A novel synthetic compound that interrupts androgen receptor signaling in human prostate cancer cells

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Abstract

The purpose of this study was to determine the effects of 6-amino-2-[2-(4-tert-butyl-phenoxy)-ethylsulfonfyl]-1H-pyrimidine-4-one (DL3), a novel synthetic compound with small-molecule drug properties, on androgen-regulated gene expression and cell growth in human prostate cancer cells. LNCaP, 22Rv1, and LAPC-4 cells were used in the studies. Expression of prostate-specific antigen (PSA) and androgen receptor (AR) was determined by ELISA, Western blotting, real-time reverse transcription-PCR, nuclear run-on, and/or promoter luciferase reporter assays. Effects of DL3 on cell growth were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining. DL3 inhibited dihydrotestosterone (DHT)-induced PSA expression in a dose-dependent fashion. The inhibitory effects of DL3 were more potent than those of flutamide, nilutamide, and bicalutamide. Moreover, DL3 blocked the stimulatory effects of nilutamide on PSA expression in LNCaP cells. Unlike the three classic antiandrogens, DL3 did not show intrinsic AR agonist activity. Nuclear run-on and PSA promoter reporter assays revealed that DL3 blocked DHT-induced PSA gene transcription. Consistent with its effects on PSA expression, DL3 inhibited DHT-stimulated cell growth with a potency significantly superior to flutamide, nilutamide, or bicalutamide. Furthermore, cells resistant to flutamide or nilutamide were as susceptible as their parental counterparts to the inhibitory effects of DL3 on both PSA expression and cell growth. DL3 did not inhibit AR nuclear localization and the NH2- and COOH-terminal interaction of AR induced by DHT. These data show that DL3 is a novel inhibitor of the AR signaling axis and a potentially potent therapeutic agent for the management of advanced human prostate cancer. [Mol Cancer Ther 2007;6(7):2057–64]

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Introduction

Prostate cancer is the most common cancer and the second most common cause of cancer death among men in the United States (1). As detection techniques improve, more patients are diagnosed with localized disease and can be cured by either surgery or radiation therapy. Metastasis in many patients, however, still occurs before the initial diagnosis. Hormonal therapies, commonly with combinations of antiandrogens (flutamide, nilutamide, or bicalutamide) and androgen deprivation, are the mainstay treatment for advanced diseases. These therapies, however, only delay tumor progression by an average of <18 months, followed by the development of hormone-refractory disease (2). A discontinuation of an antiandrogen therapy often results in clinical improvement and a decrease in serum prostate-specific antigen (PSA) in many patients with hormone-refractory disease (i.e., antiandrogen withdrawal syndrome), which is partially caused by the intrinsic androgenic activity of the antiandrogens (3). Therefore, there is an urgent need to identify and develop more effective therapeutic agents for prostate cancer.

Androgen receptor (AR), a member of the steroid receptor superfamily, is a ligand-dependent transcription factor that mediates androgen action in cells. AR is composed of three major domains: an NH2-terminal transcriptional activation domain (NTD), a central DNA-binding domain, and a COOH-terminal ligand-binding domain (LBD; 4, 5). AR is associated with cellular chaperones in the cytosol in its inactive state (6). After binding to androgens, such as testosterone and, more potently, dihydrotestosterone (DHT), AR translocates to the nucleus, binds to androgen response elements of AR target gene promoter, and regulates expression of AR target genes (4, 7). AR hypersensitivity, as a result of AR gene mutation and/or amplification, overexpression of coactivators, and AR cross-talking with other signal transduction pathways, often occurs and plays crucial roles in prostate cancer development, progression, and androgen-independent growth (7–9). Therefore, AR and its signaling axis are the most important targets for therapies against advanced prostate cancer (7–9).

