Published in final edited form as:

J Cell Physiol. 2011 November ; 226(11): 2731–2739. doi:10.1002/jcp.22758.

Rapid Communications (2nd Revision) Modulation of the tumor cell death pathway by androgen receptor in response to cytotoxic stimuli

Michael Frezza, Huanjie Yang, and Q. Ping Dou*

The Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, and Departments of Oncology, Pharmacology and Pathology, School of Medicine, Wayne State University, Detroit, Michigan 48201, USA

Abstract

Despite an initial response from androgen deprivation therapy, most prostate cancer patients relapse to a hormone-refractory state where tumors still remain dependent on androgen receptor (AR) function. We have previously shown that AR breakdown correlates with the induction of cancer cell apoptosis by proteasome inhibition. However, the involvement of AR in modulating the cell death pathway has remained elusive. To investigate this, we used an experimental model consisting of parental PC-3 prostate cancer cells that lack AR expression and PC-3 cells stably overexpressing wild type AR gene. Here, we report that both chemotherapeutic drugs (cisplatin) and proteasome inhibitors induced caspase-3-associated cell death in parental PC-3 cells whereas non-caspase-3 associated cell death in PC3-AR cells. The involvement of AR in modulating tumor cell death was further confirmed in PC-3 cells transiently expressing AR. Consistently, treatment with the clinically used proteasome inhibitor Bortezomib (Velcade/PS-341) of (AR+) LNCaP prostate cancer cells caused AR cleavage and cell death with low levels of caspase activation. However, co-treatment with Bortezomib and the AR antagonist Bicalutamide (Casodex) caused significant decrease in AR expression associated with an increase in caspase-3 activity in both LNCaP and PC3-AR cells. Thus our results provide compelling evidence for involvement of AR in deciding types of tumor cell death upon cytotoxic stimuli, and specifically, blockade of AR activities could change necrosis to apoptosis in tumor cells. Our findings may help guide clinicians based on AR status in the design of favorable treatment strategies for prostate cancer patients.

Keywords

Androgen receptor; proteasome inhibitor; cell death; caspase-3; necrosis; calpain

Introduction

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among American males (Jemal et al., 2008). Prostate cancer can be effectively treated by androgen-deprivation therapy (ADT) through medical or surgical castration. However, disease is rarely eliminated and the majority of prostate cancer patients eventually relapse to a hormone refractory state that remains dependent on the AR signaling axis (Zegarra-Moro et al., 2002). AR is a member of the steroid superfamily of ligand activated transcription factors and plays an important role in the development and progression of

Corresponding Author, Prof. Q. Ping Dou, The Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, and Department of Oncology, School of Medicine, Wayne State University, 540.1 HWCRC, 4100 John R Road, Detroit, Michigan, 48201, U.S.A. Tel: 313-576-8301; Fax: 313-576-8307; (douqp@karmanos.org).

prostate cancer by regulating various cellular processes (Chang et al., 1995). Overwhelming evidence suggests that AR undergoes alterations including gene mutation and amplification in hormone-independent prostate cancers. As a result, this allows them to become sensitive to low or no androgen levels, and become responsive to various ligands, such as growth factors, other steroid hormones, and anti-androgens (Chen et al., 2004; Culig et al., 1994). Therefore, strategies aimed at modulating AR function are likely to induce responses to multidrug-resistant prostate cancer.

Apoptosis functions by the proteolytic processing and sequential activation of various cysteine proteases, known as caspases (Degterev et al., 2003). Propagation of the cell death signal ultimately leads to the activation of effector caspases-3, -6 and/or -7, culminating in the cleavage of various protein substrates that display the typical biochemical and morphological hallmarks of apoptosis (Fulda and Debatin, 2006). Conversely, necrosis is commonly referred to as a passive and uncontrolled form of cell death and involves cell swelling, depletion of cellular energy, a pro-inflammatory response, and the absence of caspase activation (Kim et al., 2006). However increasing evidence suggests that necrosis induction may function in a more regulated fashion (Blank and Shiloh, 2007).

Most approaches used in cancer treatment, such as chemotherapy and radiation therapy kill tumor cells by inducing apoptosis, although it has been suggested that this observation could depend on the mode and degree of therapeutic insult and well as cellular context (Holdenrieder and Stieber, 2004; Okada and Mak, 2004). However, cancer cells often acquire resistance to these therapies, and no longer respond to these death signals (Hunter et al., 2007). Therefore, increasing attention has been directed toward alternative mechanisms of cell death that may help circumvent resistance to cytotoxic agents (de Bruin and Medema, 2008). Since triggering apoptotic cell death may represent a more efficient, cleaner way of targeting and eliminating tumor cells, identifying intracellular cues that may predict for the mode of cell death may help aid in clinical decision making. Moreover, detailed knowledge on the molecular events that facilitate apoptotic and non-apoptotic cell death in response to therapy could help facilitate a more rational approach to cancer treatment.

Experimental evidence has demonstrated that androgen and AR play a critical role in regulating cell death (Diallo et al., 2006; Godfrey et al., 2010; Risek et al., 2008; Yeh et al., 2000). It has been demonstrated that UV and Staurosporine-induced AR proteasomal degradation contributes to cell death in androgen-independent prostate cancer cells, and that the AR-amino terminal region was at least partially responsible for this effect (Godfrey et al., 2010). Additionally, it was shown that re-expression of AR in AR-negative prostate cancer cells confers a less aggressive phenotype by decreasing anchorage independent growth and invasiveness by blocking EGFR-mediated signaling (Bonaccorsi et al., 2008). We have previously reported that proteasome inhibitors caused downregulation of AR protein in both androgen-dependent LNCaP cells and androgen-independent C4-2B cells (Chen et al., 2007; Yang et al., 2006), and that calpain involvement is at least partially responsible for this effect (Pelley et al., 2006; Yang et al., 2008). The observation that apoptosis induction by proteasome inhibitors is accompanied by decreasing AR levels in AR-positive prostate cancer cells suggests that elimination of AR is intrinsically linked with apoptosis (Yang et al., 2008). However, whether AR stability is involved in modulating the cell death pathway in prostate cancer cells has yet to be established. To test this hypothesis, in the current study we used parental PC-3 cells that do not express AR and PC-3 cells overexpressing wild type AR gene, and investigated their response toward chemotherapy and proteasome inhibition. We found both cell lines treated with cisplatin and Velcade exhibited similar sensitivity toward growth inhibition and cell death induction during shorter exposure time (~24 h), but PC-3 AR cells showed slightly increased resistance toward these drugs during long term exposure (up to 6 days) at lower concentrations. However,

