Novel Steroid Receptor Phyto-Modulator Compound A Inhibits Growth and Survival of Prostate Cancer Cells

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Abstract

Androgen receptor (AR)– and glucocorticoid receptor (GR)–mediated signaling play opposite roles in prostate tumorigenesis: AR promotes prostate carcinoma (PC) development, whereas GR acts as a tumor suppressor. Compound A (CpdA) is a stable analogue of an aziridine precursor from the African shrub Salsola tuberculiformis Botschantzev. It was shown recently that, in model cells, CpdA inhibits AR function and strongly enhances anti-inflammatory function of GR. We determined the effects of CpdA in prostate cells with different AR/GR status: (a) RWPE-1 cells (ARlow/GRlow), (b) PC3 and DU145 cells (GR+/AR−), (c) LNCaP cells (GR−/AR+), and (d) LNCaP-GR cells expressing both receptors. Similar to steroid hormones, CpdA induces nuclear translocation of both receptors in prostate cells. Despite this, CpdA inhibits DNA-binding and transactivation potential of AR. In addition, CpdA inhibits GR-mediated transactivation but induces GR trans-repression via inhibition of several transcription factors, including nuclear factor-κB, AP-1, Ets-1, Elk-1, SRF, CRE/ATF, and NFATc. CpdA strongly decreases growth and induces caspase-dependent apoptosis in highly malignant PC3 and DU145 cells and in other AR/GR-expressing PC cells. The cytostatic effect of CpdA is receptor dependent: down-regulation of GR or AR expression drastically attenuates CpdA-induced PC cell growth inhibition. Finally, virtual docking analysis indicates that CpdA shares binding cavities in AR and GR ligand-binding domains with corresponding hormones and forms hydrogen bonds (H-bond) with the same amino acids that are involved in H-bond formation during steroid binding. Overall, our data suggest that CpdA is a unique dual-target steroid receptor modulator that has a high potential for PC therapy. [Cancer Res 2008;68(12):4763–73]

Introduction

Signaling mediated through the steroid hormone receptors plays a pivotal role in the development of prostate cancer (PC). Androgens and androgen receptor (AR) promote the development and progression of PC at the stage of hormone-dependent cancer (1–4). Thus, androgen ablation or blockade of AR function has been the cornerstone of treatment of advanced PC (1–4). In contrast, we and others showed that signaling mediated by the glucocorticoid receptor (GR) plays a tumor suppressor role in prostate (5–9). PC cells expressing GR are sensitive to the growth-inhibitory effect of glucocorticoids in vitro and in vivo when grown as xenografts in athymic mice (6–9). The activation of GR signaling results in differentiation of PC cells and “normalization” of expression pattern of PC markers, such as hepsin, AMACR, and maspin (7). Glucocorticoids also inhibit angiogenesis and lymphogenesis in PC cell–derived tumors (8, 9).

AR and GR are closely related transcription factors (TF) that belong to a superfamily of nuclear hormone receptors (10, 11). After binding to corresponding steroid hormones, AR and GR dissociate from chaperone proteins in cytoplasm, form homodimers, and translocate to the nucleus where they bind DNA through hormone-responsive elements (HRE) to activate gene expression (transactivation; refs. 10–12). In addition to the genomic, DNA-binding dependent transactivation, there are DNA-binding independent, nongenomic mechanisms of gene regulation by steroid receptors that are chieﬂy mediated via cross-talk with other TFs. The indirect regulation of gene expression by GR is better studied. It was shown that GR interacts with numerous TFs, including AP-1, nuclear factor-κB (NF-κB), signal transducer and activator of transcription-5 (STAT5), and SMAD3 (7, 10, 13–15). Most of such GR-TF interactions repress the activity of partner TFs and their target genes (transrepression). One more mechanism of indirect negative gene regulation by GR involves inhibition of mitogen-activated protein kinases. This mechanism is used in different cells, including PC cells (5, 7, 13, 15, 16). Indirect, DNA-independent mechanisms of GR gene regulation seem to be critical for the anti-inflammatory effects of glucocorticoids (13). Our work indicates that tumor suppressor effects of GR/glucocorticoids also involve gene transrepression (7, 16). The interaction between AR and other TFs is much less studied. However, there is growing evidence that AR interacts with other TFs, including AP-1, NF-κB, and Ets family members, and this interaction can modulate activity of those TFs and consequently the transcription of their target genes (3, 10, 17, 18).