In the present report, we identified 6-amino-2-[2-(4-tert-butyl-phenoxy)-ethylsulfonfyl]-1H-pyrimidine-4-one (DL3), a novel synthetic small molecule with potent anti-AR signaling activities. DL3 inhibited expression of PSA, a widely used serologic marker for prostate cancer burdens and an indicator of therapeutic efficacy and recurrence (10, 11). DL3 also inhibited androgen-stimulated growth in prostate cancer cells. Moreover, DL3 did not show any detectable intrinsic AR agonist activity and inhibited PSA expression and in vitro growth of prostate cancer cells resistant to flutamide or nilutamide.
Materials and Methods

DL3 Compound

During a high-throughput screening of a chemical library (ChemBridge Co.), we identified the novel compound DL3 (Fig. 1A), which selectively inhibited expression of genes regulated by androgen response element–driven promoter.

Tumor Cells and Culture

The well-characterized LNCaP (androgen responsive; refs. 12, 13) and 22Rv1 (androgen weakly responsive; refs. 13, 14) cell lines were purchased from American Type Culture Collection. HeLa cells were also obtained from American Type Culture Collection. The cells were maintained as a monolayer culture in MEM supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, vitamin A, and glutamine (C-MEM). LAPC-4 cells, which express the wild-type AR and are androgen responsive (13, 15), were generously provided by Dr. Karen Knudson, with the approval of Dr. Robert Reiter. LAPC-4 cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum and 10 nmol/L DHT. LNCaP cells were cultured in C-MEM containing 20 μmol/L flutamide or nilutamide for >2 months to derive LNCaP-Flut-R and LNCaP-Nilut-R cells, respectively. Cells in exponential growth phase were harvested by a 1- to 3-min treatment with a 0.25% trypsin-0.02% EDTA solution. The flasks were tapped to detach the cells, MEM-10% fetal bovine serum was added, and the cell suspension was gently agitated to produce a single-cell suspension. The cells were washed in HBSS and resuspended in growth medium. Only suspensions of single cells with viability exceeding 95% (ascertained by trypan blue exclusion) were used.

PSA Detection

LNCaP cells were plated into 96-well plates at 2 × 10⁴ per well in S-MEM (MEM medium supplemented with 10% charcoal-dextran treated fetal bovine serum) or C-MEM. After 24-h incubation, the cells were treated for 48 h as detailed in Results. PSA in the culture supernatants was quantified with an ELISA kit (United Biotech, Inc.) following the manufacturer’s instruction. Intracellular PSA was detected by Western blotting as described below.

Western Blot Analysis

Cells (2 × 10⁶ per 60-mm dish) were washed and scraped into a lysis buffer and analyzed by Western blotting, as described in our previous study (16), with PSA and AR-specific antibodies obtained from DakoCytomation and Santa Cruz Biotechnology, Inc., respectively. The immunoreactive signals were revealed with the enhanced chemiluminescence Western blotting detection system (Michigan Diagnostic LLC) and visualized in a KODAK Image Station IS4000MM Digital Imaging System (Eastman Kodak Co.). Expression of PSA, AR, and β-actin was quantitated in the linear range of exposure.
Quantitative Real-time PCR
Cells were seeded in 60-mm dishes (2 × 10⁶ per dish). After treatment as desired, the cells were washed and total RNA was extracted with TRIzol reagent. Expression of PSA and AR was analyzed by real-time reverse transcription-PCR (RT-PCR) as detailed in our previous studies (17). The cycle threshold values were used to calculate the normalized gene expression against β-actin using the Q-Gene software.

Nuclear Run-On Assay
RT-PCR-based nuclear run-on assay was done as detailed in our previous study (17). Briefly, cells in 15-cm dishes were rinsed with cold PBS and scraped in lysis buffer on ice, followed by addition of the same volume of 0.6 mol/L sucrose buffer. Cell nucleus pellets, obtained by centrifugation at 500 × g for 10 min, were incubated for 30 min at 30°C in 500 μL of transcription reaction buffer (pH 7.9) containing 50 mmol/L Tris, 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, and 0.5 mmol/L nucleotide triphosphate. RNA was extracted and followed by DNase treatment and real-time RT-PCR. Samples without transcription reaction were used as controls.

