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Piperlongumine induces rapid depletion of the androgen receptor in human prostate cancer cells

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Abstract

BACKGROUND—Androgen receptor (AR) signaling is regarded as the driving force in prostate carcinogenesis, and its modulation represents a logical target for prostate cancer (PC) prevention and treatment. Natural products are the most consistent source of small molecules for drug development. In this study, we investigate the functional impact of piperlongumine (PL), a naturally occurring alkaloid present in the Long pepper (Piper longum), on AR expression in PC cells and delineate its mechanism of action.

METHODS—Expression and transcriptional activity of AR was examined by Western blotting and luciferase reporter assay, respectively. CellTiter Blue assay was utilized to quantify cell proliferation. Reactive oxygen species (ROS) generation was examined by staining cells with a ROS indicator CM-H_2DCFDA, followed by flow cytometry analysis.

RESULTS—The results of our experiments demonstrate that PL rapidly reduces AR protein levels in PC cells via proteasome-mediated ROS-dependent mechanism. Moreover, PL effectively depletes a modified AR lacking the ligand-binding domain, shedding light on a new paradigm in the treatment approach to prostatic carcinoma that expresses mutated constitutively active AR. Importantly, PL effectively depletes AR in prostate cancer cells at low micromolar concentrations, while concurrently exerting a significant inhibitory effect on AR transcriptional activity and proliferation of prostate cancer cells.

CONCLUSIONS—Our investigation demonstrates for the first time that PL induces rapid depletion of the AR in prostate cancer cells. As such, PL may afford novel opportunities for both prevention and treatment of prostatic malignancy.

Keywords

Piperlongumine; ROS; proteasome; androgen receptor; prostate cancer

INTRODUCTION

Prostatic growth, both benign and malignant, is exquisitely sensitive to the male hormonal milieu. Testosterone is the primary circulating androgen in men, and its manipulation, either by suppressing release or blocking the binding to the AR, has represented a mainstay treatment for prostate cancer (PC). Nevertheless, classic androgen deprivation therapy has significant limitations, as malignant cells gain castration resistant status through a number of mechanisms [1–3]. Therapeutic failure is often accompanied by AR overexpression as well as the presence of constitutively active AR variants lacking the AR ligand-binding domain (ARΔLBD) [4–7]. Furthermore, mutated ARs can be activated by non-androgenic ligands,

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such as anti-androgens [8]. These findings reinforce arguments that direct targeting of AR rather than its ligands could be an advanced strategy for both prevention and treatment of PC [9]. Indeed, recent clinical studies demonstrate that inhibition of AR signaling reduces PC risk [10].

Naturally-occurring products provide consistent source of drug development. A multitude of antineoplastic agents, including docetaxel, vindesine, vinorelbine, etoposide, and topotecan, have progressed to clinical use based on developments of natural products. A number of studies suggest that dietary constituents may play an important role not only in cancer management but also its prevention [11,12]. Peppers, from the genus Piper (Piperaceae), are the most common spices consumed worldwide and have a wide array of biologically active secondary compounds [13]. Piperlongumine (PL), a natural alkaloid abundantly present in the fruit of the Long pepper (Piper longum), shows several noteworthy biological activities. This compound has insecticidal and antibacterial properties in addition to its ability to inhibit ethanol-induced gastric lesions in experimental animal models [14,15]. Recent studies demonstrate that PL can also inhibit growth of tumor cells of various origins both *in vitro* and *in vivo* [13,16]. Importantly, administration of PL does not cause any obvious adverse effects [13,16].

Our work demonstrates for the first time that PL induces rapid AR depletion in prostate cancer cells through a proteasome-mediated ROS-dependent pathway, which coincides with reduced functional activity of AR signaling. Thus, PL has the potential to inhibit prostate carcinogenesis at both initiation and advanced disease stages via depletion of the AR alone or in combination with androgen deprivation therapy (ADT) regimens, affording novel therapeutic opportunities.

MATERIALS AND METHODS

Cells and culture conditions

LNCaP and PC-3 human prostate cancer cells were obtained from ATCC (Rockville, MD). Cells were cultured in RPMI 1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), gentamicin (50 mg/l), sodium pyruvate (1mM) and non-essential amino acids (0.1mM) under conditions indicated in the figure legends.

Antibodies and Reagents

Antibody to actin, N-Acetyl Cysteine, bisphenol A (BPA) and arsenic (III) oxide were obtained from Sigma (St. Louis, MO). Antibodies to androgen receptor, Akt and ubiquitin were obtained from Cell Signaling Technology (Beverly, MA). Piperlongumine was obtained from Indofine Chemical Company (Hillsborough, NJ). MG132 and bortezomib were obtained from Biomol (Plymouth Meeting, PA).