Taking into consideration the opposite roles of AR– and GR–mediated signaling in prostate tumorigenesis, the multitarget steroid receptor modulators that act as antiandrogens but at the same time induce GR-mediated transrepression hold a great potential for PC treatment. Our work presented here is centered on a novel AR/GR ligand, 2-(4-acetoxyphenyl)-2-chloro-N-ethylammonium chloride, also called compound A (CpdA). CpdA is a synthetic analogue of a hydroxyphenyl aziridine precursor found in the African shrub Salsola tuberculiformis Botschantzev (19–22). It was shown that CpdA, like the active plant extracts, interacts with steroidogenic enzymes and steroid-binding globulins in plasma displacing the endogenous steroids and affects the bioavailability of these hormones in different target tissues (19, 20, 22). It was discovered recently that CpdA directly interacts with...
steroid receptors AR and GR in model cells (19, 23). Remarkably, CpdA inhibited AR activity via mechanisms similar to ones used by clinically used antiandrogens flutamide and cyproterone acetate (19, 24–27). CpdA was also reported to act as a GR ligand with “dissociated” properties: CpdA did not affect GR transactivation but induced GR-mediated transactivation evaluated by inhibition of NF-κB and NF-κB–dependent genes (23). Interestingly, in animal experiments, CpdA acted as a strong anti-inflammatory compound with reduced side effects such as hyperglycemia (23).

Overall, the literature data suggested that CpdA is a unique modulator of steroid hormone receptors that inhibits AR function and acts as a dissociated GR ligand. Our work was designed to analyze CpdA docking into ligand-binding domains (LBD) of AR and GR in comparison with steroids, to evaluate the effect of CpdA on AR and GR function in prostate cells, and to assess its effect on growth and apoptosis in a panel of prostate cells with different AR/GR status. Our experiments suggest that CpdA can compete with corresponding steroids for LBD binding. We also revealed strong cytostatic and apoptotic effect of CpdA specifically in highly malignant PC cells PC3 and DU145.

Materials and Methods

Cell cultures and treatments. LNCaP, PC3, and DU145 PC cells [American Type Culture Collection (ATCC)] were cultured as described (7) in RPMI 1640 (Life Technologies) with 10% fetal bovine serum (referred thereafter as complete medium; HyClone). LNCaP-GR cells and empty virus–expressing (LNCaP-V) control cells were generated by lentivirus infection as described (7). RWPE-1 normal prostate cells (ATCC) were cultured in serum-free, low Ca2+ medium supplemented with pituitary extract (Invitrogen Corp.). Cells were treated with CpdA (see below), fluocinolone acetonide (FA; Sigma-Aldrich), dihydrotestosterone (DHT; Sigma-Aldrich), antiandrogen Casodex (provided by AstraZeneca), anti-glucocorticoid RU486 (Sigma-Aldrich), tumor necrosis factor-α (TNF-α; 10 ng/mL; BioSource, Inc.), and interleukin-1 (IL-1; 1 μg/mL; BioSource). Synthesis of CpdA. The 2-(4-acetoxyphenyl)-2-chloro-V-methyl-ethyl-ammonium chloride (CpdA) was synthesized from (±)-Synephrine and acetyl chloride in glacial acetic acid by a modification of the original method of Breitschneider (28).

Western blot analysis. Whole-cell protein extracts were prepared by radioimmunoprecipitation assay buffer; cytoplasmic and nuclear protein fractions were prepared using nuclear isolation kit (Panomics, Inc.) as described (7). Proteins were resolved by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes (Bio-Rad). The following antibodies were used: anti-GR, anti-AR, anti-p65, and anti-c-Jun (all from Santa Cruz biotechnology and/or anti-mouse donkey Cy3-conjugated secondary antibody (Cell Signaling Technology). Enhanced chemiluminescence reagent (Amer sham Pharmacia Biotech) and anti–poly(ADP-ribose) polymerase (PARP; BD PharMingen). Membranes were blocked with 5% Blotto in TBS and incubated with primary antibodies overnight at 4°C followed by peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (Cell Signaling Technology). Enhanced chemiluminescence reagent (Amer sham Pharmacia Biotech) was used for the band visualization. To verify equal loading and adequate transfer, the membranes were probed with anti-actin and/or anti-β-tubulin antibodies and, if necessary, histone deactetylase-1 (HDAC-1) as nuclear protein marker (all from Santa Cruz Biotechnology). To quantify the signals, images were scanned and digitized using ImageJ software (NIH, Bethesda, MD).

Transient transfections and luciferase assay. The Firefly luciferase reporters TAT-Luc, MMTV-Luc, and x3GRE-Luc were described in previous publication (7). AP-1, CRE/ATF, NFATC, SRF, Ets-1, and Elk-1 reporters were obtained from Promega Corporation. The transfection efficiency was normalized using cotransfections with Renilla luciferase under minimal promoter (Promega Corp.; ref. 29).

Prostate cells at 70% confluence were transfected in 48-well plates with the indicated plasmids using Effectene reagent (Qiagen, Inc.) according to the manufacturer’s protocol. Each well contained 0.2 μg of the plasmid DNA. All experimental groups contained at least three wells. The cells were harvested 32 h after transfection and luciferase activity was measured using Dual Luciferase kit (Promega). Cells were treated with AR and GR ligands or corresponding vehicle (0.01% DMSO as a control for CpdA; 0.01% ethanol as a control for other treatments) for 32 h. Treatments with TNF-α or IL-1 were performed for 32 h.