significantly higher levels of caspase-3 and apoptotic indices were observed in PC-3 cells compared to PC-AR cells in response to both cytotoxic agents. Although a rapid onset of cell death was apparent in PC3-AR cells, this effect was not associated with significant caspase-3 activity and apoptosis. Moreover, the observation that parental PC-3 cells, but not PC3-AR cells, undergo a caspase-associated cell death was further confirmed by transiently expressing AR in PC-3 cells. Consistently, treatment of AR-positive LNCaP cells with the clinically used proteasome inhibitor Bortezomib (Velcade/PS-341) resulted in cell death without high levels of caspase 3 activity. However, co-treatment with the AR antagonist Bicalutamide (Casodex) and Bortezomib resulted in production of significantly higher levels of caspase 3 activity and apoptosis, associated with decreased AR protein level and increased AR cleavage. Our study suggests that AR has a regulatory role in modulating the cell death pathway in response to therapeutic stimuli and that the presence of AR in prostate cancer cells may be predictive of response toward therapeutic stress. These results are clinically significant and could hold potential value in the design of therapeutic strategies for the treatment of prostate cancer by targeting AR.

Materials and Methods

Materials

Velcade (Bortezomib) was obtained from LC Laboratories (Woburn, MA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), DMSO and other chemicals were purchased from Sigma Aldrich (St. Louis, MO). Bicalutamide (Casodex) was purchased from Toronto Research Chemicals Inc. (North York, Ontario Canada). RPMI-1640, penicillin, streptomycin, pEntr vector, and pLenti-6/V5-Dest vector were purchased from Invitrogen (Carlsbad, CA). Fluorogenic peptide substrates, Suc-LLVY-AMC (for the proteasomal chymotrypsin-like activity) and Ac-DEVD-AMC (for Caspase-3 activity) was purchased from Calbiochem (San Diego, CA). Mouse monoclonal antibody against human poly(ADP-ribose)polymerase (PARP) was purchased from BIOMOL International LP (Plymouth Meeting, PA). Mouse monoclonal antibodies against caspase-8, caspase-9, rabbit polyclonal against Androgen Receptor (AR-N20), goat polyclonal antibody against actin, and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Cell death ELISA detection kit was purchased from Roche Applied Sciences (Indianapolis, IN). A full-length AR plasmid was purchased from Open Biosystems (Lafayette, CO). Transfection reagent, ExpressFect, was purchased from Denville Scientific (Metuchen, NJ).

Cell culture and whole-cell extract preparation

PC-3 cells overexpressing wild type Androgen Receptor (AR) were obtained from Dr. Fazlul Sarkar (Wayne State University, Detroit, MI). Human prostate cancer PC-3 and LNCaP cells were obtained from American Type Culture Collection (Manassas, VA). These cell lines were grown in RPMI-1640 and supplemented with 10% fetal bovine serum and 100 units/mL of penicillin and 100 µg/mL of streptomycin. All cells were grown at 37° C in a humidified incubator with a 5% CO₂-enriched atmosphere. A whole-cell extract was prepared as previously described (Daniel et al., 2005).

Proteasome activity assay in intact human prostate cancer PC-3 and PC-AR cells

Human prostate PC-3 or PC3/AR cells were grown to 70%–80% confluency, treated with indicated compound or DMSO as vehicle control under various conditions, harvested, and used for whole-cell extract preparation. Ten micrograms of cell extract was incubated with 20 µmol/L of the fluorogenic substrate Suc-LLVY-AMC in [50 µmol/L Tris-HCL, pH 7.5] for 2h at 37°C (for the proteasomal chymotrypsin-like activity). After incubation, production

of hydrolyzed AMC groups was measured with a Wallac Victor³ multilabel counter with an excitation filter of 365 nm and emission filter of 460 nm.

Caspase-3 Activity Assay

Cells were treated with indicated agent, harvested, and lysed as described previously (Daniel et al., 2005). Ac-DEVD-AMC (40 μ mol/L) was then incubated with the prepared cell lysates for 24 h and the caspase-3 activity was measured as described above.

Cell proliferation assay

Prostate cancer cells were seeded in triplicate in a 96-well plate and grown, followed by treatment with Velcade, cisplatin or DMSO (as a control) for 24 h or up to 6 days. Following drug exposure time, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done as previously described (Daniel et al., 2005).

Western Blot analysis

PC-3, PC3-AR, and LNCaP prostate cancer cells were treated, harvested, and lysed. Cell lysates (40–50 μ g) were separated by SDS-PAGE and transferred to a nitrocellulose membrane followed by visualization using the HyGLO chemiluminescent HRP detection reagent from Denville Scientific (Metuchen, NJ), as previously described (Chen et al., 2005).

Cellular morphology analysis

A Zeiss Axiovert 25 microscope was used for all microscopic imaging with phase contrast for cellular morphology. Magnification X100.

Quantification of apoptosis

The Cell Apoptosis ELISA Detection Kit (Roche Applied Sciences) was used to detect apoptosis in PC-3 and PC3-AR cells according to the manufactures protocol.

Expression vector and transient transfection

A full-length AR plasmid was purchased from Open Biosystems (Lafayette, CO). Subsequently, it was subcloned into a pEntr vector (Invitrogen) and recombined into pLenti-6/V₅-Dest vector (Invitrogen). The sequence was confirmed by sequencing with CMV and V₅ primers.

