Differential targeting of androgen and glucocorticoid receptors induces ER stress and apoptosis in prostate cancer cells

A novel therapeutic modality

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Androgen (AR) and glucocorticoid (GR) receptor signaling play opposing roles in prostate tumorigenesis: in prostate, AR acts as an oncogene, and GR is a tumor suppressor. Recently, we found that non-steroidal phyto-chemical compound A (CpdA) is AR/GR modulator acting as anti-inflammatory anti-androgen. CpdA inhibits AR and prevents GR transactivation while enhancing GR transrepression. GR and AR are controlled by proteasomal degradation. We found that prolonged exposure of LNCaP, LNCaP-GR, DU145 and PC3 prostate carcinoma (PCa) cells to proteasome inhibitor Bortezomib (BZ) caused AR degradation and GR accumulation. BZ enhanced CpdA ability to inhibit AR and to augment GR transrepression. We also found that CpdA+BZ differentially regulated GR/AR to cooperatively suppress PCa cell growth and survival and to induce endoplasmic reticulum stress (ERS). Importantly, CpdA+BZ differentially regulated GR-responsive genes. CpdA+BZ blocked activation of glucocorticoid-responsive pro-survival genes, including SGK1, but activated BZ-induced ERS-related genes BIP/HSPA5 and CHOP/GADD153. Using ChIP, we showed that SGK1, BIP/HSPA5 and CHOP regulation was due to effects of CpdA and CpdA+BZ on GR loading on their promoters. We also found that AR and GR are abundant in advanced PCa from patients treated by androgen ablation and/or chemotherapy: 56% of carcinomas from treated patients expressed both receptors, and the other 27% expressed either GR or AR. Overall, our data validate the concept of dual AR/GR targeting in prostate cancer (PC) and suggest that BZ combination with dual-target steroid receptor modulator CpdA has high potential for PC therapy.

Introduction

Steroid hormone receptors are pivotal in the development of prostate cancer (PC). The androgen receptor (AR) promotes tumorigenesis, and androgen ablation has been the cornerstone treatment for local and metastatic PC.[1-4] In contrast, glucocorticoid receptor (GR) acts as a tumor suppressor in the prostate.[5-9] Here, we propose a novel strategy of simultaneous inhibition of AR and activation of GR signaling for PC treatment.

GR and AR are closely related transcription factors from a superfamily of nuclear hormone receptors.[1,10,11] Upon binding their cognate steroid ligands, AR and GR dissociate from the cytoplasmic chaperone proteins and translocate to the nucleus, where they form homodimers and bind palindromic hormone response elements (HRE) to activate gene expression (transactivation).[1,10-13] Negative regulation of gene expression (transrepression) by steroid hormone receptors typically does not require DNA binding.[14-16] Instead, GR directly interacts with multiple

transcription factors (TFs), including NFκB, AP-1 and p53.[14-16] It is widely accepted that GR transrepression underlies anti-inflammatory activity of glucocorticoids.[11,15] We recently showed that GR transrepression is critical for its tumor suppressive effects.[9,17] AR also interacts with some TFs, including NFκB and AP-1, and modulates their activity.[18,19]

Given AR and GR opposing roles in prostate tumorigenesis, dual steroid receptor modulators that act as anti-androgens and simultaneously promote transrepression by GR would make ideal therapy for PC. We and others recently characterized a novel non-steroidal AR/GR ligand, Compound A (CpdA), with desired properties, a synthetic analog of the compound from Namibian shrub *Salsola tuberculatiformis* Botschantzev.[20-22] CpdA inhibits AR function and prevents GR homodimerization/transactivation but activates GR-mediated transrepression.[20,23-25] We found that CpdA strongly inhibits proliferation and viability of prostate carcinoma (PCa) cells through altering the activity of both AR and GR.[20] Importantly, in vivo CpdA preserves therapeutic activity of the glucocorticoids but has fewer side effects.[21,23,25]

The 26S proteasome is a central component of the ubiquitin-proteasome system responsible for the degradation of damaged misfolded cellular proteins.[26,27] In addition, proteasome is also involved in control of expression of numerous proteins with high turnover, including steroid hormone receptors GR and AR.[28-33] Since proteasome inhibitors were reported to inhibit AR and to activate GR,[29,33] we hypothesized that they may augment CpdA's effects as a dual AR/GR modulator and enhance its toxicity for PCa cells.

In this study, we assessed the effect of the first-in-class FDA-approved proteasome inhibitor Bortezomib (BZ) on (1) the AR and GR stability and function in the presence of CpdA and AR/GR steroid ligands, (2) CpdA properties as a dual AR/GR modulator and (3) CpdA's effect against PC.

We showed that BZ, indeed, increased AR blockade by CpdA and further enhanced CpdA-driven GR transrepression. We also revealed a striking cooperation between CpdA and BZ in suppressing growth and survival of PCa cells by modulating GR and AR. Moreover, we discovered that CpdA induced endoplasmic reticulum stress (ERS), which was also augmented by BZ. This cooperation was due to unexpected differential regulation of promoter occupancy by GR. CpdA alone and in combination with BZ was unable to induce glucocorticoid-responsive pro-survival genes, such as SGK1, that are activated by glucocorticoids. In contrast, CpdA further increased BZ activation of GR-dependent transcription of ERS-related genes BIP/HSPA5 and CHOP/GADD153. In addition, AR and GR were expressed at high levels in advanced PCa from the patients treated with androgen ablation and chemotherapy. Overall, our data provide the compelling rationale for the proposed concept of AR/GR targeting in PC and suggest that the combination of BZ with a dual-target steroid receptor modulator CpdA may be successful for PC therapy.

Results

AR and GR are highly expressed in human PCa after therapy. To validate our proposed concept of PC treatment by dual AR/ GR targeting, it was critical to analyze AR and GR expression in PC patients. AR is highly expressed in most PCa regardless of the disease stage.[1-3] Recently, we and others found that GR expression was lost in most PCa from untreated patients.[9,34] However, GR expression in PCa from the patients that underwent therapy remains unknown. Using immunostaining, we analyzed AR and GR expression in PCa from 45 patients after hormone ablation (flutamide, casodex) or chemotherapy (docetaxel and dexamethasone) received at Northwestern University or at the National Cancer Research Center (**Fig. 1 and Sup. 2**).

In stark contrast to untreated PCa, GR was expressed at high levels, with nuclear localization in ~60% of treated tumors regardless of Gleason score (**Fig. 1B**). Importantly, we detected that 56% PCa obtained from treated patients expressed both receptors. Moreover, AR and GR were frequently co-expressed within the same tumor compartment (**Fig. 1C**). The other 27% PCa expressed either GR or AR (**Fig. 1C**). This finding strongly supports the feasibility of proposed AR/GR targeted therapy for advanced PC.

Differential AR and GR regulation by Bortezomib contributes to Bortezomib cytotoxicity. The stability and transcriptional activity of AR and GR can be affected by proteasome inhibitors.[28-33] However, their effect on AR and GR in PCa cells simultaneously expressing both steroid receptors has never been studied. Most of the currently known PCa cell lines express only one of the two receptors (**Sup. 4**). Therefore, we used a model cell line LNCaP-GR that expresses endogenous AR and exogenous GR introduced by lentiviral transduction.[9]

BZ differentially regulated AR and GR protein levels in LNCaP-GR and in other PCa cells in a concentration range between 10^{-6}–10^{-8} M (**Fig. 2A.1**). Prolonged 24–32 h BZ treatment caused significant GR upregulation and drastic downregulation of nuclear and cytoplasmic AR (**Fig. 2A.2**).