Expression of ARΔLBD in prostate cancer cells

To generate the ARΔLBD expression vector, the truncated AR ORF was amplified with specific forward 5′-ATCTTGGGATCCATGGAAGTGCAGTTAGGGCTGG-3′ and reverse 5′-ATCTTGATCGATTTATCCCAGAGTCATCCCTGCTTCATAAC-3′ primers carrying BamH1 and Cla1 restriction sites (underlined) using as template pEGFP-C1-AR vector (Plasmid ID: 28235) containing full-length AR (Addgene, Cambridge MA) and then cloned into BamH1/Cla1 sites of pEBB-HA vector as previously described [17]. Cells were transfected with ARΔLBD expression vector using the TransIT-Prostate transfection kit (Mirus Bio, Madison, WI).

Immunoprecipitations and Western Blot Analysis

Following treatment, LNCaP cells were lysed in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA). For immunoprecipitation (IP) whole-cell lysate (1 mg) was precipitated with either anti-AR antibody (4 μg) or normal mouse IgG at +4°C overnight. Immunocomplexes were recovered by incubating with 60 μl of Protein A/G Plus-Agarose (Santa Cruz Biotechnology) for 1h at +4°C and washed 5 times in RIPA buffer. Samples were subjected to SDS-PAGE, followed by standard immunoblot analysis. Whole cell lysates preparation and Western Blot Analysis were performed as described previously [18].

Real Time PCR

Total RNA was isolated from LNCaP cells using Mini RNA isolation II Kit (Zymo Research, Orange, CA) and purified using RNA Clean and Concentrator Kit (Zymo Research). Total RNA (1 μg) was reverse transcribed in a final volume of 20 μl with 100 U of Superscript III Reverse Transcriptase (Invitrogen, Gaithersburg, MD) and 75 ng of random hexamer primers according to the manufacturer's instructions. After reverse transcription, cDNA samples were diluted 40 times and 5 μl of diluted cDNA was amplified by real time PCR using AR TaqMan Gene Expression Assay (ID# Hs00171172_m1). Custom GAPDH Mini qPCR assay (IDT DNA Technologies, Coralville, IA) was used as an internal amplification control. The amplicon was detected with a forward primer 5′-ACATCGCTCAGACACCATG, reverse primer 5′-TGTAGTTGAGGTCAATGAAGGG and probe 5′-AAGGTCGGAGTCAACGGATTTGGTC labeled with 6-FAM and quenched with Jowa-Black FQ. Each sample was run in triplicate for both AR and GAPDH in 20 μl reaction mix using TaqMan Gene Expression Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Reactions were carried out in an Applied Biosystems 7500 Real-Time PCR System. Analysis of relative AR expression was carried out using the$2^{-\Delta\Delta Ct}$ method.

Measurement of cell proliferation

Effect of piperlongumine on cell proliferation was analyzed by CellTiter Blue assay (Promega, Madison, WI). Effective doses (ED) were calculated using XLfit, a Microsoft Excel add-in.

Luciferase reporter assay

The luciferase reporter vector was designed by subcloning the ARR2PB promoter into the pGL3-basic vector. The pBluescript-II SK+ containing the ARR2PB promoter (a kind gift from Dr. Robert J. Matusik) (15) and pGL3-basic vectors were digested with BamH1 and Xho1 restriction enzymes, respectively. After the ends were blunted with T4-DNA polymerase both plasmids have been digested with Kpn1. The insert from pBluescript-II SK + then was cloned into the pGL3-basic vector. LNCaP cells were transfected with pGL3-AR-luc and phRL-TK (Renilla) plasmids. Twenty-four hours after transfection cells were treated with PL (10μM) in the presence or absence of R1881 (0.1 nM), a synthetic androgen agonist, in medium supplemented with charcoal-stripped serum for 6 hours. Samples were assayed for firefly and renilla luciferase activities using the Dual-Glo Luciferase assay (Promega, Madison, WI).

Analysis of ROS generation

Cells were re-suspended in PBS containing 5μM CM-H_2DCFDA (Invitrogen, Carlsbad, CA), incubated at 37°C in the dark for 15 minutes and analyzed by flow cytometry. Analysis was performed using FACScan (Becton Dickinson, Franklin Lakes, NJ). Individual fluorescent populations were determined through the use of acquisition and analysis software (Cell Quest, Becton Dickinson).

Statistical analysis

Statistical analysis was performed using a two-sided Student's t-test. A p-value of <0.05 was considered statistically significant.

RESULTS

PL induces depletion of AR in prostate cancer cells

AR signaling is regarded as the main oncogenic driver in prostate carcinogenesis. Thus, modulation of AR activation and/or expression represents a logical target for PC prevention and treatment. AR-mediated functions are not completely abrogated by existing hormone therapies. Therapeutic failure is often accompanied by molecular alterations of the AR, including AR overexpression as well as expression of constitutively active AR variants lacking the AR ligand-binding domain (ARΔLBD). To establish the effect of PL (Fig. 1) on AR levels in prostate cancer cells, Western blot analysis was performed using cell lysates of androgen-dependent LNCaP cells treated with increasing concentrations of PL. As demonstrated in Figure 2A, PL effectively depletes AR in LNCaP cells at low micromolar concentrations (≥ 5 μM). Furthermore, depletion of the AR was evident as soon as 3 hours following PL administration (Figure 2B). A parallel experiment was conducted with LNCaP cells transfected with a modified androgen receptor lacking the ligand-binding domain (ARΔLBD) (Fig. 2C). As demonstrated in Figure 2D, PL effectively depletes ARΔLBD, shedding light on a unique new pathway in the treatment approach to prostatic carcinoma that expresses mutated AR domains. To strengthen our findings, we conducted a similar experiment using castration resistant PC-3 prostate cancer cell line transfected with ARΔLBD construct. These results, presented in Figure 2D, demonstrate that PL-mediated modulation of AR is not cell line specific.