Transient Transfection of AR

PC-3 cells were grown to 70%–80% confluency followed by addition of the DNA complex using the ExpressFect reagent as mentioned in the manufacturers' protocol. Briefly, 1–2 μ l ExpressFectTM was added to 30 μ l of DMEM media, mixing gently to create the polymer solution. Thirty μ l of the polymer solution was added to 30 μ l of the DNA solution to create the polymer/DNA complex. The concentration of reagents used was based on the final volume of cell culture medium. After 24 h, cells were treated with indicated drug and cell lysates were prepared as previously discussed.

Results

Cisplatin-induced cytotoxicity elicits caspase-3-associated apoptotic cell death in parental PC-3 cells and non-caspase-associated cell death in PC-3 cells stably overexpressing AR

In our current study we investigated how AR may influence the molecular events in prostate cancer cells in response to chemotherapy or proteasome inhibition. Toward this goal, we used parental PC-3 prostate cancer cells that are AR-negative and PC-3 cells stably

expressing wild type AR. PC3 and PC3-AR cells were first treated with cisplatin for 24 h followed by measurement of cell proliferation, caspase 3 activity, apoptosis-specific PARP cleavage, and cellular morphological changes (Fig. 1). The solvent DMSO was used as solvent control. We found that growth inhibition by cisplatin at 25 μ M displayed a similar sensitivity profile between both PC-3 cell lines (Fig. 1A). Although both cell lines showed a similar decrease in growth inhibition at low concentrations, PC-3-AR cells were marginally more resistant to treatment of cisplatin at 50 μ M-100 μ M (Fig. 1A). Additionally, we measured growth inhibition of both PC3 and PC3-AR cells toward cisplatin over longer exposure times (4 to 6 days) at lower concentrations (5 μ M-25 μ M; Fig. 2). Our results show that PC3-AR cells were considerably more resistant toward cisplatin at 5 μ M treatment compared to parental PC-3 cells from 4 to 6 day exposure (Fig. 2A–C). However, both cell lines showed similar sensitivity to cisplatin at higher concentrations (15–25 μ M) after 4 to 6 day treatment (Fig. 2A–C).

We next investigated whether the overexpression of AR in PC-3 cells could influence the molecular events associated with the cell death program in response to cisplatin. To test this hypothesis, both PC-3 and PC3-AR cell lines were treated with 25 μ M-100 μ M cisplatin for 24 h, followed by measurement of caspase-3 activity and PARP cleavage. Associated with growth inhibition (Fig. 1A), cisplatin elicited significantly higher levels of caspase 3 activity in parental PC-3 cells than in PC-AR cells (Fig. 1B). For example, treatment of PC-3 and PC-3-AR cells with cisplatin at 75 μ M induced activation of caspase-3 by 12- and 3-fold, respectively (Fig. 1B). Consistently, the cleaved, active form of caspase-3 fragment was found mainly in PC-3 cells after cisplatin treatment (Fig. 1C). It has been shown that PARP can be cleaved by caspase-3 into its characteristic p85 fragment and is characteristic of apoptosis (Lazebnik et al., 1994). Associated with higher levels of caspase 3 in PC-3 cells was p85 PARP cleavage at higher concentrations (75–100 μ M) of cisplatin (Fig. 1C). Although there was little apparent morphological changes in PC-3 cells after treatment with cisplatin at low concentrations (25–50 μ M; Fig. 1D), these cells were considerably rounded up, shrunken, and detached after higher concentrations (75–100 μ M; Fig. 1D), consistent with apoptosis induction (Fig. 1C). Importantly, even though PC-3 AR cells exhibited much lower levels of caspase-3 activation and PARP cleavage after cisplatin treatment than parental PC-3 cells (Fig. 1B–C), PC-3 AR cells displayed higher levels of morphological cell death, as shown by an increased detached, and rounded up cell population after only 25 μ M treatment of cisplatin (Fig. 1D). The dose-dependent death of PC-3 AR cells induced by cisplatin matched with the profile of growth inhibition in these cells after cisplatin treatment (Fig. 1A).

To further confirm the different modes of cell death in PC-3 and PC-3-AR cells induced by chemotherapy, we performed a detailed kinetics experiment by treating both cell lines with cisplatin at 75 μ M from 2 to 24 h. Again we found significantly higher levels of caspase 3 activity after 8 h treatment in PC-3 cells compared to PC3-AR cells (Fig. 3A). For example, we found 8.4- and 11.8-fold increase in caspase 3 activity after 18 h and 24 h exposure of cisplatin, respectively in parental PC-3 cells compared its AR-containing counterpart (Fig. 3A). Consistent with the time-dependent caspase activity increase in PC-3 cells was the appearance of PARP cleavage during later time points (Fig. 3B). In contrast, the levels of caspase 3 activity and p85/PARP fragment with PC3-AR cells were increased by only about 2-fold after 24 h treatment with cisplatin (Fig. 3A–B).

We also found that the appearance of aberrant morphological changes for PC-3 cells were mostly during later time points associated with caspase activation (Fig. 3C vs. A–B), whereas PC3-AR cells exhibited significant levels of cell death as judged by morphological changes after even 2 h treatment (Fig. 3C), irrespective of the lack of caspase activation in these cells (Fig. 3A–B). These results further suggest that upon cisplatin treatment PC-3

cells undergo cell death in a caspase-associated process (characteristic of apoptosis), whereas PC3-AR cells undergo a different type of cell death which is not associated with high levels of caspase activity.