Remarkably, BZ-induced downregulation of AR in both parental LNCaP and in LNCaP-GR cells as well as upregulation of GR in LNCaP-GR cells correlated with the levels of BZ-induced apoptosis assessed by PARP cleavage (**Fig. 2C**). Moreover, downregulation of GR in LNCaP-GR cells by GR-shRNA delivered by lentivirus (LNCaP-GR-shGR cells) significantly attenuated BZ-induced apoptosis (**Fig. 2D**). The role of steroid hormone receptors in BZ's anticancer effect has never been considered, and our experiments are the first to suggest AR and GR as critical mediators of BZ therapeutic effect.

Bortezomib increases compound A anticancer activity. Since BZ downregulated AR and induced GR accumulation, we hypothesized that (1) BZ may further enhance the effect of AR/GR dual modulator CpdA in PCa cells, and (2) combining BZ and CpdA may decrease their effective doses. To determine optimal concentrations for the combined treatment, we evaluated dose-dependent cytostatic and cytotoxic effects of CpdA and BZ (10^{-5}–10^{-10} M) in LNCaP-GR cells (**Fig. 3A.1**). In all further experiments, we used concentrations causing 20–25% growth inhibition (5 x 10^{-7} M CpdA and 10^{-8} M BZ).

We observed remarkable cooperation of CpdA and BZ to induce growth suppression and caspase-dependent apoptosis (**Fig. 3**). In LNCaP-GR, DU145 and PC3 cells' growth inhibition

Figure 1. AR and GR are highly expressed in PCa from patients after androgen ablation or chemotherapy. Expression of AR (A.1–A.6) and GR (B.1–B.6) in benign prostate (A.1 and B.1), prostate carcinoma from untreated patient (A.2 and B.2) (as in ref. 9 or **Sup. 2**), prostate carcinomas from patients treated with androgen ablation or chemotherapy (A.3–A.6 and B.3–B.6). (C) AR and GR expression in PCa from treated patients. The number of prostate epithelial cells with either GR or AR nuclear signal was evaluated by + to +++ scoring. Tumors with GR/AR staining intensity ++/+++ were considered GR+ or AR+ respectively. Note: (1) extensive nuclear AR and GR expression in benign prostate; absence of GR in untreated PCa and high GR and AR nuclear staining in treated PCa tissues; (2) overlapping patterns of expression of AR and GR in PCa from treated patients.

and apoptosis due to CpdA+BZ combination far exceeded that caused by FA+BZ (**Fig. 3B and C and Sup. 5**).

We also found that GR strongly contributed to cytotoxic and cytostatic effects of CpdA+BZ combined treatment. This was suggested by higher sensitivity of AR+/GR+ LNCaP-GR cells to CpdA+BZ growth inhibition compared with AR+/GR- LNCaP-V control cells (**Fig. 3A.2**). Furthermore, 50–60% GR knockdown in LNCaP-GR-shGR cells diminished growth inhibition and apoptosis by CpdA+BZ (**Fig. 3A.2 and B.1**).
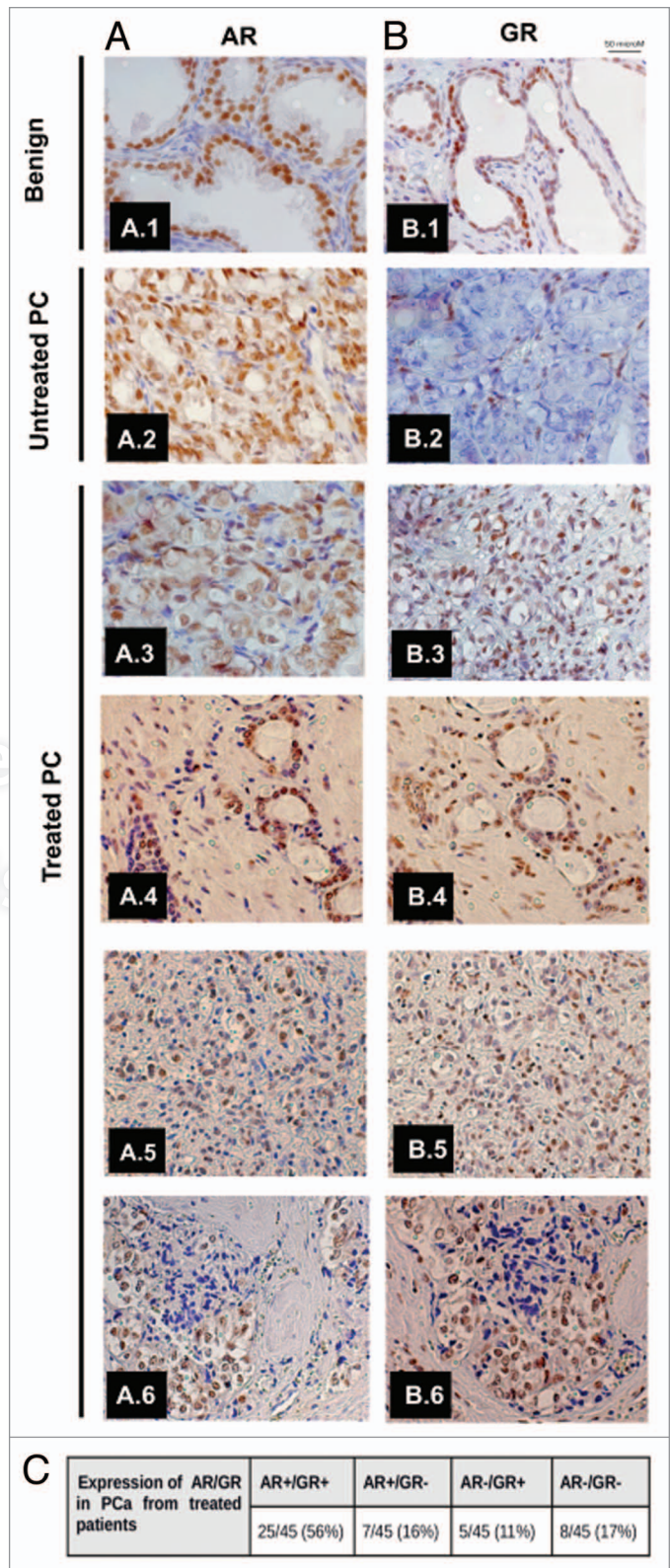
Overall, our data demonstrate significant anticancer effects of low doses of CpdA and BZ that have weak effect/no effect in stand-alone treatments. In addition, we show for the first time that both receptors are important targets for BZ+CpdA in PC.

Bortezomib enhances compound A properties as an AR inhibitor and selective GR modulator. Next, we tested our hypothesis that BZ enhances CpdA ligand properties as an anti-androgen and selective GR modulator. We found that in LNCaP-GR and LNCaP-V cells, BZ downregulated AR activity, which reflected AR protein decrease (**Fig. 4A.2, B.2 and C.2**). Consistent with the literature,[34] DHT protected AR from downregulation by BZ and largely restored its functional activity (**Fig. 4**). In contrast, CpdA did not preclude AR degradation and failed to restore its function in the presence of BZ (**Fig. 4A.2–C.2**).

BZ alone induced GR nuclear translocation. However the increase in GR activity was modest, significantly lower than that induced by glucocorticoid FA (**Fig. 4A.1, B.1 and C.1**), suggesting that a large portion of GR accumulated in BZ-treated cells remained inactive in the absence of ligand. Exposure to FA+BZ caused nuclear accumulation of fully functional GR with high DNA binding and transcriptional activity (**Fig. 4A.1–C.1**). In contrast, the treatment with CpdA+BZ resulted in decreased GR nuclear translocation, low DNA binding and transactivation potential (**Fig. 4A.1–C.1**).