Proliferation assay. The proliferation was measured by direct cell counting using Reicheet hemocytometer. Cells were plated at 105 per well onto 12-well plates and cultured in complete medium in the presence of CpdA, FA, or vehicle (0.1% ethanol and/or DMSO) for 1 to 12 d. LNCaP-GR and LNCaP-V cells were cultured in the presence of 6 μg/mL blastciclinc to maintain the selection pressure. Each experimental and control group consisted of three wells.

Apoptosis detection. To evaluate apoptosis, we used Western blot analysis of PARP cleavage and ApoAlert Caspase Assay (BD Biosciences Clontech). Cells were plated on 10-cm dishes and treated with FA, CpdA, and vehicle (0.1% ethanol and 0.1% DMSO) for 2 to 10 d, and adherent and detached floating cells were collected. Apoptosis was induced by TNF-α treatment for 24 h. PARP cleavage was estimated by Western blot analysis with anti-PARP antibody using whole-cell protein extracts. ApoAlert Caspase Assay was performed according to the manufacturer’s protocol using whole-cell lysates, and caspase activity was measured by a Victor plate reader (Perkin-Elmer) with 380-nm excitation and 460-nm emission filters.

Colony formation in soft agar. The colony-forming assay was performed as described previously (7). Forty-eight hours after plating, cells were treated with FA, CpdA, or vehicle (0.1% ethanol and/or DMSO) for 2 wk. Complete medium was changed and new reagents were added twice weekly. Stably infected LNCaP-GR and LNCaP-V cells were cultured in the presence of 6 μg/mL blastciclinc to maintain the selection. Images of entire wells were taken and colonies with diameter of >50 μm were counted using AxioVision LE Rel. 4.5 software (Carl Zeiss MicroImaging, Inc.). Each experimental group consisted of six wells.

Immunostaining. Cells plated on sterile coverslips in 24-well plates (2.0 × 105 per well) were treated for 16 to 48 h with FA, DHT, CpdA, and vehicle (0.1% ethanol and/or DMSO). To induce NF-κB and AP-1 nuclear translocation, TNF-α or IL-1 was added to cell cultures for 30 min after 48-h pretreatment with FA and CpdA. The cells were fixed for 15 min in 2% formaldehyde, permeabilized for 4 h with 50% acetone/50% methanol at −20°C, rinsed in PBS, and blocked for 1 h in 20% donkey serum. Primary antibodies were applied overnight at 4°C. Anti-rabbit donkey FITC-conjugated and/or anti-mouse donkey Cy3-conjugated secondary antibodies (both from Jackson Immunoresearch) were applied and the staining was analyzed using Zeiss Axioplan2 microscope with AxioCam MRc camera. 4′,6-Diamidino-2-phenylindole (Vector Laboratories, Inc.) was used to identify the nuclei. Images of stained cells were analyzed with AxioVision LE Rel. 4.5 software.

Electrophoretic mobility shift assay. Cells plated on 10-cm dishes were treated with FA, DHT, CpdA, and vehicle (0.1% ethanol and/or DMSO) for 16 h. Nuclear proteins were isolated by commercial Nuclear Protein Isolation kit (Panomics). The binding reaction for electrophoretic mobility shift assay (EMSA) contained 10 mmol/L HEPES (pH 7.5), 80 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 6% glycerol, 0.5 μg of poly(deoxinosinic-deoxycytidylic acid), 0.5 μg of sonicated salmon sperm DNA, 2 pmol double-stranded oligonucleotide of AR/GR HRE conjugated with IR fluorophore IRDye 700 (LI-COR), and 10 μg of the nuclear extract. To verify specificity of AR (GR) protein-DNA complexes, binding reaction was performed at increasing concentration of nonconjugated HRE (cold probe) and, alternatively, with mutated HRE conjugated with IR fluorophore IRDye 700. DNA-binding reaction was performed at 16°C for 30 min in a final volume of 20 μL. DNA-protein complexes were analyzed by Tris-borate EDTA electrophoresis on 5% polyacrylamide gels containing 0.5% glycerol. DNA-protein complexes were visualized by an Odyssey IR scanner (LI-COR) with a 680-nm scanner channel and linear graphical manipulations were made by the LI-COR Odyssey software for quantitative image analysis (LI-COR).

Short interfering RNA technology. Validated short interfering RNA (siRNA) against GR (siRNA Ambion ID 3909) and validated negative control inactive siRNA (scrambled siRNA; Silencer Cy3-labeled Negative Control
siRNA) labeled with Cy3 were obtained from Ambion. GR-specific and negative Cy3-labeled siRNAs were transfected into LNCaP-GR and PC3 cells using HiperFect siRNA transfection reagent (Qiagen) at a final concentration of 40 nmol/L according to the manufacturer’s protocol. The siRNA transfection efficiency was ~90% to 95% in both PC3 and LNCaP-GR cell cultures. To ensure long-term siRNA presence in the cells, cells were retransfected with additional 20 nmol/L of GR-specific and scrambled siRNAs every 5 d.