Transient Transfection and Luciferase Assays
LNCaP cells (5 × 10⁴ per well in 48-well plates) were cotransfected for 24 h with PSA promoter luciferase reporter plasmid (pGL3-PSA-luc) and internal control Renilla luciferase vector (pRL-CMV-luc) using the Lipofectamine 2000 transfection reagent (Invitrogen). Luciferase activities were measured by using the dual-luciferase reporter gene assay system (Promega) following the manufacturer’s instruction. Final results were normalized for transfection efficiencies using the Renilla luciferase assay value (16, 17).

The mammalian two-hybrid assay was done to evaluate ligand-induced AR NH₂- and COOH-terminal interaction (18–20). Briefly, HeLa cells (10⁵ per well in 12-well plates) were cotransfected for 24 h with GAL4-luc (a luciferase reporter driven by the GAL4 binding site), GAL4-LBD (a plasmid encoding GAL4 DNA-binding domain fused with the LBD of AR), and VP16-NTD [a plasmid encoding etoposide (VP16) transactivation domain fused with the NTD of AR]. GAL4-LBD and VP16-NTD were generously provided by Dr. Karen Knudson. The cells were treated with DHT, flutamide, or DL3 in S-MEM for 24 h, followed by luciferase as detailed above.

Cell Density Assay
LNCaP cells were plated into 96-well plates at 1,000 per well. After an overnight incubation, the cells were treated, as detailed in Results, for 4 days and viable cells in the wells were stained with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (17, 21). During the final 2 h, 0.4 mg/mL MTT (Sigma) was added. After removing the medium, dark-blue formazan was dissolved in DMSO and the absorbance at 570 nm was measured with a FLUOstar Optima microplate reader (BMG LABTECH, Inc.). Inhibition of cell growth was calculated by the following formula: growth inhibition (%) = (1 − A₅₇₀ of treated / A₅₇₀ of control) × 100.

Immunofluorescent Staining of AR
Control and treated LNCaP cells were permeabilized with 0.25% NP40 in PBS, fixed in 3.7% formaldehyde in PBS for 10 min, blocked in PBS containing 5 mg/mL bovine serum albumin and 0.1% Tween 20, and incubated with the antibody against AR in the blocking buffer for 2 h; then, cells were washed in PBS-0.1% Tween 20 and incubated with Texas red–conjugated antirabbit antibody in the blocking buffer for 1 h. After wash in PBS-0.1% Tween 20, the slides were mounted and examined under a fluorescence microscope. The images were captured with a Spot cooled charge-coupled device camera and digitized to a computer.

Statistical Analysis
All experiments were done at least twice. Data shown are the mean ± SE. Differences between means were compared by the two-tailed Student t test and were considered significantly different at P < 0.05.

Results
Inhibitory Effects of DL3 on PSA Expression
After starvation for 48 h in S-MEM, LNCaP cells were treated for 48 h with DHT in the absence or presence of
DL3, flutamide, nilutamide, or bicalutamide. The normalized data in Fig. 1B show that LNCaP cells secreted very low basal levels of PSA in the absence of DHT, which was significantly elevated in cells incubated with 1 nmol/L DHT (Fig. 1B). Treatment with 20 μmol/L DL3 did not induce PSA production and inhibited the DHT-induced PSA production by ~90% (Fig. 1B). Unlike DL3, treatment with 20 μmol/L of flutamide or bicalutamide and, much more potently, with nilutamide stimulated PSA production under androgen-free conditions (Fig. 1B), which is consistent with the observations reported previously by others (3, 22–26). Flutamide and bicalutamide, but not nilutamide, reduced DHT-induced PSA production (Fig. 1B). The inhibitory effects of DL3 on DHT-induced PSA production were dose-dependent, with a 50% inhibition at ~5 to 10 μmol/L DL3 and were partially reversed by increasing concentrations of DHT, suggesting that the inhibition was due to AR blockade rather than nonspecific toxicity (Fig. 1C). Data in Fig. 1D show that stimulatory effects of nilutamide on PSA production were dose dependent and were also blocked by DL3.