To extend these findings, we assessed how BZ combined with different GR ligands affects the expression of endogenous GR target genes (**Fig. 5A and B**). Using promoter screening for putative GR binding sites, we selected the following four genes: the GR and AR regulator FKBP5,[35] pro-survival kinase SGK1[36] and ERS regulators central for BZ-dependent apoptosis BIP/HSPA5 and CHOP/GADD153.[37,38] All four promoters contained GR biding sites within 2,500 bp of the start codon (**Sup. 3**).

As shown by SQ-RT-PCR, the expression of FKBP5 and SGK1 was unaffected or weakly induced by CpdA or CpdA+BZ but strongly induced by FA or FA+BZ (**Fig. 5A**). BZ activated expression of early ERS marker BIP, whose expression was further increased by both GR ligands (**Fig. 5A**). Unexpectedly, the expression of known BZ target ERS- and apoptosis-related CHOP/GADD153 was stimulated by CpdA (**Fig. 5A**) despite its known inability to activate GR-dependent genes.[20,23,25] Moreover,
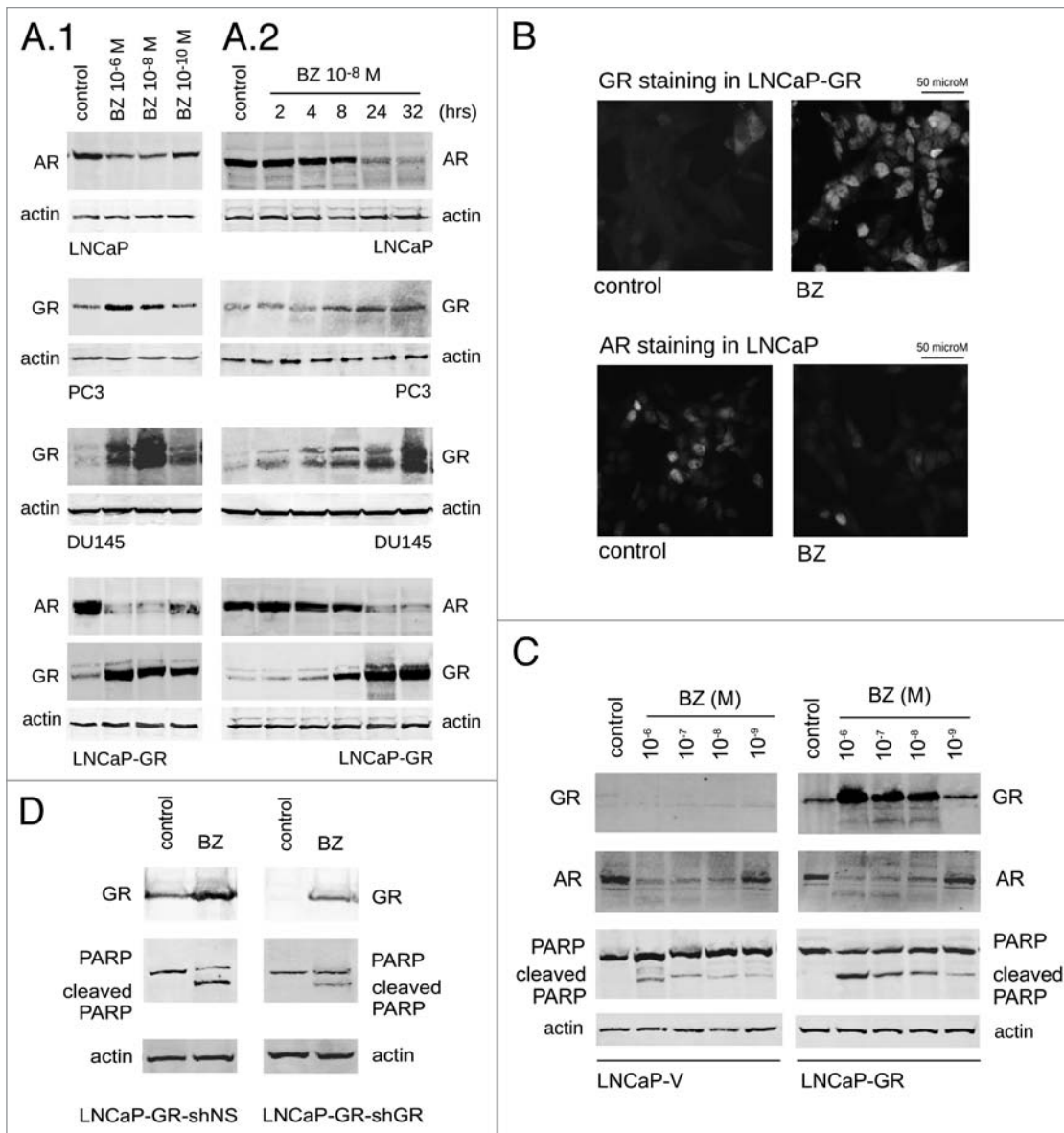
Figure 2. Differential AR and GR regulation by Bortezomib correlates with PC cell apoptosis. (A) Western blot analysis of AR/GR in whole-cell lysates of LNCaP, LNCaP-GR, PC3 and DU145 treated with 10^{-6}–10^{-10} M BZ for 24 h (A.1) and 10^{-8} M BZ for 2–32 h (A.2). (B) Immunostaining for AR/GR in cells treated with 10^{-8} M BZ (24 h). (C and D) Western blot analysis of PARP cleavage. (C) LNCaP and LNCaP-GR treated with 10^{-6}–10^{-9} M BZ (32 h). (D) LNCaP-GR-shNS (expressing non-silencing shRNA) and LNCaP-GR-shGR treated with 10^{-8} M BZ (32 h). Note: (1) GR upregulation and AR downregulation after BZ correlates with PARP cleavage; (2) decreased PARP cleavage in LNCaP-GR-shGR cells.

CpdA augmented BZ induction of CHOP protein (see below, **Fig. 7**), while FA had the opposite effect.

Using ChIP, we proved that differential effects of CpdA and CpdA+BZ on SGK1, BIP and CHOP expression reflected GR occupancy of the predicted GREs in their promoters (**Fig. 5B.1**). GR loading on SGK1 promoter was increased by FA, while in the presence of CpdA ± BZ, the GR loading was very weak (**Fig. 5B.1**). In contrast, CpdA ± BZ greatly enhanced GR loading on the CHOP/GADD153 promoter, whereas FA ± BZ, reduced GR loading of the same CHOP promoter (**Fig. 5B.1**).