**Radioligand-binding assay.** Freshly harvested PC cells expressing endogenous receptors were homogenized by sonication on ice in the binding buffer [10 mmol/L Hepes (pH 7.5), 1 mmol/L EDTA, 5 mmol/L DTT, 20 mmol/L sodium molybdate] as described previously (30). The homogenate was centrifuged at 4°C, 100,000 × g for 1 h, and the supernatant (cytosol fraction) was used for the binding reaction. The binding assay was performed as described (30). Briefly, 200 µL of cytosol (200 µg protein per reaction) were incubated with 10 nmol/L [3H]Dex-methasone ([3H]Dex) or [3H]DHT (both from Amersham Pharmacia Biotech) for 24 h at 4°C. Bound and free hormones were separated by the incubation with dextran-coated charcoal (Sigma-Aldrich), and the binding was assessed by liquid scintillation spectroscopy. For competition-binding assays, cytosol was incubated with 10 nmol/L [3H]Dex or [3H]DHT and 100-fold molar excess of corresponding radioligand ligands, such as Dex, DHT, or CpdA, for 24 h at 4°C. All binding reactions were performed in triplicate. The competition between steroid hormones and CpdA for AR/GR binding is presented as percentage to the inhibition of [3H]Dex or [3H]DHT binding by the corresponding cold steroid hormones.

**Virtual docking.** For virtual docking, we used PDB files of recently published crystal structures of AR (PDB identifier 1T7T; ref. 31) and GR (PDB identifier 1P93; ref. 32) LBDs available from the Protein Bank Database Web site and virtual docking software Molegro (Molegro Aps; ref. 33). As an additional control, we compared our data and published results for AR/DHT and GR/Dex docking. Our data on steroid docking into GR and AR LBDs, including identification of binding cavities, positions for ligand binding, and points of hydrogen bond (H-bond) formation, were identical to previously published results obtained with other docking software (Fig. 1; refs. 34–36).

**Statistical analysis.** All experiments were repeated at least three times. Mean and SE values were calculated using Microsoft Excel software and compared using paired Student’s t test. A P value of <0.05 was considered statistically significant.

**Results**

**Evaluation of ligand properties of CpdA.** CpdA has been shown to interact with AR and GR in model cells (21, 23). To further characterize CpdA ligand properties and to compare binding of CpdA and natural steroid ligands to LBDs of AR and GR in terms of potential binding cavities and amino acids involved in binding to LBDs, we performed virtual docking. For analysis, we recently used published crystal structures of AR and GR LBDs available from the Protein Bank Database Web site (AR: 1T7T and GR: 1P93; refs. 31, 32) and virtual docking software Molegro (ref. 33). As shown in Fig. 1, CpdA shares binding cavities within LBDs of GR and AR with corresponding steroid hormones Dex and DHT. Furthermore, structural modeling predicts that CpdA will form H-bonds with several amino acids lining these binding cavities of receptors, and indicates that some of those amino acids (Asn564 and Arg611 in GR; Asn356 and Arg30 in AR) are also involved in H-bond formation with steroidal ligands of AR (DHT) and GR (Dex). Overall, our virtual docking data clearly indicate that CpdA could act as a ligand for both GR and AR, and suggest that CpdA would compete with steroids for LBDs in steroid hormone receptors.

It was reported previously that CpdA indeed strongly competes with glucocorticoid Dex for GR binding (23). However, the competition between CpdA and androgen mibolerone for AR binding seemed to be weak (21). Thus, we repeated the competitive binding assay using cytosol fractions isolated from PC cells expressing endogenous AR (LNCaP cells) and GR (DU145 cells). Unlabeled hormones when added at the 100-fold molar excess inhibited binding of corresponding [3H]hormones essentially by 100%. As shown in Fig. 1D, CpdA at the same 100-fold molar excess inhibited [3H]Dex binding to GR by 85% and [3H]DHT binding to AR by 30% compared with corresponding radioligand hormones.

**Effect of CpdA on function of steroid hormone receptors.** In the next experiments, we assessed CpdA effect on the function of endogenous receptors in a set of prostate cell lines with different AR/GR status: androgen-dependent LNCaP PC cells (AR+/GR–), two highly malignant androgen-independent cell lines DU145 and PC3 (both are AR+/GR–), and nontransformed prostate cell line RWPE-1 with low AR expression (37). We also used in our work stably transfected LNCaP-GR cells expressing both receptors that we recently characterized in detail (7). Although the AR/GR status for RWPE-I, LNCaP, DU145, and PC3 cells was previously published, we performed Western blot analysis of whole-cell protein extracts to directly compare the AR and GR protein levels in different cells under study. As shown in Supplementary Data 1, parental LNCaP and LNCaP-GR cells expressed significant amount of AR, and DU145, PC3, and LNCaP-GR cells expressed significant amount of GR; the level of both receptors was low in RWPE-1 cells. As only LNCaP-GR cells expressed significant amount of both receptors, we extensively used those cells to compare the effect of CpdA on AR and GR function.

