic effects when PXD101 is added to a medium supplemented with CF-FCS (androgen deprivation culture condition) or when this inhibitor acts together with BCLT may be explained...
considering that prostate cancer cells may use different mechanisms to compensate for low androgen levels.

In addition, we demonstrated that PXD101 was able to reduce AR expression in AR-positive CRPC cell lines and this was associated with the increased sensitivity to androgens and to the restored bicalutamide effectiveness. It is well known that the development of CRPC requires continued androgen signaling in prostate cancer is patient independent mechanisms. The consequence of this continued androgen signaling in prostate cancer is patient relapse with castration-resistant disease (Lonergan & Tindall 2011, Wang & Tindall 2011). Therefore, decreasing the level of AR within prostate cancer cells using HDACi could enhance the ability of hormonal management to inhibit the growth of prostate cancer and possibly delay the emergence of CRPC. Alternatively, the reduction in AR signaling caused by bicalutamide or androgen withdrawal may be sufficient to sensitize the AR-positive CRPC cells to PXD101-induced apoptosis.

<table>
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<tr>
<th>Table 1</th>
<th>Antitumor activity of PXD101 in 22rv1 xenografts.</th>
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<tr>
<td>Category</td>
<td>Treatment</td>
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<td>A</td>
<td>Saline</td>
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<tr>
<td>B</td>
<td>Belinostat 20</td>
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<tr>
<td>C</td>
<td>Castration</td>
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<td>D</td>
<td>Belinostat 40</td>
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<tr>
<td>E</td>
<td>BCLT 50</td>
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<td>F</td>
<td>Belinostat + BCLT 40 bid + 50</td>
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| A vs B | P = 0.088 | | | | |
| A vs C | P = 0.042 | | | | |
| A vs E | P = 0.177 | | | | |
| C vs D | P = 0.192 | | | | |
| B vs D | P = 0.032 | | | | |
| E vs F | P = 0.003 | | | | |
| B vs F | P = 0.476 | | | | |
| D vs F | P = 0.020 | | | | |

Bold indicates statistical significance (P < 0.05).

<table>
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<tr>
<th>Table 2</th>
<th>Antitumor activity of PXD101 in PC3 xenografts.</th>
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<td>Category</td>
<td>Treatment</td>
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<tr>
<td>A</td>
<td>Saline</td>
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<td>B</td>
<td>Belinostat 20</td>
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<td>F</td>
<td>Belinostat + BCLT 40 bid + 50</td>
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</table>

| A vs B | P = 0.057 | | | | |
| A vs C | P = 0.185 | | | | |
| A vs E | P = 0.904 | | | | |
| C vs D | P = 0.032 | | | | |
| B vs D | P = 0.494 | | | | |
| E vs F | P = 0.016 | | | | |
| B vs F | P = 1.000 | | | | |
| D vs F | P = 0.472 | | | | |

Bold indicates statistical significance (P < 0.05).
Interestingly, HDAC4 and HDAC6 silencing enhanced the androgen responsiveness of C-81 androgen-independent cell model. On the contrary, HDAC4 and HDAC6 knockdown did not seem to have a significant role as modifiers of androgen sensitivity in androgen-dependent models. Differently, from HDAC4, which did not influence the AR, HDAC6 silencing resulted in AR, HER2, EGFR, and AKT downregulation. The reasons for which HDAC4 and HDAC6 differentially enhanced the androgen responsiveness only in androgen-independent models is a matter of debate and the mechanisms responsible for these events are still unknown and require further investigation. However, some hypothesis may be taken into consideration. Recently, it has been demonstrated that valproic acid, a pan HDACi, modifies and increases androgen sensitivity of C81 cells and these findings agree with our data on PXD101 and HDAC4 and HDAC6 silencing (Chou et al. 2011). Additionally, evidence suggests the AR overexpression coupled with p21 downregulation may favor androgen resistance in PCa models (Wang et al. 2001). Thus, treatments able to decrease AR and increase p21 expression may delay the onset of androgen-independent phenotype and re-establish sensitivity to hormonal manipulation. This evidence seems to fit with our data regarding the effect of PXD101 on AR as well as p21 expression levels. It is also possible that restoration of normal AR expression may significantly modify the response to hormone manipulation of refractory PCa cells. Therefore, the downregulation of AR, together with the concomitant down-modulation of EGFR, HER2, and AKT, observed upon HDAC6 silencing in androgen-independent models may attenuate ligand-independent AR activation. This may shift the balance toward an androgen-dependent AR activation, in the presence of DHT, restoring the sensitivity to androgens. This event could also happen in the presence of low AR levels as it is known that androgen-independent tumor cells may have a hypersensitive AR, which may be triggered by low concentrations of DHT. Although the same biological modifications (down-modulation of EGFR, HER2, and AKT) were also found in androgen-dependent models, it is possible that the ligand-independent AR activation may have less importance in determining androgen responsiveness in androgen-dependent models.

The reduction in AR levels induced by PXD101 in AR-positive CRPC cells may involve translational as well as transcriptional mechanisms: i) in the absence of its ligand, ARs are localized mainly in the cytoplasm and are bound to heat-shock proteins including HSP90. Binding to the heat-shock proteins is an important step for the stabilization of the three-dimensional structure of AR in a conformation that permits androgen binding. The Hsp90 chaperone activity is ATP dependent, and inhibition of ATP binding to Hsp90 has been shown to destabilize Hsp90 client proteins ultimately resulting in their degradation. Ablation of HDAC6 using chemical inhibitors was shown to induce hyperacetylation of Hsp90 leading to abrogation of its ATP-binding activity and disruption of chaperone function, resulting in poly-ubiquitination and depletion of Hsp90 client proteins (Ball et al. 2005, Kovacs et al. 2005). ii) Increased mitogen-activated protein kinase signaling, mediated by oncogenes such as ERBB1 (EGFR) or ERBB2 can cause ligand-independent activation of AR especially when its function is not blocked by specific antagonists, such as BCLT (Chen et al. 2011, Marques et al. 2011). (iii) Akt/mTor pathway activation modulates CRM1-mediated nuclear export of proteins involved in cell cycle progression (cyclin D1 and cyclin inhibitor p21 and p27) or apoptosis (survivin) as well as of AR (Schütz et al. 2010, Zhou et al. 2010, Kong et al. 2011). CRM1 activity (Schütz et al. 2010) is mediated by the activation status of GSK3β, a downstream kinase of TORC1. When Akt expression/activity is high, GSK3β activity is low and CRM1 may support nuclear export of chaperoned proteins for recycling and activity.

Bicalutamide is able to increase Akt activity in prostate cancer cell models (Festuccia et al. 2007) whereas PXD101 is able to reduce the expression of Akt, EGFR, and Her2 able to activate Akt both at levels of Thr308 (by PDK1) and ser473 (by TORC2). Here, we report that knockdown of HDAC6 but not of HDAC4 in CRPC cell models using siRNA reduces AR expression, impairs ligand-independent nuclear localization of endogenous AR, and inhibits cell growth. HDAC6 knockdown using PXD101 also inhibited 22rv1 xenograft tumor establishment in castrated, as well as in testes-intact, nude mice treated or not with bicalutamide.

So, we believe the best therapeutic setting, in which the HDAC functional knockdown may better act, is when HDACi are administered in the presence of competitive AR antagonists and androgen deprivation condition rather than in the presence of androgen deprivation conditions only. Our data suggest that PXD101 may differentially recover the antitumor response, in terms of therapeutic efficacy, of competitive AR antagonists or androgen deprivation therapy providing a new treatment option to counteract disease progression and improve and prolong the effects under hormonal treatment.
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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