Bortezomib enhances compound A capability to induce GR transrepression. The transrepression by GR is chiefly mediated by protein-protein interaction between GR and other transcription factors, such as AP-1 and NFκB, which inhibits their transcriptional activity.[9,14-16] We and others showed previously that CpdA shifts GR activity toward transrepression.[20,23] Using Luciferase reporter assay, we found that in AR+/GR− LNCaP-V, AR+/GR+ LNCaP-GR and AR−/GR+ PC3 cells, CpdA+BZ inhibited basal, and IL-1 induced NFκB- and AP1-dependent transcription (**Fig. 6**). Importantly, combined CpdA+BZ suppression of the TFs activity was more pronounced in LNCaP-GR than in GR-negative LNCaP-V or parental LNCaP cells (**Fig. 6B**), suggesting the leading role of GR in CpdA+BZ inhibition of NFκB and AP-1 activity.
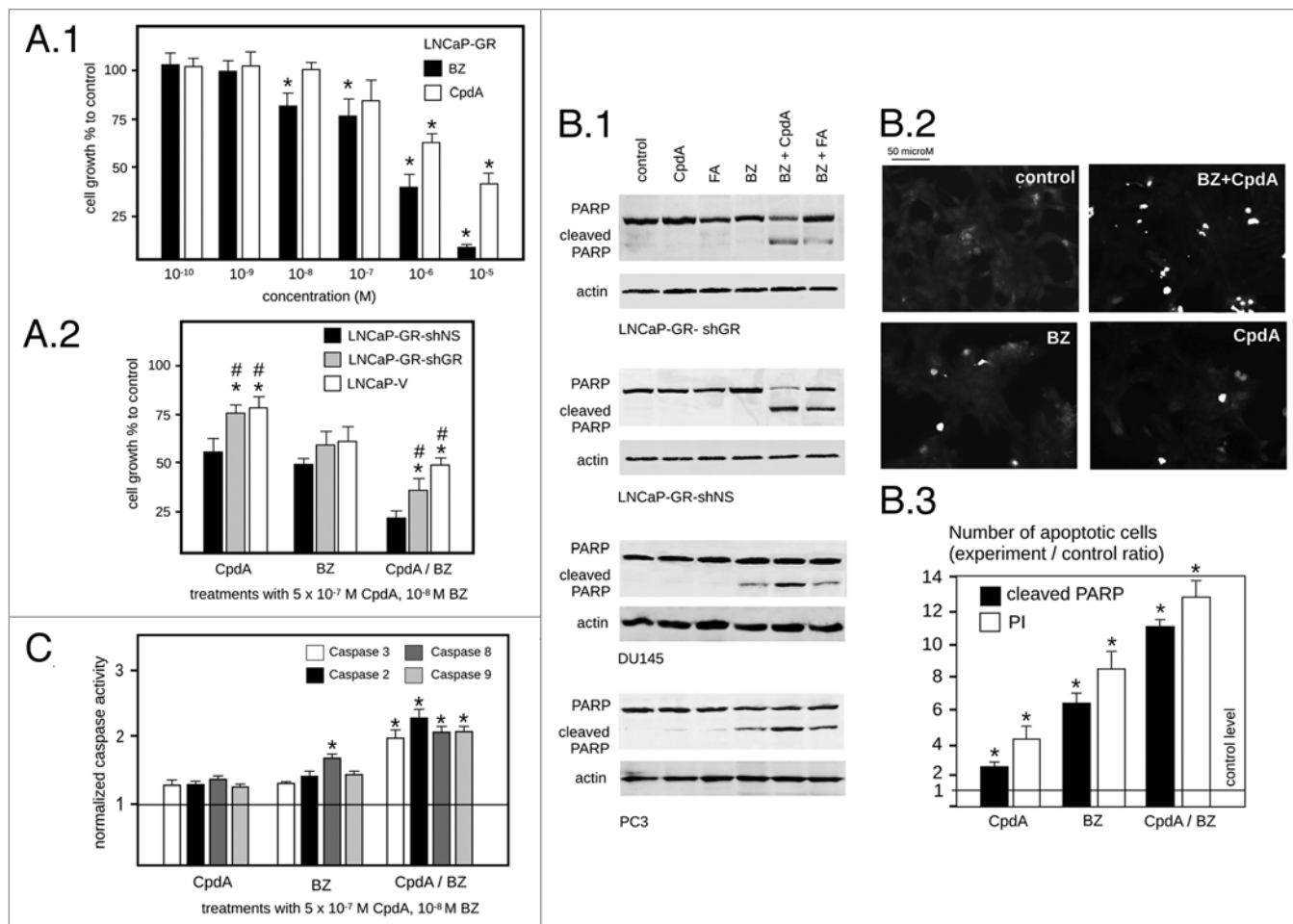
Figure 3. Bortezomib increases anti-PC activity of CpdA. (A.1) Proliferation of LNCaP-GR cells treated with BZ or with CpdA (10^{-5}–10^{-10} M, 4 d). (A.2) Proliferation of LNCaP-GR-shGR, LNCaP-V and LNCaP-GR-shNS cells treated with 5 x 10^{-7} M CpdA and 10^{-8} M BZ (4 d); (B) Apoptosis after treatment with 10^{-8} M BZ and 5 x 10^{-7} M CpdA (24 h) in LNCaP-GR-shGR, LNCaP-GR-shNS, PC3 and DU145 as measured by (1) PARP cleavage using western blotting (B.1), (2) immunofluorescent microscopy with anti-bodies against cleaved PARP (B.2) and (3) quantitative analysis of cleaved PARP staining and propidium iodide (PI) incorporation (B.3) using Celligo cell analyzer (Cyntellect Inc.). (C) Caspase activity in LNCaP-GR treated as described in (B); data were normalized to control (0.01% DMSO). (A.1, B.3 and C) Statistically significant differences compared with vehicle control; (A.2) statistically significant differences compared with LNCaP-GR-shNS cells. Note: CpdA+BZ cooperate at low, singly ineffective doses to suppress PCa cell growth and induce apoptosis.

We conclude that BZ enhances CpdA ligand properties as anti-androgen and selective enhancer of GR transrepression. In addition, we discovered that CpdA together with BZ induces expression of central pro-apoptotic factor CHOP/GADD153.

Combined CpdA+BZ treatment triggers endoplasmic reticulum stress in PCa cells via CHOP. To further characterize molecular mechanisms underlying the cooperation between CpdA and BZ, we focused on GR-dependent ERS response, a critical component of the anticancer action of proteasome inhibitors.[26,27,37-39] We found that CpdA and BZ cooperatively induced ERS in LNCaP-GR cells, as was indicated by upregulation of BIP/HSPA5, ATF2 phosphorylation and nuclear translocation, and by induction of downstream pro-apoptotic ERS effector CHOP/GADD153 (**Fig. 7A and B**). The latter was also observed in PC3 cells (**Fig. 7B.3**).

Since CHOP/GADD153 was differentially activated by CpdA and BZ+CpdA via GR, we analyzed its role in anticancer effects of CpdA ± BZ. We found that BZ alone (10^{-8} M–10^{-9} M) and CpdA+BZ cooperatively upregulated cytoplasmic and nuclear CHOP in LNCaP-GR, LNCaP and PC3 cells (**Fig. 7B**). CHOP induction was more pronounced in GR-positive than in GR-negative LNCaP cells (**Fig. 7B.1 and B.2**), emphasizing the GR role in CHOP regulation. In contrast, steroids FA and DHT combined with BZ downregulated cytoplasmic and nuclear CHOP compared with BZ ± CpdA (**Fig. 7B.1 and B.2**) pointing to the ability of the androgens and glucocorticoids to counteract ERS in PCa cells.