Immunostaining and Western blot analysis revealed that, similar to androgen DHT, CpdA induced AR nuclear translocation in LNCaP-GR cells with endogenous AR (Fig. 2A). However, in contrast to DHT, CpdA inhibited both constitutive and DHT-induced AR DNA binding and AR transcription activity in luciferase test (Fig. 2B). In this respect, the effect of CpdA on AR activity closely resembles the effect of known antiandrogen Casodex (Fig. 2B; refs. 38, 39). The effect of CpdA on AR transcriptional activity depended on concentration and plateaued at 10−5 to 10−7 mol/L. This correlates well with the biologically effective CpdA concentration range used in previous work (21–23).

The same strategy was used to study the effects of CpdA on GR localization and function in PC cells expressing endogenous (DU145 and PC3) and exogenous (LNCaP-GR) GR. According to Western blot analysis and immunostaining, in LNCaP-GR cells, CpdA induced GR nuclear import, although at a significantly less extent than glucocorticoid FA (Fig. 2C). CpdA inhibited constitutive and FA-induced GR-DNA binding and GR transcription activity in LNCaP-GR cells (Fig. 2D) as well as in PC3 and DU145 cells (Supplementary Data 2). Moreover, the inhibitory effects of CpdA and known glucocorticoid antagonist RU486 (23, 28) on hormone-induced GR transcriptional activity were alike in luciferase test (Fig. 2D).

In addition to the DNA-binding dependent gene activation, there are DNA-binding independent mechanisms of gene regulation by GR mostly mediated via negative interaction between GR and other TFs, including NF-κB, AP-1, and p53 (10, 13, 14). Thus, in the next set of experiments, we assessed how CpdA affected GR transcriptional activity in DU145 cells with a panel of luciferase reporters that we used previously (7). Remarkably, the activity of numerous TFs, including NF-κB, AP-1, Ets-1, Elk-1, SRF, CRE, and NFATc, was reduced in LNCaP-GR cells.

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1 http://www.PDB.org
Figure 1. Analysis of CpdA binding to AR and GR. A and B, virtual docking was performed using PDB files of GR and AR LBDs obtained from the Protein Bank Database Web site and Molegro virtual docking software. Virtual docking of CpdA (pink) as an active ligand and Dex (green) or DHT (yellow) as reference ligands at GR (A) or AR (B) LBDs. Red dotted lines, H-bonds formed by CpdA; black dotted lines, H-bonds formed by Dex or DHT. Left, lists of amino acids potentially involved in H-bond formation with steroids and CpdA in GR (A) and AR (B). Predicted binding cavities for CpdA and steroid hormones in GR and AR LBDs are marked as cavity. Note: CpdA shares binding cavities (cavity) within LBDs of GR and AR with corresponding steroid hormones. C, chemical formulas of DHT, Dex, and CpdA. D, competition between CpdA and steroid hormones for binding to receptors. Two hundred microliters of cytosol (200 µg protein per reaction) were incubated with 10 nmo/L [3H]Dex or [3H]DHT for 24 h at 4 °C in the presence of 100-fold molar excess of corresponding radiolabeled ligands: Dex, DHT, and CpdA. The competition between steroid hormones and CpdA for AR/GR binding is presented as % to the inhibition of [3H]Dex binding to GR by cold Dex and [3H]DHT binding to AR by cold DHT.
CpdA inhibits transcription activity of AR and GR. AR and GR function was analyzed in LNCaP-GR cells. A and C, immunofluorescent staining and Western blot analysis of nuclear translocation of AR (A) and GR (C) in cells treated for 16 h with DHT (10\(^{-6}\) mol/L), FA (10\(^{-6}\) mol/L), and CpdA (10\(^{-5}\) mol/L). B and D, effect of CpdA on AR (B) and GR (D) activity. For luciferase reporter assay, cells were transfected with MMTV-Firefly luciferase (B) or TAT-Firefly luciferase (D) reporters and Renilla luciferase reference reporter. Firefly luciferase activity was normalized against Renilla luciferase activity to equalize for transfection efficacy. Cells were treated with CpdA (0.1–10 \(\mu\)mol/L), DHT (10\(^{-6}\) mol/L), FA (10\(^{-6}\) mol/L), AR antagonist Casodex (Cdx; 10\(^{-5}\) mol/L), and GR antagonist RU486 (10\(^{-5}\) mol/L) for 32 h. Columns, mean results of one representative experiment (three wells per experimental group); bars, SD. DNA binding of AR (B) and GR (D) measured by EMSA. Cells were treated as in A and C for 16 h. HRE (oligonucleotide with HRE) labeled with IR dye was used to detect GR or AR DNA binding.