The proteasome inhibitor bortezomib induces caspase-3-associated apoptosis in PC-3 cells and non-caspase-associated cell death in PC-3 AR cells

We next tested whether a proteasome inhibitor, similar to cisplatin (Figs. 1–3), could also cause different types of cell death in the absence or presence of AR expression. Bortezomib (or Velcade) is a clinically used proteasome inhibitor for the treatment of myeloma and mantle cell lymphoma and also being tested against solid tumors (Kane et al., 2003; Kane et al., 2007). In this experiment, both PC-3 cells and PC-3-AR cells were treated with Velcade at various concentrations for 24 h, followed by an MTT assay. Our results show, although Velcade inhibited cell proliferation in both cell lines in a dose-dependent fashion after 24 h, PC-AR cells appeared to be slightly more sensitive to Velcade at lower concentrations (50 nM to 0.50 μ M; Fig. 4A). However, this trend narrowed between both cell lines in response to higher doses of Velcade, rendering a similar growth inhibitory profile (Fig. 4A). In the longer treatment (4 to 6 days) experiment, although under some conditions both cell lines showed a similar growth inhibitory profile (Fig. 2A–C), as seen after 24 h treatment (Fig. 4A), after 6 day's treatment, PC3-AR cells appeared to be more resistant toward Velcade at both 15 and 25 nM (Fig. 2A–C).

To test the effect of Velcade on the proteasome activity levels in both cell lines, PC-3 and PC-AR cells were treated with Velcade (0.25 μ M–5 μ M) for 24 h followed by measurement of the proteasomal chymotrypsin-like activity. We found that both cell lines showed a similar sensitivity pattern toward treatment of Velcade at all concentrations tested compared with DMSO control treatment (Fig. 4B).

We next investigated whether the presence of AR could influence the molecular events associated with the cell death program in response to Velcade. Both PC-3 cell lines were treated with different concentrations of Velcade (0.25 μ M–5 μ M) or DMSO solvent control for 24 h, followed by various assays including caspase activity, PARP cleavage, morphological changes, and histone-DNA ELISA. Significantly higher caspase-3 levels were visible in PC-3 cells treated with Velcade, reaching about 26 fold increase compared to DMSO-treated cells (Fig. 4C). In comparison, only about 6 fold increase in caspase-3 activity was visible in PC3-AR cells treated with Velcade (Fig. 4C). This striking difference in caspase-3 activity was verified by Western blot analysis, which showed a dose-dependent increase in levels of cleaved caspase-3 and cleaved p85 PARP protein in PC-3 cells but not PC-3 AR cells treated with Velcade (Fig. 4D).

We then investigated the levels of upstream initiator caspases, caspases-8 and -9, in both PC-3 and PC-3 AR cells in this experiment. We found that upon treatment of Velcade at increased concentrations, parental PC-3 cells first displayed decreased levels of pro-caspase-8 protein which disappeared at higher concentrations, associated with production of high levels of cleaved caspase-8 (Fig. 4D). In contrast, the levels of pro-caspase-8 protein in PC-3 AR cells remained high with very low levels of active caspase-8 fragment produced (Fig. 4D). Similarly, decreased levels of pro-caspase 9 protein and increased levels of cleaved caspase-9 were visible in PC-3 cells, but not PC-3 AR cells after Velcade treatment (Fig. 4D).

To further confirm that Velcade induces apoptosis apparently in PC-3 cells but not PC-3 AR cells, a histone-DNA ELISA assay was performed which measures apoptotic cell death. Figure 4E shows a significant increase in apoptotic cells (~7 fold) in PC-3 cells treated with

Velcade. In contrast, treatment of PC3-AR cells under the same experimental conditions resulted in only minimal levels of apoptotic cells (Fig. 4E).

We also observed cell death-associated morphological changes (cell shrunken, and rounded up) in both PC-3 and PC3-AR cells treated with increasing concentrations of Velcade (data not shown). However, although PC3-AR cells shows significantly lower levels of caspase 3 compared to parental cells (Fig. 4C–E), abnormal cell morphology was more pronounced at the lowest concentrations of Velcade tested (data not shown), signifying a shift to an alternative form of cell death.

Transient expression of AR inhibits caspase 3-associated cell death induced by proteasome inhibitors and cisplatin

Our results thus far have suggested that the overexpression of AR in prostate cancer cells can influence regulatory events that switch the cell death program from apoptotic cell death to a form of cell death that is not associated with caspase-3 activity in response to therapeutic stimuli (such as cisplatin and Velcade). To further confirm this finding, we performed transient transfection of the AR gene into PC-3 cells. Both parental PC-3 cells (or empty vector-transfected PC-3 cells) and PC-3 cells transiently expressing AR were then treated with cisplatin, Velcade, or MG-132, another proteasome inhibitor for 24 h, followed by measurement of caspase-3 activity (Fig. 5). Consistent with our finding from cells stably expressing AR (Figs. 1–4), both proteasome inhibitors induced significantly lower levels of caspase 3 activity in the PC-3 cells transiently expressing AR compared to the parental PC-3 cells (Fig. 5B) or in the empty vector-transfected PC-3 cells (data not shown). For example, we found exposure to Velcade and MG-132 for 24 h induced the activation of caspase-3 activity about 2.5- fold and 4-fold lower, respectively, in the PC3 cells transiently transfected with AR than the parental PC-3 cells (Fig. 5B), consistent with the idea that AR might be a suppressor of caspase-3 activation induced by proteasome inhibition. We also found that when cisplatin was used, the caspase-3 activity level in the PC3 cells transiently transfected with AR was only slightly lower than in the parental PC-3 cells (Fig. 5B). However, a decrease in AR levels (compared to the control lane, Fig. 5A) may at least partially account for this weak inhibitory effect (Fig. 5B). All these data support the hypothesis that AR protein acts as a suppressor of the caspase-3 pathway and switches the cell death program from apoptosis to one that is not heavily dependent on caspase-3.

Combination of an AR antagonist and proteasome inhibition induced AR cleavage and caspase-3 activation in AR-positive LNCaP cells and PC3 cells stably expressing AR

We previously reported that cleavage of endogenous AR protein in LNCaP cells is associated with induction of apoptotic cell death by proteasome inhibitors at effective concentrations (Chen et al., 2007; Yang et al., 2006). Consistently, the treatment of LNCaP cells with Velcade at lower concentrations failed to induce any significant levels of caspase-3 activation and PARP cleavage (Fig. 6A–B). We also found that a small portion of AR protein was cleaved into a detectable ~80 kDa fragment under the tested conditions, suggesting the involvement of calpain (Fig. 6B). It has been shown that calpain activation was responsible for the generation of ~80 kDa AR fragment that appears to contain elevated transcriptional activity (Libertini et al., 2007).