ShRNA knockdown of endogenous CHOP in LNCaP-GR cells (LNCaP-GR-shCHOP cells) confirmed the importance of CHOP for the CpdA+BZ effects on PC (**Fig. 7C and data not shown**). The cooperative CpdA+BZ growth suppression and induction of apoptosis were significantly stronger in LNCaP-GR-CHOP compared with LNCaP-GR cells with low endogenous CHOP expression. Since LNCaP-GR-CHOP cells became
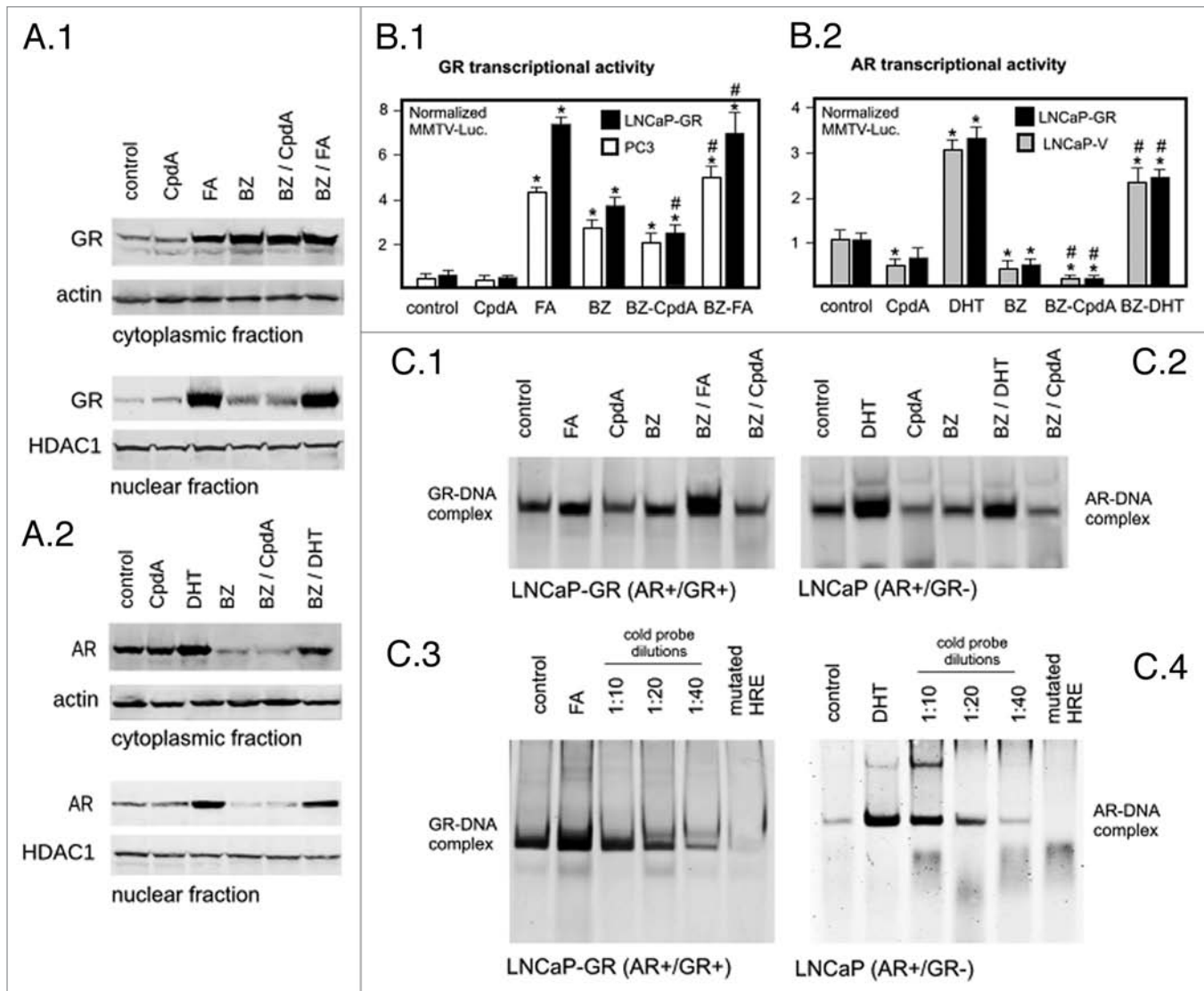
Figure 4. Effect of Bortezomib on AR and GR transcription activity. (A) Western blot of cytoplasmic and nuclear AR/GR in LNCaP-GR (A.1) and LNCaP (A.2) treated with CpdA (5×10^{-7} M), DHT (10^{-7} M), FA (10^{-7} M) and BZ (10^{-8} M) for 16 h. (B) Assessment of GR activity (B.1) and AR activity (B.2) using MMTV-Luciferase reporter in LNCaP-GR, PC3 and LNCaP cells treated as in (A) for 24 h; *p < 0.05 statistically significant difference compared with vehicle control; #p < 0.05 difference compared with BZ treated cells. (C) DNA binding by GR in LNCaP-GR (C.1) and AR in LNCaP-V (C.2) cells treated as in (A) for 16 h (EMSA). AR/GR-binding hormone responsive element (HRE) labeled with infrared dye IR-680. (C.3 and C.4) Confirmation of DNA complexes formed with GR in LNCaP-GR cells stimulated with FA (C.3) and AR in LNCaP cells stimulated with DHT (C.4) using dilutions with non-labeled HRE and mutated HRE as described in Materials and Methods. Note: CpdA+BZ strongly inhibit AR DNA-binding and transcriptional activity.

sensitive to apoptosis, in this sets of experiments we used low BZ concentrations (10^{-9} M, **Fig. 7B.4 and C**). Growth suppression and apoptosis induction by CpdA+BZ were drastically diminished in LNCaP-GR-shCHOP cells with endogenous CHOP depleted by 65–70% (**Fig. 7C.2 and 3**).

We previously hypothesized that the inability of CpdA to induce expression of pro-survival GR targets, such as SGK1, improves its anticancer activity over glucocorticoids.[20,36] In this study, neither CpdA, nor CpdA+BZ upregulated SGK1 and FKBP5 at mRNA or protein levels (**Figs. 5A, B and 7A**, and data not shown). At the same time, those genes were readily upregulated by FA ± BZ.

Discussion

We previously showed that dual AR/GR modulator CpdA strongly inhibits growth and viability of PCa cells in AR/GR-dependent fashion.[20] Here, we further explored a novel AR/GR-targeted strategy for PC treatment and corroborated our hypothesis that proteasome inhibitors enhance CpdA ligand properties and anticancer activity.