Cold probe, unlabeled HRE; HRE mut, mutated HRE.
treated with CpdA for 32 h in comparison with control cells treated only with the vehicle (Fig. 3A). Moreover, the comparison of glucocorticoid FA and CpdA in luciferase assay revealed a noteworthy similarity in the overall negative effect of those GR ligands on TF activity: activity of most studied TFs (five of seven) was inhibited by both FA and CpdA in LNCaP-GR cells. The only exceptions were AP-1 and NFATc factors, whose function was not significantly affected by FA in LNCaP-GR cells at 32-h time point when we measured luciferase activity. We further characterized CpdA effects on NF-κB and AP-1 in other PC cells, as negative interaction between GR and NF-κB and AP-1 is central for anti-inflammatory and possibly for tumor suppressor effects of glucocorticoids. In all GR-expressing prostate cells, CpdA inhibited both basal and inducible NF-κB activity in luciferase test (Fig. 3A). For NF-κB induction, we used transfection of PC cells with IκB kinase β (IKKβ), an upstream activating kinase (40). In a similar way, CpdA inhibited basal and IL-1-induced (Fig. 3) or TNF-α-induced (data not shown) AP-1 activity in LNCaP-GR and PC3 cells.

The detailed analysis of nuclear translocation of major NF-κB protein p65 in LNCaP-GR cells activated by TNF-α revealed that p65 nuclear import was inhibited by CpdA (Fig. 3B). The similar results were obtained for AP-1 subunit c-Jun in LNCaP-GR cells activated by IL-1 (Fig. 3B; data not shown). These data suggest that cytoplasm retention of NF-κB and AP-1 could be the leading mechanism underlying CpdA-mediated inhibition of those factors in prostate cells. Interestingly, the cytoplasm retention of NF-κB and AP-1 was specific only to CpdA, as in cells treated with glucocorticoid FA the nuclear import of those TFs was not altered (Fig. 3B).

Altogether, these results indicate that CpdA combines properties of antiandrogen and selective GR modulator that shifts GR activity toward transrepression.

CpdA inhibits cell growth and induces apoptosis in PC cells.

We and others showed that AR blockage (by androgen ablation in androgen-free medium) as well as activation of GR signaling, especially GR-mediated transrepression, result in the inhibition of PC cell growth (1–3, 6–9). This suggested that CpdA may also possess a cytostatic potential in PC cells.

We indeed found that, in PC3, DU145, and parental LNCaP cells, CpdA acted as a potent growth suppressor. The CpdA effective concentrations were in the range of $10^{-5}$ to $10^{-6}$ mol/L (Supplementary Data 3) that correlated well with the range of concentrations that affected AR and GR function (see Fig. 2). Remarkably, highly malignant androgen-independent DU145 and PC3 cells seemed to be especially sensitive to CpdA: at the log phase, their growth was inhibited by ~70% to 90% compared with the vehicle-treated control cells (Fig. 4A). Interestingly, the cytostatic effect of CpdA in GR-expressing PC3 and DU145 cells was much stronger than the effects of GR agonist glucocorticoid FA and GR antagonist RU486 (data not shown).

In addition to experiments with monolayer cultures, we also assessed the effect of CpdA on growth of LNCaP cells (LNCaP-V

![Figure 3. CpdA induces GR transrepression. A, effects of CpdA on TF activity in LNCaP-GR cells. Cells were cotransfected with indicated Firefly luciferase reporters and Renilla luciferase reference plasmid and treated with DMSO (control), CpdA ($10^{-5}$ mol/L), or FA ($10^{-6}$ mol/L) for 32 h. Firefly luciferase activity was normalized against Renilla luciferase activity. Columns, mean results of one representative experiment (three wells per experimental group); bars, SD. NF-κB activity was induced by transfection with IKKβ; AP-1 activity was induced by IL-1 (1 μg/mL). B, effect of CpdA on nuclear translocation of p65 (NF-κB) and c-Jun (AP-1). LNCaP-GR cells were pretreated with CpdA ($10^{-5}$ mol/L) and FA ($10^{-6}$ mol/L) for 48 h and stimulated with TNF-α (10 ng/mL × 30 min). p65 and c-Jun nuclear translocation was assessed by immunofluorescence or Western blot analysis of nuclear extracts. HDAC-1 was used as a nuclear protein loading control. Note: CpdA decreases NF-κB and AP-1 nuclear translocation induced by TNF-α.](cancerres.aacrjournals.org)
cells infected with empty virus) in colony-forming assay. As shown in Fig. 5B, CpdA significantly reduced anchorage-independent growth of these PC cells.

To further evaluate cytotoxic potential of CpdA, we studied its effect on survival of DU145 and PC3 cells that are resistant to multiple proapoptotic stimuli (40). The analysis of the PARP cleavage revealed that long-term exposure of cells to CpdA (4–8 days) induced significant apoptosis (Fig. 4B). The treatment of these cells with CpdA for 2 to 3 days did not induce apoptosis per se but significantly sensitized DU145 and PC3 cells to TNF-α–induced apoptosis (Fig. 4B; data not shown). Interestingly, the apoptotic effect of CpdA was much more pronounced than the effect of glucocorticoid FA (Fig. 4B). The apoptosis induced in DU145 and PC3 cells by CpdA was caspase dependent: similar to TNF-α, CpdA induced activity of several caspases, especially caspase-2 and caspase-3 (Fig. 4C).