We next investigated whether pretreatment with an AR antagonist could rescue caspase-3-dependent cell death in LNCaP cells. Casodex acts as an anti-androgen by binding to the AR and preventing the activation of its target genes (Furr, 1996). In addition, casodex accelerates the degradation of the androgen receptor (Waller et al., 2000). LNCaP cells were untreated or pretreated with casodex for 24 h, followed by co-treatment with Velcade at different concentrations (0.25, 1 or 2.5 μ M) for an additional 24 h and measurement of

caspase 3 activity, PARP cleavage, and AR levels. We found that while Velcade or casodex alone under the experimental conditions induced no or low levels of caspase-3 activity and PARP cleavage, co-treatment of Velcade and casodex elicited a significant increase in caspase 3 activity reaching 4.8-fold increase (Fig. 6A), which was associated with increased PARP cleavage (Fig. 6B). Furthermore, levels of AR, both full length and ~ 80 kDa fragment forms, were significantly decreased in cells co-treated with casodex and Velcade compared to each treatment alone (Fig. 6B).

Similarly, co-treatment of the AR stably transfected PC3-AR cells with casodex and Velcade resulted in a 12-fold increase in caspase-3 activity (Fig. 6C), higher than each treatment alone, associated with production of p85 PARP fragment and ~80 Kd AR fragment (data not shown). These results strongly suggest that inhibition of AR function shifts noncaspase-3 associated cell death to caspase-3-associated apoptosis in prostate cancer cells.

Discussion

Many reports have suggested that AR is a critical molecular determinant in driving prostate cancers from a hormone-sensitive to a hormone-refractory state following ablation of steroidal androgens (Attard et al., 2009; Cohen and Rokhlin, 2009; Taplin, 2008). We have previously reported that proteasome inhibitors caused downregulation of AR in both androgen-dependent LNCaP cells and androgen-independent C4-2B cells (Chen et al., 2007; Yang et al., 2006), and that calpain involvement is at least partially responsible for this effect (Pelley et al., 2006; Yang et al., 2008). Consistently, it has been reported most recently that calmodulin protects androgen receptor from calpain-mediated breakdown in prostate cancer cells (Sivanandam et al., 2010). The finding that AR degradation by proteasome inhibition is associated with apoptotic cell death stimulated our investigation into examining the regulatory events of AR in facilitating tumor cell death in the current study.

Clinical studies have shown that most anticancer agents induce apoptosis, and that inhibition of the apoptotic program can trigger chemoresistance (Okada and Mak, 2004). Therefore, targeting alternative forms of cell death may provide effective means for maximal tumor ablation. It is likely that both apoptotic and non-apoptotic pathways contribute to cell death depending on the cell type and degree of therapeutic insult. It has also been shown that many current anticancer therapies, including DNA-alkylating agents can induce necrosis by activation of PARP-1 (Zong et al., 2004). Increasing evidence suggests that death receptor adaptors, including receptor-interacting protein kinase (RIPK1) and tumor necrosis factor (TNF) receptor associated factor 2 (TRAF2), as important regulators of necrotic cell death (Chan et al., 2003; Holler et al., 2000). These findings open the possibility of exploiting these as potential molecular targets in cancer therapy. It has also been shown that breast cancer cells treated with tamoxifen can induce autophagy by accumulating autophagic vesicles before tumor cell death (Bursch et al., 1996; Bursch et al., 2000). However, the molecular events by which tumors cells can control their fate has remained unanswered. Therefore, assessment of the expression of protein signatures associated with cell death may have significant implications in the selection of treatment strategies for cancer (Bruckheimer and Kyprianou, 2000).

Our current study reports that parental AR-negative PC-3 cells and PC-3 cells transfected with AR gene showed similar responses to chemotherapy and proteasome inhibition (Figs. 1–4). However, significantly higher caspase-3 activity was seen in parental PC-3 cells compared to PC3-AR cells associated with apoptotic indices in response to both therapeutic agents (Fig. 1–4). These results suggest that parental PC-3 cells undergo caspase-dependent

apoptosis, whereas PC3-AR cells undergo a caspase-3 independent cell death (i.e. necrosis, or autophagy). While there was a report that AR-containing PC-3 cells could somehow have increased sensitivity toward apoptotic stimuli (Davis et al., 2003), their experimental design (measuring pan caspase activation elicited by paclitaxol and radiation-induced apoptosis) was significantly different from our system. Our finding that AR could act as a suppressor of caspase-3-dependent cell death was further validated in PC-3 cells transiently expressing AR as well as in AR(+) LNCaP cells (Figs. 5, 6).

Since AR plays critical role in the transition of hormone sensitive to hormones resistant prostate cancer (Taplin and Balk, 2004), elimination of AR may provide therapeutic benefit in drug resistant disease. Since our data suggest that AR presence appears to be important in shifting the cell death program from a caspase-independent to a caspase-dependent (apoptosis) mechanism, we investigated whether decreasing AR levels could regenerate the apoptotic process. We found that proteasome-inhibitor-mediated cell death in LNCaP cells was associated with low caspase 3 activity under the experimental condition (Fig. 6A), and co-treatment with Casodex and Velcade was accompanied by higher levels of caspase 3, PARP cleavage, and AR elimination (Fig. 6A, B). Therefore, we propose that treatment with proteasome inhibitor (Velcade) leads to the activation of calpain (Fig. 7). Proteasome-inhibitor-mediated calpain activation leads to the degradation of the androgen receptor, which can ultimately trigger prostate tumor cell death via apoptosis. In an effort to augment the efficacy of proteasome inhibitor (PI)-induced AR degradation, the use of an AR antagonist (casodex) in combination with proteasome inhibitor could significantly enhance AR breakdown and ultimately trigger apoptotic cell death (Fig. 7). This finding was further supported in PC-3 cells stably expressing AR (Fig. 6C; data not shown). We have also recognized the limitations of our current experiments. First, although higher caspase activity was observed in co-treated cells, accompanied by higher levels of cleaved AR (~80 Kd), a complete elimination of AR was unsuccessful. Secondly, a complete knockdown of overexpressed AR in PC-3 was also unachievable even at 250 $\mu\text{mol/l}$ of AR antagonist Casodex. Our data also suggest that the direct target of AR is not caspase-3, since AR expression also inhibits activation of caspase-8 and caspase-9 (Fig. 3) and since AR from LNCaP cell extracts failed to inhibit the activity of caspase-3 *in vitro* (data not shown). Further studies are being performed regarding the direct target of AR in inhibiting the apoptotic pathway.