PL induces AR depletion at post-transcriptional level

To explore the mechanism of PL-mediated AR depletion, we examined whether PL modulates expression levels of AR mRNA in prostate cancer cells. As shown in Figure 3A and B, decrease of AR mRNA coincided with depletion of AR protein level as measured both on concentration and time scales.

To discriminate whether PL promotes AR degradation or prevents AR synthesis, LNCaP cells were pre-incubated with emetine, a known inhibitor of protein synthesis, followed by treatment with PL. At various times following treatment, LNCaP cells were harvested and expression of the AR was examined by Western blotting followed by densitometry scanning (Fig. 4A and B). Treatment with emetine alone for up to six hours had no visible effect on AR protein levels. In contrast, treatment with PL, induced rapid depletion of the AR in LNCaP cells, suggesting that PL induces active degradation of AR protein rather than blocking its synthesis. Notably, PL-mediated effect was selective for the AR, while not having any impact on the expression of other Hsp90 client proteins, namely estrogen receptor beta (ER-β) and Akt (Fig. 4A). Similar results were obtained with PC-3 cells transfected with ARΔLBD (Fig. 4C and D).

A potential mechanism explaining PL-induced decrease of the AR at the protein level may relate to its increased degradation via the ubiquitin-proteasome pathway. To investigate this hypothesis, we pre-incubated LNCaP cells with two proteasome inhibitors, MG-132 and bortezomib. The results presented in Figure 4E demonstrate that both inhibitors induce accumulation of slowly migrating forms of the AR with typical ubiquitinated patterns, implying that PL-mediated depletion of AR occurs via a proteasome-mediated mechanism. Our hypothesis was further validated by additional experiments employing the IP assay demonstrating ubiquitinization of AR following administration of PL (Fig. 4F).

PL down-regulates AR transcriptional activity and suppresses proliferation of LNCaP cells

Next, we assessed the effect of PL on functional potential of PC cells. Our data provide evidence that PL-mediated depletion of the AR coincides with considerable inhibition of AR transcriptional activity in LNCaP cells (Fig. 5A). Furthermore, our experiments demonstrate that PL exerts a significant inhibitory effect on proliferation of LNCaP cells as determined by CellTiter Blue assay (Fig. 5B).

PL induces AR depletion via ROS-dependent mechanism

Recent study by Raj et al. elucidates that PL-induces apoptotic cell death in sarcoma and mammary tumors is directly linked to elevated levels of ROS [16]. Indeed, the addition of PL causes a marked rise in ROS levels in LNCaP cells (Fig. 6A). N-acetyl-L-cysteine (NAC) is an anti-oxidant known to lower cellular ROS levels. Co-administration of NAC and PL reversed PL-mediated increase of ROS and completely blocked degradation of AR protein (Fig 6A and B). Additionally, we examined alternate compounds, bisphenol A and arsenic trioxide, known to raise the level of ROS in cells [19,20]. They were administered to LNCaP cells, with the resultant Western blot showing evidence of increased AR degradation (Fig. 6C). This finding offers additional support to our hypothesis that an increase in ROS levels plays a pivotal role in PL-mediated AR protein depletion in prostate cancer cells.

DISCUSSION

Prostate cancer harbors profound sensitivity to the body's androgen milieu and is considered the most endocrine-dependent solid neoplasm [21]. Since androgens are essential oncogenic promoters in prostatic carcinoma, modulation of androgen signaling pathways represents a rational approach to prostate cancer therapy. Although ADT remains the mainstay therapy for patients with both localized and advanced disease, in most patients PC eventually progresses to a castration-resistant form (CRPC) [22]. Aberrant signaling of androgen-dependent pathways likely plays a major role in the mechanism of ADT resistance. For instance, several xenograft models have demonstrated that AR up-regulation is invariably the most consistent alteration that takes place during progression to CRPC (18). AR up-regulation can be modulated in the short-term with administration of non-steroidal anti-androgens, such as bicalutamide [23]. Despite short-term biochemical benefits of AR targeting with bicalutamide, true clinical benefits of this therapy in patients with CRPC are inconsequential.