Cytostatic effect of CpdA depends on steroid receptor expression. PC cells sensitive to CpdA have markedly different phenotype in terms of the expression of steroid hormone receptors. The most sensitive DU145 and PC3 express only GR, whereas LNCaP cells with moderate sensitivity to CpdA express only AR. To prove that GR plays an important role as a mediator of CpdA toxicity, we performed two types of experiments. First, we compared effect of CpdA in LNCaP cells stably infected with GR-expressing lentivirus and LNCaP cells infected with empty virus (LNCaP-V). Importantly, LNCaP-GR cells appeared to be significantly more sensitive to growth inhibition by CpdA than LNCaP-V cells both in monolayer and in colony-forming assay (Fig. 5).

Second, we inhibited GR expression by 70% to 80% in PC3 and LNCaP-GR cells using siRNA approach (Fig. 6A and B). As shown in Fig. 6, GR blockage has resulted in a drastic loss of sensitivity to CpdA in both PC cell types.
To delineate AR role in CpdA effects, we have generated a clone of androgen-independent LNCaP cells with low AR expression (LNCaP-ARlow), which was derived from parental LNCaP cells during selection in androgen-deprived medium. These cells are analogous to previously reported androgen-independent LNCaP clones (41). LNCaP-ARlow cells that express only residual amounts of AR and no GR (data not shown) seemed almost completely resistant to CpdA (Fig. 5A).

Finally, we used RWPE-1 prostate cells expressing very low levels of AR and GR proteins (Supplementary Data 1). As shown in Fig. 4A, these cells were the most resistant to the growth-inhibitory effect of CpdA at all tested concentrations.

Overall, our results suggest an important role for both AR- and GR-mediated signaling in the cytostatic effect of CpdA in PC cells.

Discussion

A large body of work published by different groups, including our laboratory, suggests that signaling mediated via two closely related steroid hormone receptors, AR and GR, plays opposite role in PC formation. AR promotes PC development and growth at the stage of hormone-dependent cancer, whereas GR acts as a tumor suppressor in prostate (1–9, 16). Therefore, our study explores a novel strategy for PC treatment through simultaneous inhibition of protumorigenic and activation of antitumorigenic signaling mediated by steroid hormone receptors using dual specificity ligands that modulate activity of both AR and GR. One of the potential candidates for such approach to PC treatment is CpdA, a novel phyto-modulator of steroid hormone receptors (21, 23).

The chemical structure of CpdA significantly differs from most known AR/GR antagonists and GR-dissociated ligands (11, 19, 27, 41, 42): CpdA is small molecule with only one benzene ring (Fig. 1C). Using receptor ligand virtual docking analysis, we predict that CpdA could bind to LBDs of both AR and GR at the binding pockets of natural steroid ligands. Moreover, CpdA can form H-bonds with the same amino acids that are involved in H-bond formation during steroid binding (Fig. 1). These data suggest that CpdA would compete with corresponding steroids for receptor binding. Indeed, whole-cell binding assays revealed a strong competition between CpdA and glucocorticoid Dex for endogenous GR (Fig. 1D and ref. 23). The competition between androgens and CpdA has not been found previously (21). However, our data suggest that CpdA competes for AR binding with androgens, although at a lesser extent than it competes with glucocorticoids for GR (Fig. 1D). The difference in our results and previous data in respect to CpdA binding to AR may reflect the significant variations in experimental conditions, as Tanner and colleagues performed whole-cell binding assay and incubated cell cultures with a different radiolabeled androgen mibolerone for a short period of time.

Using prostate cell lines with different status of receptor expression, we found that CpdA induces nuclear translocation of both receptors in PC cells. Nevertheless, it inhibits basal and hormone-induced AR and GR DNA binding and gene transactivation. Remarkably, CpdA inhibits activity of numerous proproliferative and antiapoptotic TFs in a way similar to glucocorticoids. CpdA strongly inhibits growth of PC cells and induces caspase-dependent apoptosis of highly malignant PC3 and DU145 cells that are resistant to many anticancer drugs. Cytostatic effects of CpdA seemed to be AR and GR dependent.
Inhibitory effect of CpdA on hormone-induced AR transactivation reported here agrees with the results of previous studies (21). In addition, in prostate cells, CpdA negatively affected AR in the absence of hormone treatment (Fig. 2). Previously, Tanner and colleagues (21) described mechanisms of AR down-regulation by CpdA that included (a) altered interaction between AR and steroid receptor coactivator SRC-1 and (b) inhibition of ligand-induced interaction between NH2-terminal transactivation domain (AF1) and COOH-terminal transactivation domain (AF2) essential for normal AR function. These and our observations indicate that mechanisms of action of CpdA and widely used antiandrogens flutamide, Casodex, and cyproterone acetate are similar (Fig. 2B; refs. 27, 31, 36, 39).