In cancer treatment, the ideal outcome is to trigger tumor-selective cell death, such as apoptosis, a death from within. In contrast, tumor cell necrosis, a “messy” type of cell death, may affect the adjacent normal tissue, causing inflammation. Therefore, the mechanism responsible for controlling apoptotic death can have important clinical significance in determining the treatment efficacy of specific treatments (Sellers and Fisher, 1999). From a clinical perspective, the identification of biomarkers that may predict the mode of tumor cell death induced by chemotherapy may help aid clinicians to better tailor treatment regimens that may lead to an improved clinical outcome.

Thus the first clinical significance of our findings is that the AR status in prostate cancer patients may serve as a clinical molecular marker that could predict their possible outcomes after chemotherapeutic treatment: tumor growth inhibition without inflammation (such as *via* caspase-dependent apoptosis induction) or tumor suppression with an inflammatory response (such as *via* non-caspase necrosis death pathway). In this case, AR-negative prostate cancer patients would have a significant clinical benefit compared to AR-positive patients. Our results clearly show that prostate cancer cells that lack AR expression undergo cell death through a caspase-dependent mode (apoptosis), and re-engagement of the AR signaling axis switches the cell death program to a caspase-independent form of cell death (i.e. necrosis, autophagy).

The second clinical significance of our results is that co-treatment with casodex and velcade could switch the mode of cell death from a caspase-independent form (i.e. necrosis) to a caspase-dependent mechanism, which may highlight a favorable strategy that selectively induces apoptosis in prostate cancer cells that harbor AR expression. In the second scenario, AR-positive prostate cancer patients would benefit from the combinational treatment of an AR inhibitor and a chemotherapeutic drug.

Selectively inducing apoptotic cell death *in vivo* by targeting AR expression or function may avoid the buildup of an inflammatory response and the collateral damage to normal tissues which is often associated with necrosis. In addition to the necrosis-mediated damage to normal surrounding tissues, a sustained and chronic inflammatory response could result in the production of prosurvival cytokines and growth factors such as high-mobility group box 1 protein (HMGB1) and hepatoma-derived growth factor (HDGF), which in turn can engage signaling pathways that mediate cell outgrowth in damaged areas, and trigger tumor growth and development, thus confounding the intended clinical response (Ricci and Zong, 2006). Furthermore, autophagy induction by anticancer drugs has been demonstrated. However this type of cell death can act as both a “cell death” and a “cell survival” strategy, thus potentially confounding the clinical outcome (Amaravadi and Thompson, 2007).

Although it is likely that within a heterogeneous population of tumor cells that engagement of both apoptotic and non-apoptotic programs are needed for tumor ablation, our finding that AR may act as a signature to predict the mode of tumor cell death could help aid in the clinical treatment decisions of prostate cancer patients.

Finally, since the majority of hormone resistant prostate cancer (HRPC) remains highly dependent on AR function, inhibition or elimination of AR and selective induction of apoptotic cell death may prove to be a viable therapeutic strategy in the treatment of HRPC.

In summary, our results demonstrate the intrinsic value of AR in modulating the cell death pathways in response to therapeutic stimuli and these findings could have important clinical implications in the design of therapeutic strategies by targeting AR for the treatment of prostate cancer.

Acknowledgments

This work was partially supported by grants from Karmanos Cancer Institute 2009 Pilot project funding (to QPD) and the National Cancer Institute (1R01CA120009 and 3R01CA120009-04S1, to QPD) as well as a training grant “Ruth L. Kirschstein National Service Research Award (T32-CA009531, to MF). We greatly thank Dr. Fazlul Sarkar for the PC-3 AR cell line, Dr. Shumei Zhei and Dr. Haijun Zhang for assistance in the gene cloning and recombination, and the laboratory of Dr. Ramzi Mohammad for the apoptosis ELISA assay.

Abbreviations

AR	androgen receptor
Cas-3	caspase 3
Cas	Casodex (bicalutamide)
cis	cisplatin
DMSO	Dimethylsulfoxide
MTT	-[4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
PARP	poly(ADP-ribose)polymerase
PC3 AR	PC-3 cells stably overexpressing wild type AR