Novel therapeutic regimens targeting the AR axis have been investigated in the recent years including abiraterone acetate and MDV3100. Abiraterone acetate serves as an irreversible inhibitor of cytochrome P450-17 (CYP 17) with concomitant 17α-hydroxylase blockade resulting in an inhibitory effect on both adrenal and intra-tumor androgens (20, 21). Despite its apparent efficacy, abiraterone predisposes patients to certain toxicities resulting from homeostatic adjustments to testosterone loss. The response entails up-regulation of the hormonal milieu, importantly adrenocorticotropic hormone, which triggers elevations in blood pressure. This particular side effect is generally controlled by administration of corticosteroids, which themselves lead to fluid retention, liver function abnormalities, hypopotassemia and cardiac dysfunction. In contrast, MVD3100 has no direct effect on hormone synthesis, while exerting its effect via several alternate mechanisms. Its action pivots on competing with testosterone for binding to AR, blocking nuclear translocation and DNA binding of the hormone-receptor complex [24]. Because MVD3100 has no effect on hormone synthesis, it can be given without concomitant administration of steroids, reducing the potential for side effects [24]. Nevertheless, not all patients respond to MDV3100 treatment. In fact, resistance develops in many initial responders potentially due to the expression of drug-resistant AR mutants [25].

Compelling evidence suggests that direct targeting of AR rather than its ligands could be a novel and effective strategy for both primary and secondary prevention of PC [9]. Indeed, mutations in the AR can result in altered ligand specificity and AR amplification. In addition, AR hyper-activation can occur without any androgen binding due to expression of constitutively active AR variants lacking the carboxy-terminal ligand-binding domain (2, 21). Furthermore, mutated AR can be activated by non-androgenic ligands such as anti-androgens [8]. A handful of natural products have been shown to induce AR degradation. Recent reports demonstrate that some phytochemicals such as berberine and isosilybin B induce AR degradation in PC cells [26,27]. Berberine acts by disrupting interaction of AR with heat shock protein 90 (Hsp90), a major chaperone for the AR [27], whereas isosilybin B functions via PI3K-Akt-Mdm2-mediated pathway [26]. Albeit promising, these agents likely do not possess the most favorable bioavailability and cytotoxicity profiles [28–30].

Our investigation is the first to demonstrate that PL, a naturally occurring alkaloid present in the Long pepper (Piper longum), induces rapid depletion of the AR in prostate cancer cells. Degradation of AR occurs through a ROS-mediated proteasome-dependent mechanism. Importantly, PL effectively depletes AR in prostate cancer cells at low micromolar concentrations with an evident decrease as early as 3 hours, while concurrently exerting a significant inhibitory effect on AR transcriptional activity with decrease in proliferation of prostate cancer cells. Our data are in agreement with recent findings by Raj et al. demonstrating that PL inhibits growth of tumor cells of various origins both *in vitro* and *in vivo* by triggering increased ROS production [16]. Notably, administration of PL has no major impact on biochemical, hematological and histopathological parameters in experimental animals [13,16]. Although our investigation suggests that PL-mediated depletion of AR parallels a decrease in proliferation of PC cells, our data do not prove causality and, as such, do not exclude the possibility that PL affects other important cellular pathways.

CONCLUSIONS

In summary, natural products are the most consistent source of small molecules for drug development. Our work describes original findings that naturally occurring alkaloid, piperlongumine, induces AR depletion in prostate cancer cells through a ROS-mediated proteasome-dependent mechanism. Given PL's extremely favorable cytotoxic profile [16], it offers novel therapeutic opportunities for both prostate cancer prevention and treatment.

Acknowledgments

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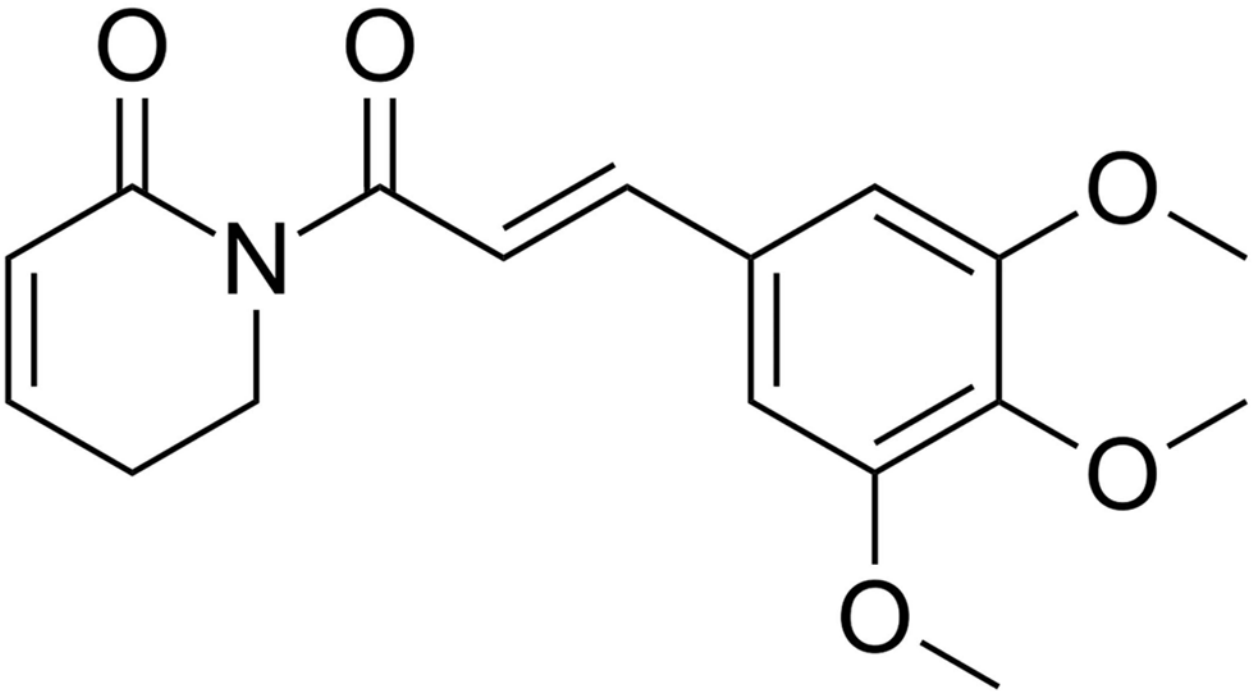