Our central hypothesis is based on the previous findings that 26S proteasome inhibitors activate GR but inhibit AR.[29,33] We confirmed this differential effect using several PCa cell lines, including one with dual AR/GR expression (**Fig. 2 and Sup. 4**).
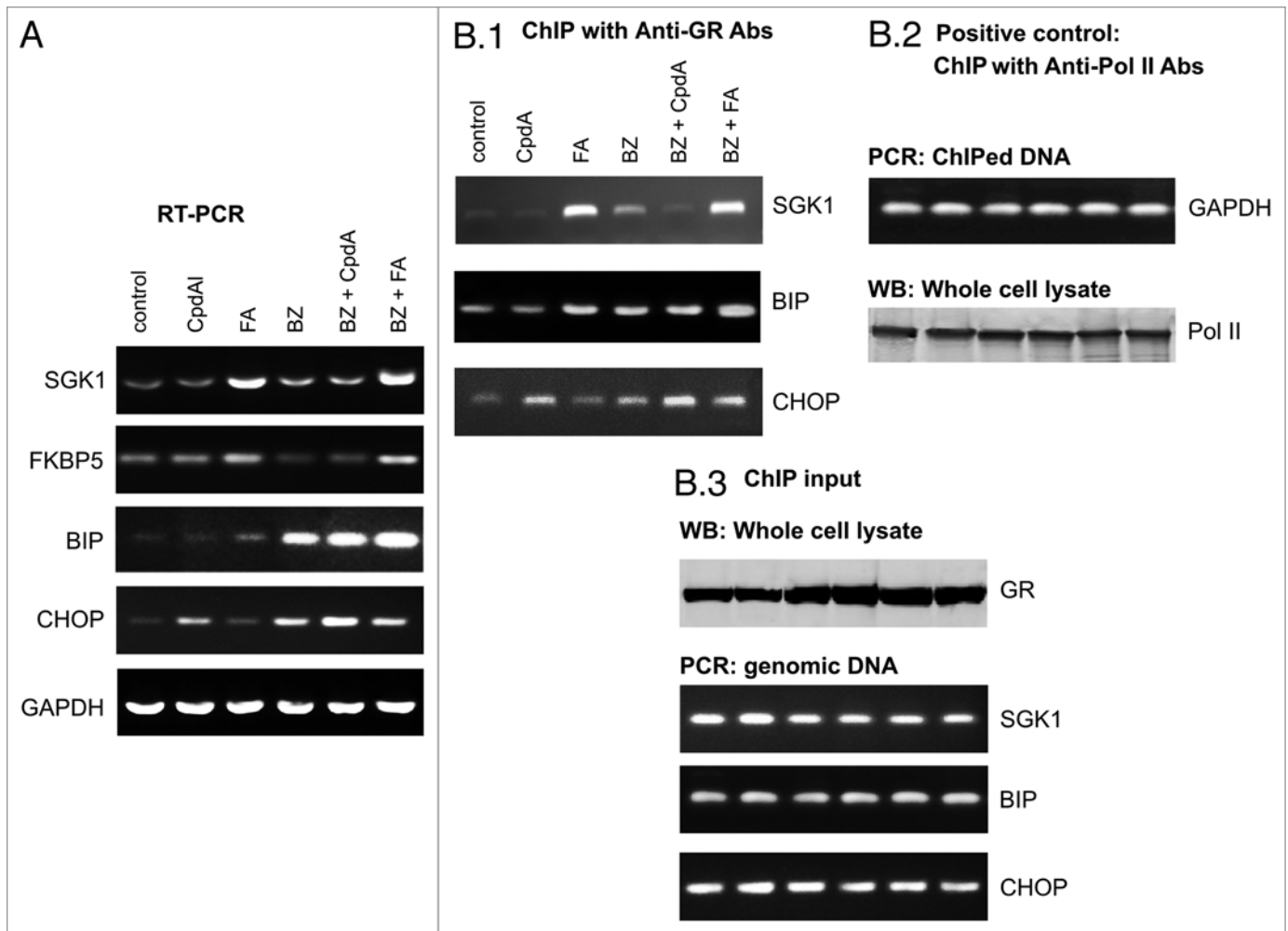
Figure 5. Bortezomib enhances CpdA ligand properties as a selective modulator of GR. (A) Semiquantitative RT-PCR analysis of GR-target gene expression in LNCaP-GR treated as in **Figure 4A** (24 h). (B.1) Analysis of GR promoter occupancy for SGK1, BIP/HSPA5 and CHOP/GADD153 in LNCaP-GR treated as in **Figure 4A** (16 h). (B.2) Positive control (**Sup. 3**): GAPDH promoter occupancy by Pol II; PCR products of ChIP-ed GAPDH promoter fragments and whole-cell Pol II analyzed by western blot. (B.3) Input: whole-cell GR analyzed by western blotting and PCR products of SGK1 and CHOP promoters before ChIP (see also **Sup. 3**). As a negative control, DNA ChIP-ed with non-specific rabbit IgG was used as described in Materials and Methods.

Proteasome regulates nuclear localization and transcriptional activity of steroid receptors via turnover of the receptors, their chaperones and co-regulators.[28-31] We and others have found that prolonged exposure to proteasome inhibitors BZ and MG-132 causes AR depletion (**Fig. 2** and reviewed in ref. 35), which may be explained by AR proteolysis by caspases 3 and 8, calpain and other non-specific serine/threonine proteases in PCa cells undergoing apoptosis.[40-42] Remarkably, unlike androgen DHT, CpdA failed to protect AR against destabilization by BZ.

In contrast to their effect on AR, proteasome inhibitors prevent hormone-induced GR downregulation, elevate its levels and enhance basal and hormone-induced GR transactivation.[30-33] The role of proteasomes in GR regulation was additionally confirmed when a highly conservative proteasome recognition motif (PEST) was discovered in GR.[31,33] Here, we demonstrate that non-steroid GR ligand CpdA and glucocorticoid FA had no effect on GR stabilization by BZ, but instead defined its functional profile. When combined with BZ, CpdA only weakly induced/modulated GR transactivation but heavily augmented transrepression (**Figs. 4–6**).

Although the ability of proteasome inhibitors to modulate the activity of steroid hormone receptors has been known, the role of AR and GR in proteasome-induced apoptosis has not been elucidated. We discovered that BZ inhibits PCa cell survival by differential modulation of GR and AR activity. We therefore establish GR and AR as novel molecular targets of proteasome inhibitors. We demonstrate here that BZ increased CpdA anticancer effects, and that CpdA+BZ cooperatively induced apoptosis. Next, we defined CpdA+BZ combinations with the best anticancer effect and demonstrated the potential for CpdA and BZ dose reduction. The latter is important because in vivo as stand-alone agents, both are effective at high, maximally tolerated doses (MTD), and in clinic BZ doses also approach MTD, with multiple adverse effects.[23,25,43]

The mechanisms of anticancer effects induced by proteasome inhibitors are broad and include ERS and subsequent
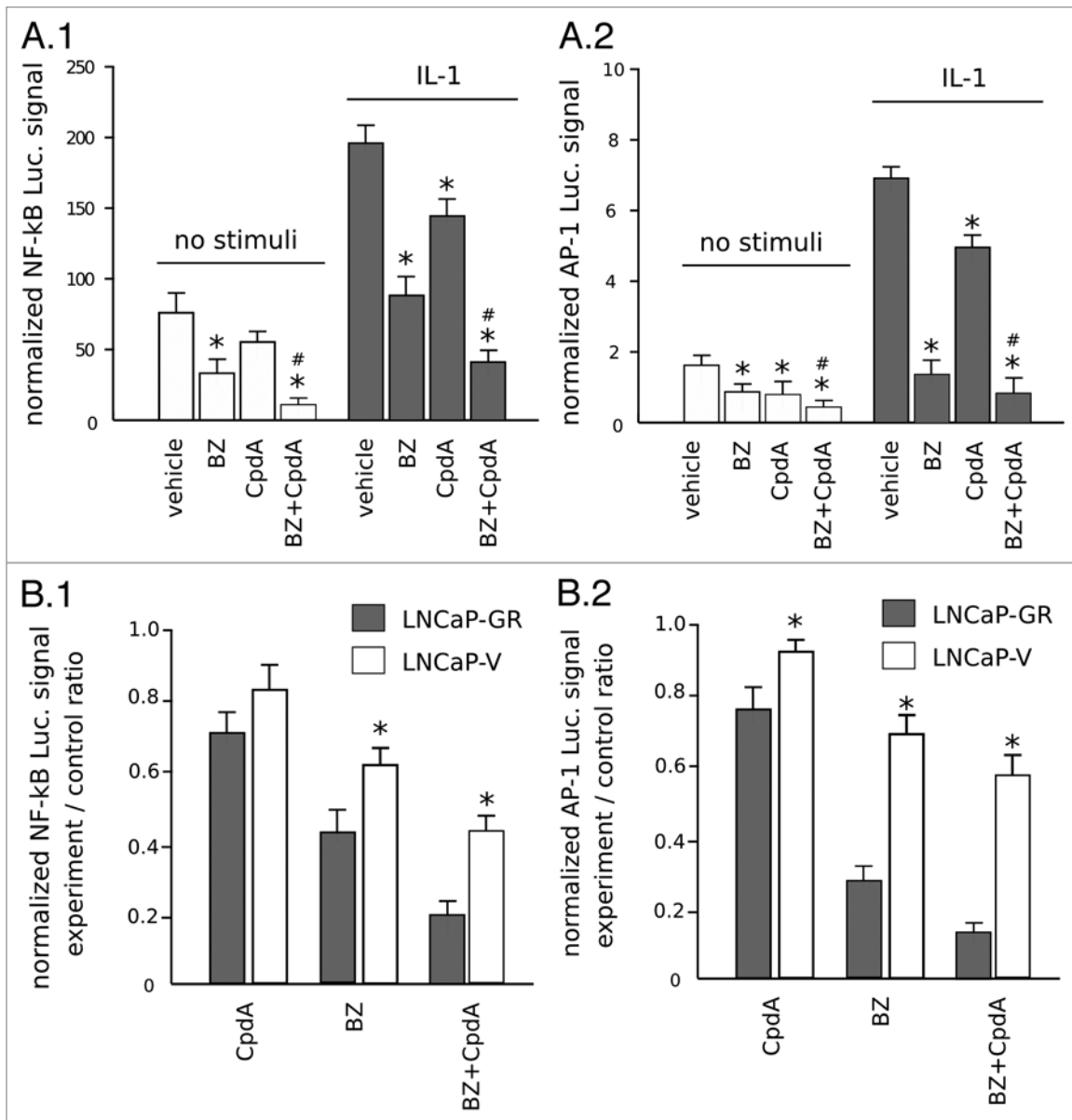
Figure 6. Bortezomib enhances CpdA ability to induce GR transrepression. (A) Evaluation of NFκB (A.1) and AP-1 (A.2) activity by Luciferase assay in LNCaP-GR cells. Cells were transfected with 3 x κB-Luciferase and AP-1-Luciferase reporters and pre-treated with 0.01% DMSO (control), 5 x 10^{-7} CpdA and 10^{-8} BZ for 16 h and, where it is indicated, induced with 1 μg/mL IL-1 for 12 h. *p < 0.05 statistically significant difference compared with control; #p < 0.05 statistically significant difference compared with CpdA alone. (B) Comparison of CpdA, BZ and BZ + CpdA effects on NFκB and AP-1 in LNCaP-V and LNCaP-GR cells induced with IL-1 and transfected as in **Figure 6A**. *p < 0.05 statistically significant difference compared with LNCaP-V; Note: (1) Bortezomib enhances CpdA ability to inhibit activity of NFκB and AP-1; (2) effect of CpdA, BZ and CpdA+BZ on NFκB and AP-1 is GR-dependent and more pronounced in LNCaP-GR cells.