Vel Velcade

Literature Cited

- Amaravadi RK, Thompson CB. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin Cancer Res*. 2007; 13:7271–7279. [PubMed: 18094407]
- Attard G, Cooper CS, de Bono JS. Steroid hormone receptors in prostate cancer: a hard habit to break? *Cancer Cell*. 2009; 16:458–462. [PubMed: 19962664]
- Blank M, Shiloh Y. Programs for cell death: apoptosis is only one way to go. *Cell Cycle*. 2007; 6:686–695. [PubMed: 17361099]
- Bonaccorsi L, Nosi D, Quercioli F, Formigli L, Zecchi S, Maggi M, Forti G, Baldi E. Prostate cancer: a model of integration of genomic and non-genomic effects of the androgen receptor in cell lines model. *Steroids*. 2008; 73:1030–1037. [PubMed: 18358509]
- Bruckheimer EM, Kyprianou N. Apoptosis in prostate carcinogenesis. A growth regulator and a therapeutic target. *Cell Tissue Res*. 2000; 301:153–162. [PubMed: 10928288]
- Bursch W, Ellinger A, Kienzl H, Torok L, Pandey S, Sikorska M, Walker R, Hermann RS. Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. *Carcinogenesis*. 1996; 17:1595–1607. [PubMed: 8761415]
- Bursch W, Hochegger K, Torok L, Marian B, Ellinger A, Hermann RS. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. *J Cell Sci*. 2000; 113:1189–1198. [PubMed: 10704370]
- Chan FK, Shisler J, Bixby JG, Felices M, Zheng L, Appel M, Orenstein J, Moss B, Lenardo MJ. A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. *J Biol Chem*. 2003; 278:51613–51621. [PubMed: 14532286]
- Chang C, Saltzman A, Yeh S, Young W, Keller E, Lee HJ, Wang C, Mizokami A. Androgen receptor: an overview. *Crit Rev Eukaryot Gene Expr*. 1995; 5:97–125. [PubMed: 8845584]
- Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL. Molecular determinants of resistance to antiandrogen therapy. *Nat Med*. 2004; 10:33–39. [PubMed: 14702632]
- Chen D, Frezza M, Shakya R, Cui QC, Milacic V, Verani CN, Dou QP. Inhibition of the proteasome activity by gallium(III) complexes contributes to their anti prostate tumor effects. *Cancer Res*. 2007; 67:9258–9265. [PubMed: 17909033]
- Chen D, Peng F, Cui QC, Daniel KG, Orlu S, Liu J, Dou QP. Inhibition of prostate cancer cellular proteasome activity by a pyrrolidine dithiocarbamate-copper complex is associated with suppression of proliferation and induction of apoptosis. *Front Biosci*. 2005; 10:2932–2939. [PubMed: 15970547]
- Cohen MB, Rokhlin OW. Mechanisms of prostate cancer cell survival after inhibition of AR expression. *J Cell Biochem*. 2009; 106:363–371. [PubMed: 19115258]
- Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res*. 1994; 54:5474–5478. [PubMed: 7522959]
- Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, Dou QP. Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells. *Breast Cancer Res*. 2005; 7:R897–908. [PubMed: 16280039]
- Davis R, Jia D, Cinar B, Sikka SC, Moparty K, Zhau HE, Chung LW, Agrawal KC, Abdel-Mageed AB. Functional androgen receptor confers sensitization of androgen-independent prostate cancer cells to anticancer therapy via caspase activation. *Biochem Biophys Res Commun*. 2003; 309:937–945. [PubMed: 13679064]
- de Bruin EC, Medema JP. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treat Rev*. 2008; 34:737–749. [PubMed: 18722718]

- Degterev A, Boyce M, Yuan J. A decade of caspases. *Oncogene*. 2003; 22:8543–8567. [PubMed: 14634618]
- Diallo JS, Peant B, Lessard L, Delvoye N, Le Page C, Mes-Masson AM, Saad F. An androgen-independent androgen receptor function protects from inositol hexakisphosphate toxicity in the PC3/PC3(AR) prostate cancer cell lines. *Prostate*. 2006; 66:1245–1256. [PubMed: 16705740]
- Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*. 2006; 25:4798–4811. [PubMed: 16892092]
- Furr BJ. The development of Casodex (bicalutamide): preclinical studies. *Eur Urol*. 1996; 29(2):83–95. [PubMed: 8717469]
- Godfrey B, Lin Y, Larson J, Haferkamp B, Xiang J. Proteasomal degradation unleashes the pro-death activity of androgen receptor. *Cell Res*. 2010; 20:1138–1147. [PubMed: 20479785]
- Holdenrieder S, Stieber P. Apoptotic markers in cancer. *Clin Biochem*. 2004; 37:605–617. [PubMed: 15234242]
- Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschoop J. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol*. 2000; 1:489–495. [PubMed: 11101870]
- Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. *Apoptosis*. 2007; 12:1543–1568. [PubMed: 17573556]
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin*. 2008; 58:71–96. [PubMed: 18287387]
- Kane RC, Bross PF, Farrell AT, Pazdur R. Velcade: U.S. FDA approval for the treatment of multiple myeloma progressing on prior therapy. *Oncologist*. 2003; 8:508–513. [PubMed: 14657528]
- Kane RC, Dagher R, Farrell A, Ko CW, Sridhara R, Justice R, Pazdur R. Bortezomib for the treatment of mantle cell lymphoma. *Clin Cancer Res*. 2007; 13:5291–5294. [PubMed: 17875757]
- Kim R, Emi M, Tanabe K. The role of apoptosis in cancer cell survival and therapeutic outcome. *Cancer Biol Ther*. 2006; 5:1429–1442. [PubMed: 17102590]
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*. 1994; 371:346–347. [PubMed: 8090205]
- Libertini SJ, Tepper CG, Rodriguez V, Asmuth DM, Kung HJ, Mudryj M. Evidence for calpain-mediated androgen receptor cleavage as a mechanism for androgen independence. *Cancer Res*. 2007; 67:9001–9005. [PubMed: 17909000]
- Okada H, Mak TW. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer*. 2004; 4:592–603. [PubMed: 15286739]
- Pelley RP, Chinnakannu K, Murthy S, Strickland FM, Menon M, Dou QP, Barrack ER, Reddy GP. Calmodulin-androgen receptor (AR) interaction: calcium-dependent, calpain-mediated breakdown of AR in LNCaP prostate cancer cells. *Cancer Res*. 2006; 66:11754–11762. [PubMed: 17178871]
- Ricci MS, Zong WX. Chemotherapeutic approaches for targeting cell death pathways. *Oncologist*. 2006; 11:342–357. [PubMed: 16614230]
- Risek B, Bilski P, Rice AB, Schrader WT. Androgen receptor-mediated apoptosis is regulated by photoactivatable androgen receptor ligands. *Mol Endocrinol*. 2008; 22:2099–2115. [PubMed: 18562628]
- Sellers WR, Fisher DE. Apoptosis and cancer drug targeting. *J Clin Invest*. 1999; 104:1655–1661. [PubMed: 10606616]
- Sivanandam A, Murthy S, Chinnakannu K, Bai U, Kim SH, Barrack ER, Menon M, Reddy GP. Calmodulin protects androgen receptor from calpain-mediated breakdown in prostate cancer cells. *J Cell Physiol*. 2010
- Taplin ME. Androgen receptor: role and novel therapeutic prospects in prostate cancer. *Expert Rev Anticancer Ther*. 2008; 8:1495–1508. [PubMed: 18759700]
- Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem*. 2004; 91:483–490. [PubMed: 14755679]