Figure 1.
Chemical structure of PL molecule.

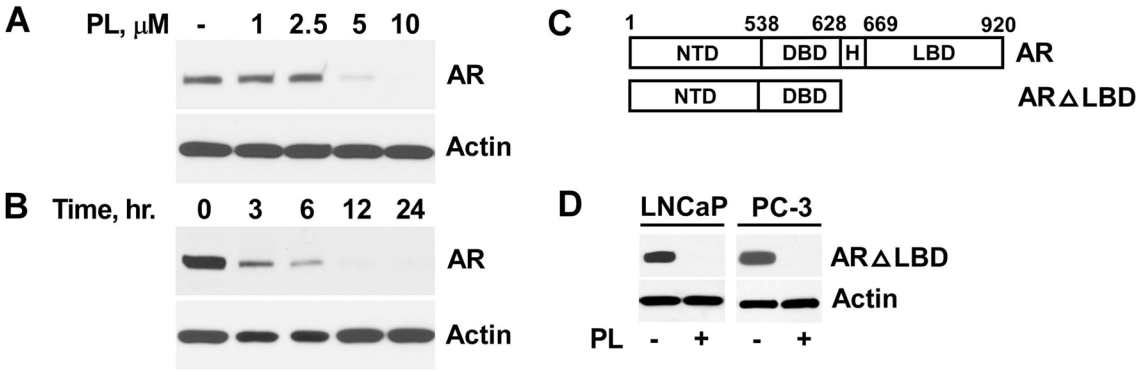

Figure 2.

PL down-regulates the expression of AR in prostate cancer cells. (A) PL induces dose-dependent reduction of AR protein levels in LNCaP cells. Cells were treated with PL for 3 hours. Cell lysates were subjected to SDS-PAGE, blotted, and probed with specific antibodies. (B) Time course of AR protein depletion in LNCaP cells treated with PL (10μM). Cells were cultured in the presence of PL for the indicated periods of time. Expression of AR and actin was detected by immunoblotting with antiAR or anti-actin antibodies respectively. (C) Structural domains of full length human AR and ARΔLBD. (D) LNCaP and PC-3 prostate cancer cells were transfected with a plasmid encoding ARΔLBD. Cells were treated with PL (10μM) for 3 hours. Expression of ARΔLBD was examined by Western blot analysis.