To determine whether the reduction of PSA in the culture supernatant of DL3-treated cells was due to a blockade in PSA secretion, we investigated intracellular accumulation of PSA by Western blotting. Data in Fig. 1E show that androgen-starved LNCaP cells expressed a low level of PSA, which was significantly increased by treatment with 1 nmol/L DHT (Fig. 1E). The DHT-induced PSA expression was reduced by DL3 in a dose-dependent manner and diminished in cells treated with 40 μmol/L DL3 (Fig. 2A). The similar inhibitory effects of DL3 on PSA expression were observed in LAPC-4 cells that express a wild-type AR (Fig. 2B, left) and 22Rv1 cells that express a mutant AR (Fig. 2B, right). Note that both LAPC-4 and 22Rv1 cells expressed much lower levels of PSA. The exposure of the membranes in Fig. 2B was five to six times longer than that in Fig. 2A.

Because down-regulation of AR could be one of the potential mechanisms by which DL3 interrupts AR signaling, we examined the effects of DL3 on AR expression. As shown in Fig. 2A and B, the expression of AR in both LNCaP and 22Rv1 cells was not significantly altered in cells treated with 5 and 10 μmol/L DL3, but reduced in cells treated with 20 and 40 μmol/L DL3. Similarly, AR expression was reduced in LAPC-4 cells treated with 20 μmol/L DL3 (Fig. 2B). Next, we compared the effects of DL3, flutamide, nilutamide, and bicalutamide on PSA and AR expression in LNCaP cells (Fig. 2C). Consistent with the data from ELISA, flutamide and bicalutamide moderately enhanced PSA expression under androgen-free culture conditions but inhibited DHT-induced PSA expression. On the other hand, nilutamide enhanced PSA expression in LNCaP cells regardless of the absence or presence of DHT. AR expression was down-regulated in cells treated with DL3 or bicalutamide, not altered by flutamide, and enhanced by nilutamide.

**DL3 Inhibited PSA Gene Transcription**

As shown in Fig. 3A, PSA mRNA levels in LNCaP cells was ~20-fold higher than that in 22Rv1 cells, which was in agreement with PSA protein levels in the two cell lines. DL3 inhibited the expression of PSA mRNA in both LNCaP and 22Rv1 cells in a dose-dependent fashion (Fig. 3A). Treatment of LNCaP cells for 24 h with 20 μmol/L DL3 reduced basal PSA mRNA level and
blocked DHT-stimulated PSA mRNA expression (Fig. 3B). In contrast, incubation with the same concentration of bicalutamide had no significant effects on the basal level of PSA mRNA and only partially inhibited DHT-stimulated PSA mRNA expression (Fig. 3B). Expression of AR mRNA in LNCaP cells was partially down-regulated by 1 nmol/L DHT, 20 μmol/L DL3 or bicalutamide, or a combination of either DHT and DL3 or bicalutamide (Fig. 3C).

Next, we carried out nuclear run-on assay to determine whether DL3 decreased the transcription of PSA. LNCaP cells were starved and treated for 24 h with 1 nmol/L DHT. Nuclei from control and treated cells were isolated and in vitro PSA mRNA transcription by the nuclear extracts was assessed. As shown in Fig. 3D, the in vitro transcription of PSA mRNA was increased by 8-fold in DHT-treated cells. Consistent with its effects on PSA protein and mRNA expression, DL3 abolished the DHT-induced transcription of PSA. To further determine the inhibitory effects of DL3 on PSA gene transcription, the PSA promoter–driven luciferase reporter activity was assessed in LNCaP cells transfected with pGL3-PSA-luc in which expression of luciferase gene was driven by 4.3 kb of human PSA gene promoter. Data in Fig. 3D show that DHT enhanced PSA promoter activity by ~5-fold, which was abolished when the cells were treated with DL3.