- Waller AS, Sharrard RM, Berthon P, Maitland NJ. Androgen receptor localisation and turnover in human prostate epithelium treated with the antiandrogen, casodex. *J Mol Endocrinol.* 2000; 24:339–351. [PubMed: 10828827]
- Yang H, Chen D, Cui QC, Yuan X, Dou QP. Celastrol, a triterpene extracted from the Chinese “Thunder of God Vine,” is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. *Cancer Res.* 2006; 66:4758–4765. [PubMed: 16651429]
- Yang H, Murthy S, Sarkar FH, Sheng S, Reddy GP, Dou QP. Calpain-mediated androgen receptor breakdown in apoptotic prostate cancer cells. *J Cell Physiol.* 2008; 217:569–576. [PubMed: 18726991]
- Yeh S, Hu YC, Rahman M, Lin HK, Hsu CL, Ting HJ, Kang HY, Chang C. Increase of androgen-induced cell death and androgen receptor transactivation by BRCA1 in prostate cancer cells. *Proc Natl Acad Sci U S A.* 2000; 97:11256–11261. [PubMed: 11016951]
- Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res.* 2002; 62:1008–1013. [PubMed: 11861374]
- Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev.* 2004; 18:1272–1282. [PubMed: 15145826]

Fig 1. Cisplatin induces dose-dependently higher levels of Caspase-3 associated with the induction of apoptosis in PC-3 compared to nonapoptotic cell death in PC3 cells stably expressing AR (PC3 AR cells). PC-3 and PC-3 AR cells were treated with cisplatin at indicated concentrations, with DMSO vehicle as control for 24 h, followed by measurement of cell proliferation by MTT assay (**A**), caspase-3 activity using the fluorogenic substrate Ac-DEVD-AMC (**B**), levels of Caspase 3, PARP, and AR (N20), and actin (as loading control) by Western blot analysis (**C**), and cellular morphological changes (X100, **D**).

Fig. 2. Inhibition of cell proliferation in PC-3 and PC-3 AR cells by cisplatin and Velcade. PC-3 and PC-3 AR cells were treated with cisplatin (5–25 μ M), Velcade (5–25 nM), or DMSO vehicle (D) as control after 4 (A), 5 (B), and 6 days (C), followed by measurement of cell proliferation by MTT assay.

Fig. 3. Kinetic effect of cisplatin-induced caspase-3 activation in PC-3 but not PC-3 AR cells. **A:** Kinetic effect of caspase 3 activity in PC-3 cells. PC-3 and PC3-AR cells were treated with cisplatin at 75 μ mol for up to 24 h, DMSO (D) used as a control, followed by measurement of caspase-3 activity (**A**), levels of PARP, and AR, and actin (as loading control) by Western blot analysis (**B**), and cellular morphological changes (X100, **C**).

Fig. 4. Proteasome inhibitor Velcade induces, in a dose-dependent manner, higher levels of Caspase-3, caspase-8 and caspase-9 activation and apoptosis in PC-3 compared to nonapoptotic cell death in PC3 AR cells. PC-3 and PC3 AR cells were treated with Velcade at indicated concentrations, with DMSO vehicle as control for 24 h, followed by measurement of cell proliferation by MTT assay (**A**), proteasomal chymotrypsin-like activity with the substrate Suc-LLVY-AMC (**B**), caspase-3 activity (**C**), levels of Caspases-3, -8 and -9, PARP, AR, and actin (as loading control) by Western blot analysis (**D**), and Apoptosis induction by histone DNA ELISA (**E**).

Fig. 5. Proteasome inhibitor induces significantly higher Caspase-3 activity in parental PC-3 cells compared to PC-3 cells transiently expressing AR. Human prostate PC-3 and PC3 cells transiently expressing AR (A) were treated with either cisplatin (50 $\mu\text{mol/L}$), Velcade (0.25 $\mu\text{mol/L}$), or MG-132 (1 $\mu\text{mol/L}$) for 24 h, followed by measurement of Caspase 3 activity (B). DMSO vehicle was used as solvent control.

Fig. 6. Casodex increases Velcade-induced caspase-3-mediated cell death in AR(+) LNCaP and PC3 cells stably expressing AR. (**A**, **B**) LNCaP cells were pretreated with casodex (at 100 μ M) or DMSO for 24 h, and then co-treated with Velcade at indicated concentrations for additional 24 h, which was followed by measurement of caspase-3 activity (**A**) and levels of PARP, and AR, and actin (as loading control) by Western blot analysis (**B**). (**C**) PC-3 AR cells were pretreated with casodex (at 250 μ M) or DMSO for 24 h, and then co-treated with Velcade (at 1 μ M) for additional 24 h, which was followed by measurement of caspase-3 activity.

Fig. 7. Schematic representation of the proposed mechanism for Casodex plus proteasome inhibitor combinational therapy for prostate cancer treatment. The combination of Casodex and Velcade should cause dual inhibition of the androgen-AR-mediated survival pathway in prostate cancer cells since Casodex-mediated inhibition is *via* its binding to AR and preventing the activation of AR signaling while Velcade's action involves activation of calpain that consequently cleaves AR protein. Such combinational strategy could significantly enhance AR signaling inhibition and ultimately prostate cancer cell death, which may provide a compelling strategy for the treatment of HRPC.