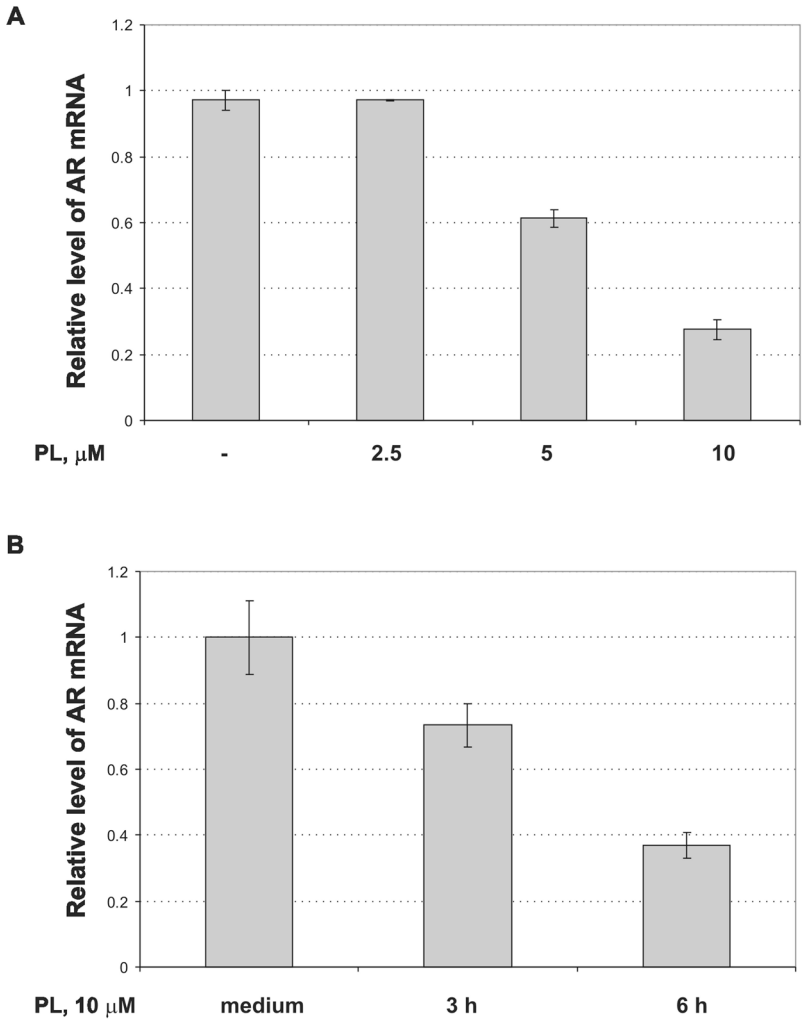

Figure 3.

Levels of AR mRNA in LNCaP cells treated with PL. (A) Cells were treated with indicated concentrations of PL. AR mRNA levels were detected by Real Time PCR using specific primers. (B) Cells were treated with PL (10μM) for the indicated periods of time. AR mRNA levels were detected by Real Time PCR.

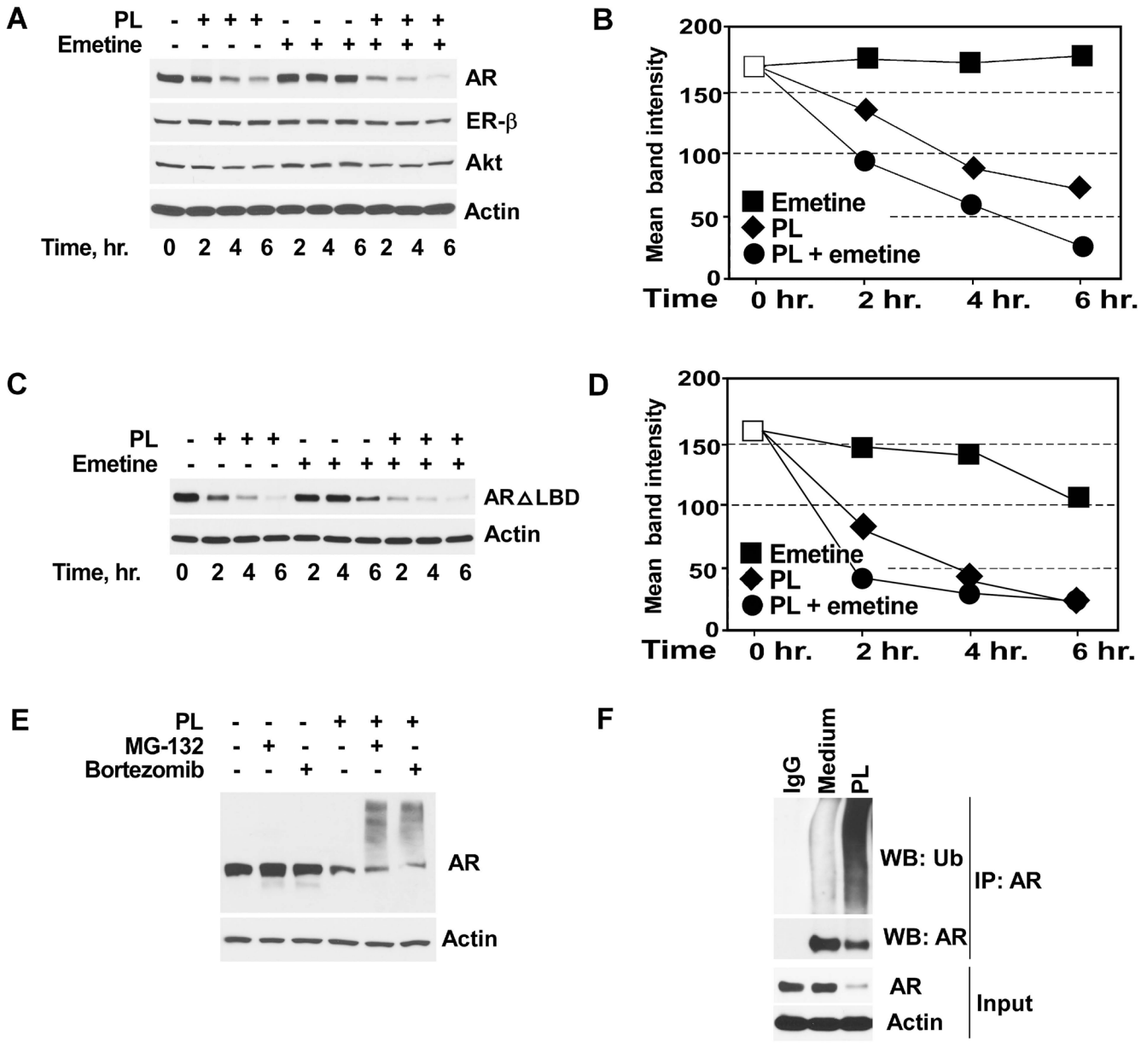

Figure 4.