CpdA differentially affects transactivation and transrepression functions of GR. Depending on the cell type, GR transactivation was not changed or was decreased to a different degree by CpdA (refs. 21, 23 and our work). Despite similarities in ligand properties between CpdA and glucocorticoids revealed by virtual docking,
CpdA failed to induce effective GR nuclear translocation and transactivation of GR (Fig. 2). This could possibly relate to inability of CpdA to induce GR phosphorylation at Ser521, which is an important step for GR nuclear translocation and transactivation by agonists (23).

In contrast to its antagonism to glucocorticoids in terms of inducing GR transactivation, CpdA was able to up-regulate GR transrepression, mimicking this important mode of glucocorticoid effects on transcription. We found that in all studied PC cells expressing GR, both CpdA and glucocorticoid FA inhibited function of several TFs, including NF-κB, AP-1, Ets-1, Elk-1, SRF, ATF/CRE, and NFATc (Fig. 3). These results correlate well with the previous observations that CpdA inhibited NF-κB-dependent gene expression in HEK293T and L929sA cells with endogenous and exogenous GR (23). The expression of functional GR in cells was a prerequisite for inhibition of NF-κB and other TFs by CpdA (ref. 23 and our not shown data). Moreover, the analysis of specific GR domains that are necessary for inhibition of NF-κB activity by CpdA has revealed the importance of LBD as well as second zinc finger in DNA-binding domain of GR (23). The same domains are critical for GR transrepression induced by glucocorticoids (43, 44). Overall, these findings strongly suggest that CpdA acts as a dissociated glucocorticoid modifying GR properties and shifting its activity toward transrepression.

Intriguingly, in prostate cells treated with cytokine TNF-α (Fig. 3) or IL-1 (data not shown), CpdA prevented translocation of p65 and c-Jun proteins into the nucleus. This indicates the important role of NF-κB and AP-1 cytoplasm retention in the inhibition of activity of these TFs specifically by CpdA, as nuclear translocation of these proteins was not inhibited by glucocorticoid FA (Fig. 3). It is possible that notably weaker nuclear import of GR induced by CpdA compared with glucocorticoids (Fig. 2C) is responsible for this NF-κB and AP-1 cytoplasm sequestration. The process of hormone-induced nuclear translocation of GR is complex and involves GR phosphorylation, substitution of protein partners in GR-chaperone complex, and recruitment of transport protein dynein (13, 44). As mentioned above, CpdA fails to induce GR phosphorylation. Other effects of CpdA on these early steps of GR activation remain to be investigated. However, our data suggest that CpdA induces only partial changes in GR conformation that are not fully sufficient for receptor nuclear translocation and its DNA binding but at the same time are permissive for GR/p65 and GR/c-Jun interaction in the cytoplasm. It is noteworthy that there is experimental evidence that GR indeed interacts with p65/p50 NF-κB dimer in cytoplasm and affects NF-κB cytoplasm/nuclear shuttling (reviewed in ref. 45).

One of the most important findings of this work is receptor-dependent strong cytostatic and apoptotic effects of CpdA in PC cells especially in highly malignant DU145 and PC3 cells that are resistant to many other growth-inhibitory and proapoptotic compounds (reviewed in ref. 40). We are currently investigating the molecular mechanisms underlying these cytostatic anticancer effects of CpdA in PC cells using different approaches, including gene arrays. Our working hypothesis is that the remarkable cytostatic potential of CpdA in PC cells is a result of the inhibition of numerous pro-proliferative and antiapoptotic TFs, such as factors from the Ets family, NF-κB, and AP-1 combined with the blocked defense response of cancer cells presumably mediated via gene activation by steroid hormone receptors. Importantly, neither glucocorticoids that are as potent as CpdA in inducing GR transrepression nor GR/AR antagonist mifepristone (RU486; refs. 7, 36) does not induce significant growth inhibition or apoptosis of PC3 and DU145 cells in vitro (Fig. 4 and data not shown).

It is currently well accepted that the anti-inflammatory therapeutic effects of glucocorticoids are mostly mediated via gene transrepression, whereas many side effects of glucocorticoids are mediated via DNA-dependent transactivation (11). Our recent studies have revealed the leading role of GR-mediated transrepression in its tumor suppressor effect in different cells, including prostate (7, 16, 46). This ties anti-inflammatory and anticancer effects of the GR signaling. Thus, selective GR modulators that preferentially induce GR transrepression with improved therapeutic-to-side effect ratio have a great potential both as anti-inflammatory and anticancer drugs. CpdA obviously represents one of such compounds and acts as GR-dissociated ligand not only in vitro but also in vivo. It was shown that CpdA possesses strong anti-inflammatory activity in mice, but in comparison with glucocorticoid, Dex does not induce such adverse effect as hyperglycemia (23).

Overall, our results and literature data clearly indicate that phyto-modulator of steroid hormone receptors CpdA has unique combination of properties acting as anti-inflammatory antiandrogen with reduced side effects. Thus, CpdA itself or as a prototype of novel class of multitarget steroid hormone receptor modulators has a great potential for PC treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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