Inhibitory Effects of DL3 on Androgen-Stimulated Cell Growth

DL3 inhibited growth of LNCaP cells in a dose-dependent manner regardless of the presence or absence of DHT (Fig. 4A). The inhibitory effects on DHT-stimulated cell growth were, however, much more potent than those on cell growth in the absence of DHT. As shown in Fig. 4A, the growth of LNCaP cells in the presence and absence of DHT was inhibited by 82% and 27%, respectively, in cells treated with 10 μmol/L DL3. Similar inhibitory effects were observed in LAPC-4 and 22Rv1 cells (data not shown). Flutamide (Fig. 4B), nilutamide (Fig. 4C), and bicalutamide (Fig. 4D) also inhibited growth of LNCaP cells in a dose-dependent manner; the magnitude of inhibition was much inferior to that of DL3. Moreover, although both flutamide and nilutamide at 40 μmol/L inhibited cell growth under DHT-free conditions, they promoted cell growth in S-MEM (the absence of androgen) at concentrations up to 20 μmol/L (Fig. 4B and C).

Effects of DL3 on Cells Resistant to Flutamide and Nilutamide

We have established LNCaP-Flut-R and LNCaP-Nilut-R cells resistant to flutamide and nilutamide, respectively. AR expression level was moderately reduced in the two sublines of drug-resistant cell (Fig. 5A). Next, we investigated effects of DL3 on PSA expression (Fig. 5B) and cell growth (Fig. 5C) in cells refractory to antiandrogens flutamide and nilutamide. LNCaP, LNCaP-Flut-R, and LNCaP-Nilut-R cells in C-MEM were treated with 10 μmol/L DL3, flutamide, or nilutamide as described above for PSA production and cell growth. As shown in Fig. 5A and B, PSA production was elevated in the resistant cells compared with that in their parental cells. DL3 abolished PSA production in all three sublines of LNCaP cells. In contrast, the inhibitory effect of flutamide on PSA
production was reduced in the drug-resistant cells compared with that in parental cells (Fig. 5B). Nilutamide enhanced PSA production by both parental and drug-resistant cells (Fig. 5B). Similar to its effect on PSA production, DL3 inhibited growth of all three sublines of cell to a similar extent (Fig. 5C). In contrast, flutamide moderately inhibited growth of parental and LNCaP-Nilut cells but promoted growth of LNCaP-Flut-R cells (Fig. 5C), whereas nilutamide moderately inhibited growth of parental cells but promoted growth of the two lines of antiandrogen-resistant cells (Fig. 5C).

**DL3 Did Not Alter the Interaction of AR and DHT**

To determine whether DL3 interfered with the interaction of DHT and AR, we examined DHT-induced nuclear localization of AR. LNCaP cells in S-MEM were plated into eight-well chamber slides at 2 x 10^5 per well. Three days later, the cells were treated for 1 h with 20 μmol/L of DL3 or flutamide, followed by incubation for 2 h with 1 nmol/L DHT. As shown in Fig. 6A, AR in untreated LNCaP cells distributed in both cytoplasm and nucleus. AR was translocated into the nucleus after treatment with DHT. DL3 treatment alone did not significantly alter the AR localization and had no inhibitory effects on DHT-induced AR translocation. In contrast, treatment with flutamide alone induced nuclear translocation of AR.

The association of androgen with AR leads to an interaction between the NH2-terminal domain and the COOH-terminal LBD of AR (i.e., the AR NH2- and COOH-terminal interaction; refs. 18, 19), which enhances the sensitivity of AR to low concentrations of androgen by increasing the stability of AR and the recruitment of coactivators (20). HeLa cells do not express AR. To further determine whether DL3 alters the interaction between DHT and AR, we investigated the effects of DL3 on the AR NH2- and COOH-terminal interaction in HeLa cells cotransfected with GAL4-luc, GAL4-LBD, and VP16-NTD. Data in Fig. 6B show that the luciferase activity was not significantly altered by either DL3 or flutamide and was increased by ~40- to 50-fold in cells treated with 1 nmol/L DHT. Flutamide inhibited DHT-stimulated NH2- and COOH-terminal interaction in a dose-dependent manner. In contrast, the DHT-induced NH2- and COOH-terminal interaction was not significantly altered by DL3 (Fig. 6B).