PL induces depletion of AR at post-transcriptional level. (A) AR protein levels were determined by Western blot analysis after incubation of LNCaP cells with PL (10μM) with or w/o emetine (10μM) for the indicated periods of time. (B) Densitometry readings of the immunoblot shown in panel A. The intensity of the signal was quantitated by ImageJ 1.45i software. (C) PC-3 cells transfected with ARΔLBD vector were incubated with PL (10μM) with or w/o emetine (10μM) for the indicated periods of time. Protein levels were determined by Western blot analysis. (D) Densitometry readings of the immunoblot shown in panel C. The intensity of the signal was quantitated by ImageJ 1.45i software. (E) Co-treatment with proteasomal inhibitors, MG-132 and bortezomib, induces accumulation of slow-migrating ubiquitinated forms of AR in PL-treated LNCaP cells. Cells were treated with PL (10μM) with or w/o MG-132 (10μM) or bortezomib (10μM) for 3 hours. Expression of AR and actin was detected by immunoblotting with anti-AR or anti-actin

antibodies respectively. (F) LNCaP cells were treated with PL (10μM) for 3 hours. Cell lysates were subjected to IP with antibodies to AR followed by Western blot analysis using antibody to ubiquitin. Aliquots of cell lysates (Input) were also subjected to Western blot analysis.

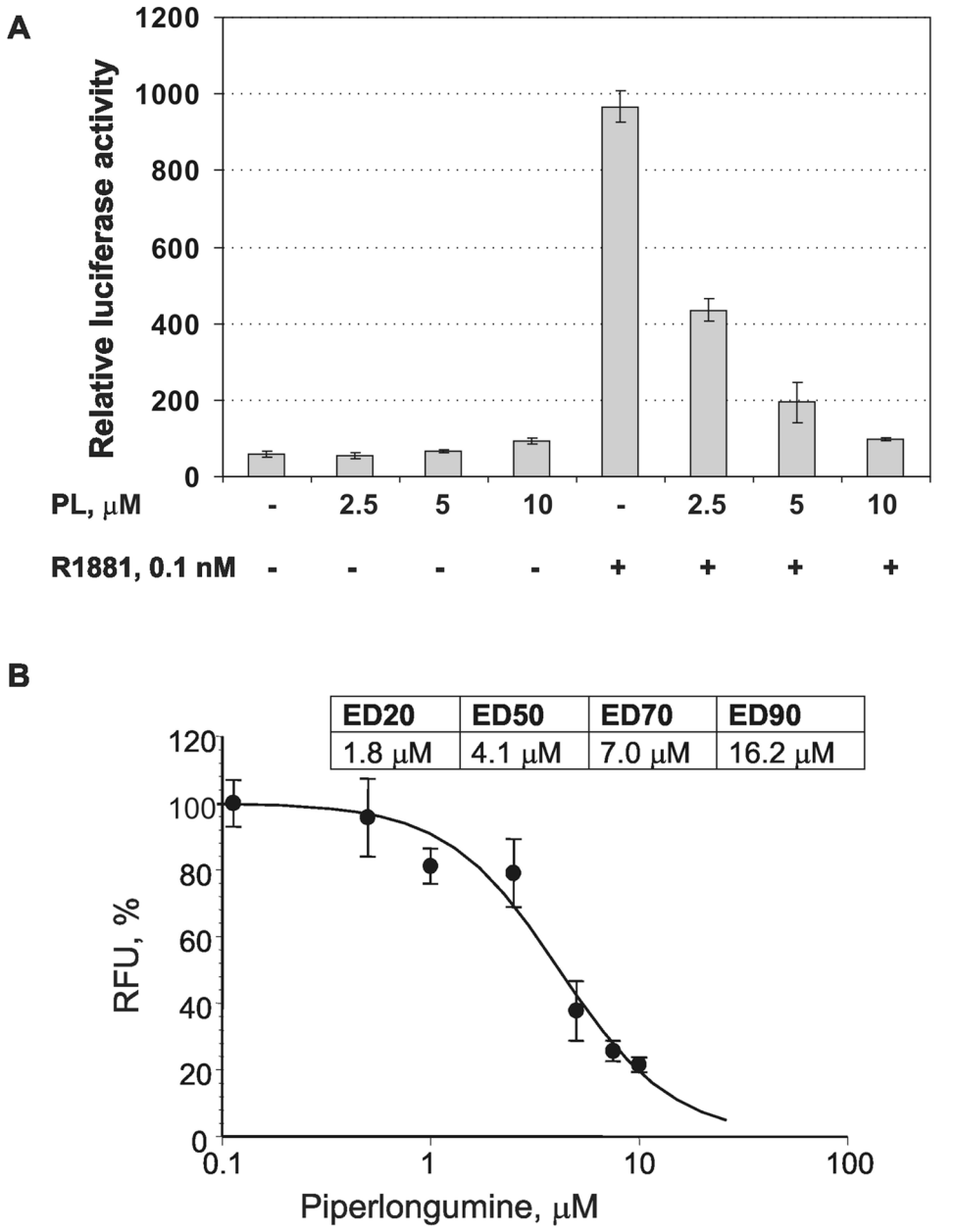

Figure 5.