apoptosis.[26,27,38,39,44] We found that CpdA induced ERS cooperatively with BZ (**Fig. 7**). Together with BZ, CpdA enhanced expression of the early ERS marker BIP/HSPA5 and its downstream effector pro-apoptotic CHOP/GADD153 (**Figs. 5 and 7**). The effects of AR and/or GR ligands on BIP and CHOP expression were previously unknown. We discovered that the promoters of both genes harbor multiple GREs (**Sup. 3**). Intriguingly, despite its typical support of transrepression, CpdA increased GR occupancy of the CHOP/GADD153 promoter and elevated CHOP expression. The increased sensitivity of LNCaP-GR-CHOP cells

to BZ cytotoxic effects highlights the unique anticancer properties of CpdA and its potential for combination therapies. This is especially significant, since apoptosis due to ERS recently emerged as a strategy for PC treatment.[45,46]

The comparison of cytostatic and cytotoxic effects of BZ combinations with FA or CpdA revealed superior potency of CpdA+BZ regimen. Glucocorticoids are known to induce expression of anti-apoptotic and self-defense genes and to desensitize cells against apoptosis by GR.[36,37,47] The advantage of CpdA was its inability to initiate the expression of pro-survival genes,
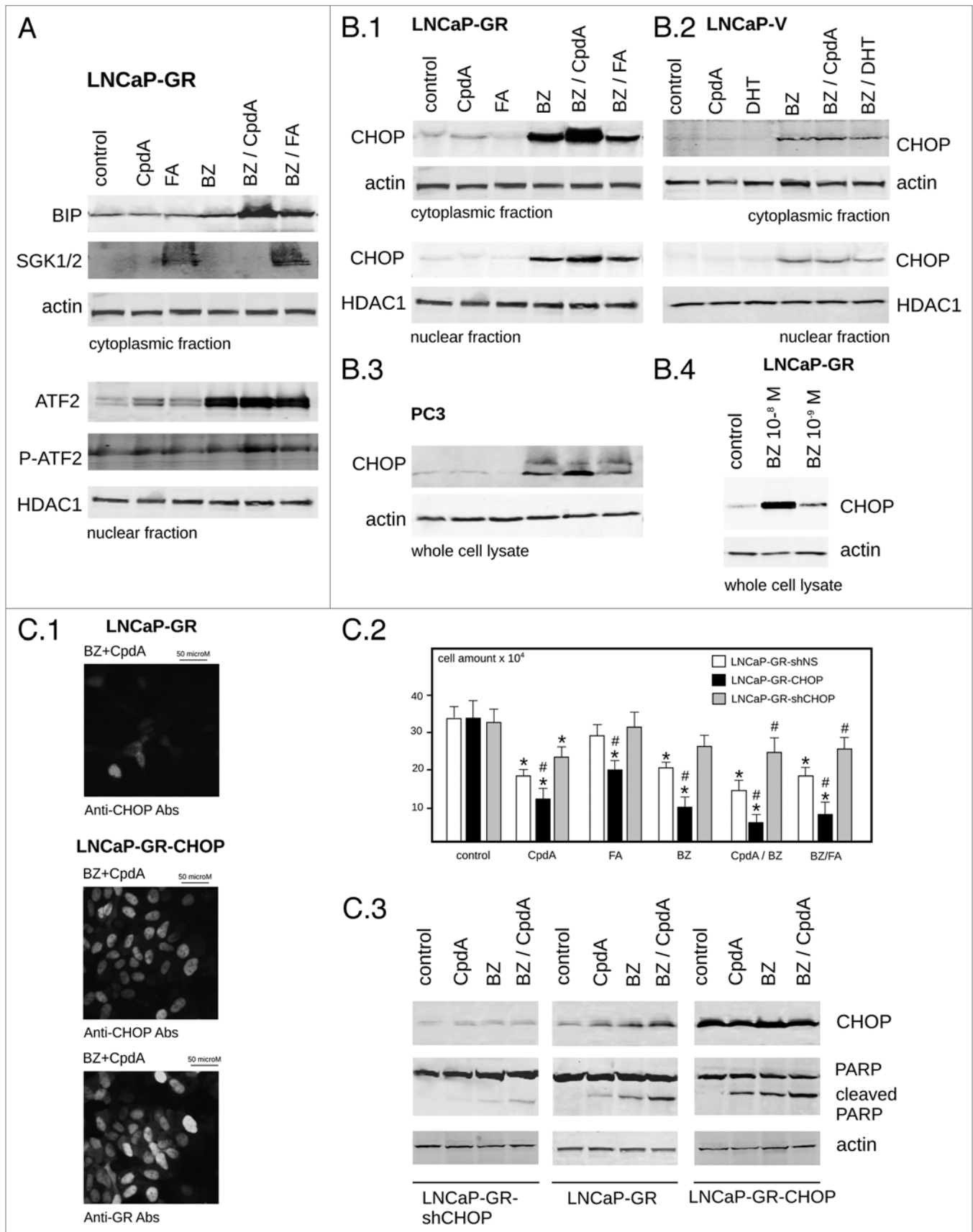
Figure 7. For figure legend, see page 404.

such as SGK1. Moreover, FA stimulated early pro-survival (BIP/HSPA5) and inhibited late (CHOP/GADD153) pro-apoptotic ERS regulators whose expression was triggered by BZ. In sharp contrast, CpdA alone and cooperatively with BZ induced and sustained late, pro-apoptotic ERS phase via GR-dependent CHOP induction.

We therefore hypothesize that the remarkable cytotoxic activity of the CpdA+BZ combination in PCa cells is manifold. First, CpdA+BZ inhibit pro-proliferative, anti-apoptotic transcription factors, such as NFκB and AP-1. Second, CpdA+BZ induce ERS and apoptosis by upregulating CHOP/GADD153. Third, CpdA+BZ block defense response of the cancer cells due to the gene expression activated by the glucocorticoid receptors. Overall, we demonstrate that combining selective GR modulators like CpdA with proteasome inhibitors could release the anti-cancer GR signaling at its maximal potential.