**Discussion**

AR signaling axis is crucial for growth of both androgen-dependent and androgen-independent prostate cancers and a key target in treatment of advanced prostate cancer (7–9). The goal of this research was to characterize the inhibitory effects of DL3, a novel synthetic small-molecule compound, on AR signaling. Among numerous androgen-regulated genes, we have chosen PSA because it is primarily regulated by AR signaling at the transcriptional level and is a well-established marker for prostate cancer (10, 11). As an initial step toward exploring therapeutic potentials of this novel compound against prostate cancer, we investigated its effects on cell growth in culture. Our data clearly show that DL3 is a potent and unique inhibitor of AR signaling axis. Not only is DL3 more potent than flutamide, nilutamide, and bicalutamide in suppressing both DHT-induced PSA expression and cell growth, but it also blocks the AR agonist activity of nilutamide on PSA expression. Moreover, DL3 inhibits PSA expression in and growth of cells resistant to flutamide and nilutamide.
Furthermore, DL3 has no detectable AR agonist activity in either stimulating cell growth or inducing PSA expression. Taken together, these data indicate that DL3 is a unique AR signaling inhibitor.

The data presented clearly show that both cellular and secreted PSA are significantly reduced in cells exposed to DL3, suggesting that the treatment with this compound down-regulates PSA production. Whether this reduction of PSA production in DL3-treated cells is caused by a reduction of PSA synthesis, its stability, or both remains to be further determined. However, given that PSA expression is regulated mainly at transcription level (10, 11) and that DL3 down-regulates PSA mRNA, PSA gene transcription in vitro, and PSA promoter activity, the blockade of PSA gene transcription is probably of major importance in DL3-regulated PSA expression.

The dose-response analysis reveals the differential effects of DL3 on AR expression and the AR-induced responses (i.e., DHT-induced PSA expression and DHT-stimulated cell growth). At concentrations of 5 to 10 μmol/L, DL3 significantly inhibits both PSA expression and cell growth but has no significant effects on AR expression. In contrast, DL3, at concentrations >20 μmol/L, down-regulates AR expression at both protein and mRNA levels. These data suggest that the inhibition of AR signaling by DL3 may be mediated by both direct interruption of AR function and down-regulation of AR expression. It is noteworthy that DL3, at concentrations that abolish DHT-stimulated cell growth or PSA expression, has minimal effects on both basal and DHT-stimulated AR NH2- and COOH-terminal interaction (19, 22, 27). Because the AR NH2- and COOH-terminal interaction relies on the association of androgen with the LBD of AR, these data suggest that the inhibition of AR signaling by DL3 may not be mediated by interfering with the interaction of AR with its ligand. This conclusion is supported by several lines of evidence. First, DL3 does not significantly alter the AR localization and has no inhibitory effects on DHT-induced AR nuclear translocation. Second, unlike the three AR antagonists (flutamide, nilutamide, and bicalutamide), DL3 has no detectable intrinsic AR agonist activity. Third, DL3 inhibits the agonist activity of nilutamide on PSA expression even when they were used at similar concentrations. Fourth, cells resistant to either flutamide or nilutamide are still susceptible to the inhibitory effects of DL3 on both PSA expression and cell growth. Whether DL3 alters the binding of DHT to the LBD of AR remains to be determined in a ligand-binding assay.

In summary, the data presented in this study reveal that DL3 is a novel AR signaling inhibitor, which can block AR-regulated gene expression and cell growth in human prostate cancer cells expressing both mutant and wild-type AR and in both parental and antiandrogen-resistant cells. The most unique property of this novel compound is that it displays no detectable intrinsic AR agonist activity. The inhibitory effects of DL3 seem to be due to interference with the interaction of AR and its ligand, but to novel mechanisms remaining to be further elucidated and to the down-regulation of AR. The molecular mechanisms, such as its effects on expression of cyclin-dependent kinases and cyclins, by which DL3 inhibits cell growth, particularly the growth in the absence of androgen, remain to be elucidated by in-depth analysis. Moreover, further studies on the in vivo antitumor effect in prostate cancer xenografts or mouse models of prostate cancer progression are required to determine therapeutic potential of DL3 in treatment of human prostate cancer.

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