PL attenuates the transcriptional activity of the AR and suppresses proliferation of LNCaP cells. (A) Analysis of transcriptional activity was performed as described in Materials and Methods. Columns, means of three different experiments; bars, SDs. (B) LNCaP cells cultured in the presence of escalating concentrations of PL for 48 hours. The effect of PL on cellular proliferation was analyzed by CellTiter Blue assay. EDs were calculated as described in Materials and Methods.

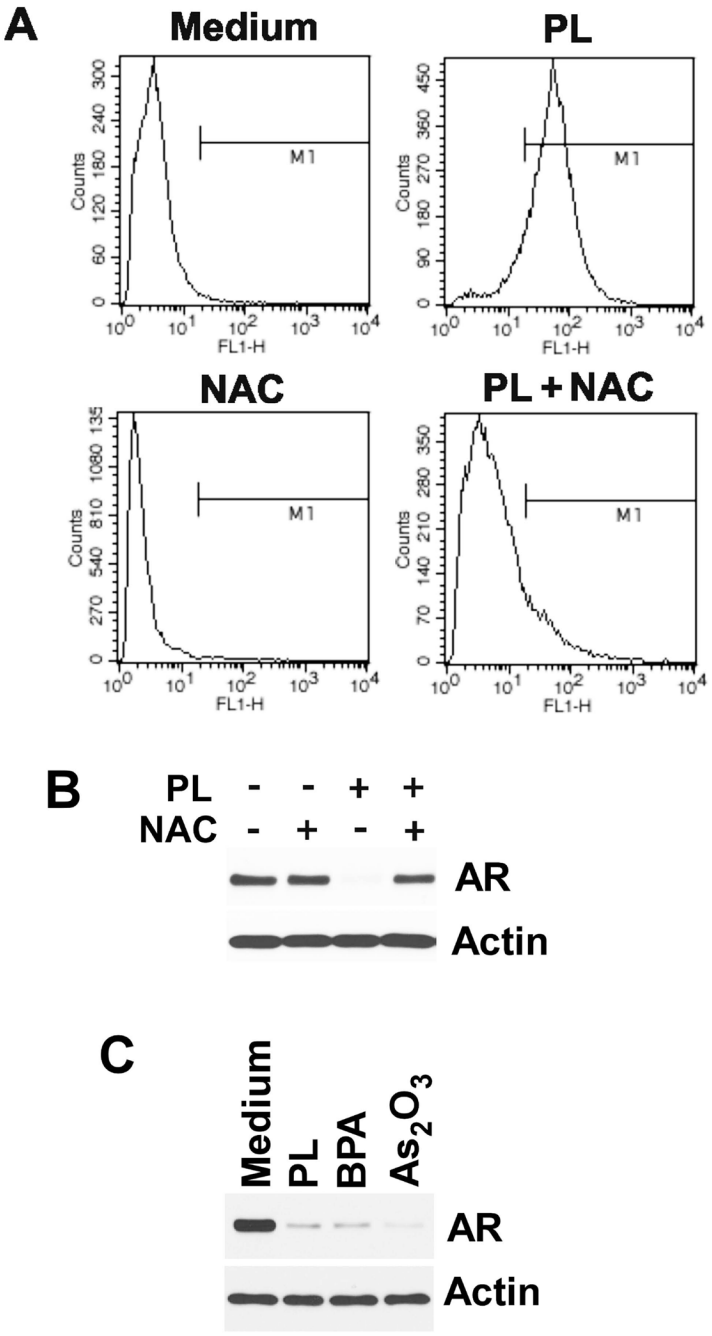

Figure 6.

PL reduces AR protein levels via ROS-dependent mechanism. (A) PL induces ROS production in LNCaP cells. LNCaP cells were treated with PL (10μM) with or w/o NAC (5mM) for 1 hour, stained with CM-H_2DCFDA and analyzed by flow cytometry as described in Materials and Methods. (B) Cells were treated with PL (10μM) with or w/o NAC (5mM) for 3 hours. Expression of AR and actin was detected by immunoblotting with anti-AR and anti-actin antibodies respectively. (C) Cells were treated with PL (10μM), bisphenol A (100μM) or arsenic (III) oxide (25μM) for 3 hours. Expression of AR and actin was detected by immunoblotting with anti-AR and anti-actin antibodies respectively.