The important finding of this study is the high expression of nuclear AR and GR in PCa following androgen ablation or chemotherapy. This was unexpected, because in primary PCa from non-treated patients, GR expression is strongly decreased.[9,34] The fact that both AR and GR are widely expressed in treated human PCa enhances the possibility of success of the proposed PC treatment strategy by simultaneous targeting of AR and GR. Overall, our novel approach of simultaneous inhibition of oncogenic AR and activation of tumor-suppressive GR signaling responds well to the growing interest in combinatorial chemo-hormonal therapies preventing PC transition from an androgen-dependent to castration-resistant phenotype.

Materials and Methods

Cell culture. LNCaP, PC3 and DU145 PCa cells (American Type Culture Collection) were cultured as described in references 9 and 20. The 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride (CpdA) was synthesized as described in reference 20. Cells were treated with fluocinolone acetonide (FA), dihydrotestosterone (DHT) (Sigma-Aldrich), Bortezomib (BZ) (Millenium Pharmaceuticals) and Interleukin-1 (IL-1, at 1 μg/mL, Invitrogen).

Lentiviral technology. Lentiviral stocks were generated as described in references 9 and 48 using lentiviral expression vectors pLOC encoding CHOP/GADD153, pGIPZ encoding shRNA against GR or CHOP (all from Open Biosystems) and PL6-V5/TOPO encoding GR (Invitrogen). Since lentiviral infection efficacy was 90%, we used bulk cell population for the establishment of the following cell lines: LNCaP-GR, LNCaP-GR-CHOP, LNCaP-GR-shGR, LNCaP-GR-shCHOP, LNCaP-GR-shNS infected with non-silencing shRNA and

control LNCaP-V infected with empty virus (all from Open Biosystems).

Western blot analysis. Western blot analysis of whole-cell extracts, cytoplasmic and nuclear fractions was performed as described in references 9 and 20. Primary Abs against GR and AR were from Santa Cruz; those against PARP, CHOP, BIP, SGK1/2, ATF2 and phospho-ATF2 were from Cell Signaling. Secondary anti-rabbit or anti-mouse IgG conjugated with IR dyes IRE-680 or IRE-800 were from LI-COR. Actin or HDAC1 were used as loading controls for cytoplasmic and nuclear proteins, respectively.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed as described in reference 20, with 2 pmol double-stranded oligonucleotide of AR/GR HRE conjugated with IRE-680 (LI-COR) and 10 μg nuclear extract. Cells were treated as indicated for 12 h. The specificity of the DNA-protein complexes was verified against increasing concentration of non-conjugated HRE (cold probe) or mutated HRE conjugated with IRE-680 (**Fig. 4C.3**). DNA-protein complexes were analyzed by Tris-borate EDTA electrophoresis on 6% polyacrylamide gels with 0.5% glycerol.

Image acquisition/analysis. Western blotting and EMSA images were obtained with Odyssey IR scanner (LI-COR) at 800-nm and/or 680-nm channels. Image digitizing and quantitative analysis were performed using the LI-COR software.

Semiquantitative RT-PCR (SQ-RT-PCR). SQ-RT-PCR was performed using reverse MLV transcriptase with random primers and PCR-Supermix (Invitrogen) with appropriate PCR primers (**Sup. 1**). Total RNA was isolated with RNAeasy kit (Qiagen). PCR products were separated on 1.5% agarose gel and visualized using automated imaging system (BioRad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control.

Immunostaining of prostate tissue samples. Immunostaining and morphological evaluation of the prostate samples are described in **Supplemental Material 2**. Tissues were obtained by transurethral prostatic resection or radical prostatectomy from consented patients (total 45 patients aged 40–82 y) treated with hormone ablation (flutamide, casodex) or chemotherapy (docetaxel/dexamethasone) at Northwestern University Hospital or at the National Cancer Research Center. AR/GR immunostaining was performed on serial paraffin sections of formalin-fixed prostate samples, as described in reference 9. The number of prostate epithelial cells with either GR or AR nuclear signal was evaluated by + to +++ scoring. Tumors with GR/AR staining intensity ++/+++ were considered GR+ or AR+ respectively.

Chromatin immunoprecipitation (ChIP). ChIP was performed as described in reference 19 with commercial reagents

(Millipore). Cells were fixed with 1% formaldehyde at 37°C for 30 min to cross-link protein-DNA complexes, lysed and sonicated to generate 300–600 bp DNA fragments. The GR-DNA complexes were precipitated with rabbit anti-GR Abs conjugated to agarose beads (Santa Cruz). The DNA fragments were isolated and GR loading on promoters of SGK1 BIP/HSPA5, and CHOP/GADD153 genes was analyzed by PCR. The promoter maps, primer sequences, positive and negative controls are presented in **Supplement 3**. As a positive control, RNA polymerase II (Pol II) loading on GAPDH promoter was assessed using anti-Pol II antibodies conjugated to agarose beads (Santa Cruz) and commercial primers for the GAPDH promoter (Millipore). As a negative control, non-specific rabbit IgG conjugated to agarose beads was used; ChIP-ed DNA fragments were analyzed by PCR with SGK1, BIP/HSPA5 and CHOP/GADD153 primers (**Sup. 3**).

Luciferase assay. The Firefly luciferase reporters TAT-Luc, MMTV.Luc, x3 κB-Luc and AP-1-Luc were from Panomics. Transfection efficacy was normalized by co-transfections with empty Renilla luciferase vector (Panomics). PCa cells at 70% confluence in 48-well plates were transfected with the indicated plasmids using Effectene reagent (Qiagen).[20] Cells were pretreated with AR and GR ligands or appropriate vehicle (0.01% DMSO and/or 0.01% ethanol) for 16 h and stimulated with IL-1 for 12 h.

Caspase activity assay. Caspase activity was evaluated with ApoAlert Assay (Clontech) according to the manufacturer's protocol. Cells in 10-cm dishes were treated as indicated for 48 h. Caspase activity was measured with a Victor plate reader (Perkin-Elmer) at 380-nm excitation and 460-nm emission.

Immunofluorescence of PCa cell cultures. PCa cells on glass coverslips were fixed, permeabilized and incubated with primary Abs (4°C overnight), followed by donkey anti-rabbit FITC-conjugated and/or anti-mouse Cy-3-conjugated secondary Abs (Jackson Immunoresearch) and 4,6-diamidino-2-phenylindole (DAPI, Vector Labs) to highlight the nuclei. Images were obtained with Zeiss Axioplan 2 microscope with AxioCaM HRC camera and analyzed using AxioVision LE Rel-4.7 software (Carl Zeiss MicroImaging).

Quantitative analysis of cell growth and apoptosis. Automatic cell analyzer Celligo (Cyntellect Inc.) was used to assess (1) proliferation and survival of living cells using Hoechst and propidium iodide (PI) and (2) apoptosis of cells immunostained with anti-cleaved PARP antibody as described above. Cells were plated at 10^4/well in 12-well plates and cultured for 1–3 d in the presence of CpdA, BZ or vehicle (0.1% ethanol and/or 0.01% DMSO). Each treatment was repeated in triplicate.

In silica analysis of TF binding sites. In silica screening of the GR responsive elements (GRE) in the promoters of FKBP5, BIP/HSP5, SGK1 and CHOP/GADD153 genes were performed using online Transcription Element Searching System TESS (www.cbil.upenn.edu/cgi-bin/tess/tess).[49]

Statistical analysis. All experiments were performed at least three times. Mean and SE values were calculated using Microsoft Excel software and compared using paired Student t-test. p-value of < 0.05 was considered significant.

Note

Supplemental material can be found at:
www.landesbioscience.com/journals/cc/article/18945

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