A novel class of pyrano coumarin anti–androgen receptor signaling compounds

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Abstract

Androgen and the androgen receptor (AR)–mediated signaling are crucial for prostate cancer development. Novel agents that can inhibit AR signaling in ligand-dependent and ligand-independent manners are desirable for the chemoprevention of prostate carcinogenesis and for the treatment of advanced prostate cancer. We have shown recently that the pyrano coumarin compound decursin from the herb Angelica gigas possesses potent anti-AR activities distinct from the anti–androgen bicalutamide. Here, we compared the anti-AR activities and the cell cycle arrest and apoptotic effects of decursin and two natural analogues in the androgen-dependent LNCaP human prostate cancer cell culture model to identify structure-activity relationships and mechanisms. Decursin and its isomer decursinol angelate decreased prostate-specific antigen expression with IC₅₀ of ~ 1 μmol/L. Both inhibited the androgen-stimulated AR nuclear translocation and transactivation, decreased AR protein abundance through proteasomal degradation, and induced G₀/G₁ arrest and morphologic differentiation. They also induced caspase-mediated apoptosis and reactive oxygen species at higher concentrations. Furthermore, they lacked the agonist activity of bicalutamide in the absence of androgen and were more potent than bicalutamide for suppressing androgen-stimulated cell growth. Decursinol, which does not contain a side chain, lacked the reactive oxygen species induction and apoptotic activities and exerted paradoxically an inhibitory and a stimulatory effect on AR signaling and cell growth. In conclusion, decursin and decursinol angelate are members of a novel class of nonsteroidal compounds that exert a long-lasting inhibition of both ligand-dependent and ligand-independent AR signaling. The side chain is critical for sustaining the anti-AR activities and the growth arrest and apoptotic effects. [Mol Cancer Ther 2007;6(3):907–17]

Introduction

Prostate cancer is the most frequently diagnosed cancer in U.S. men and second only to lung cancer as the cause of male cancer mortality (1). Androgen and the androgen receptor (AR) are crucial for the prostate function and pathobiology (2) and prostate cancer development (3, 4). Therefore, they are important targets for the chemoprevention of prostate cancer. With respect to prostate cancer treatment, hormone ablation therapies can result in several years of remission, but the disease invariably progresses into the androgen ligand-independent stage (3–5). A common feature of even these advanced hormone refractory prostate cancer is that the majority of the cases still express the wild-type AR (3–5) and they remain AR dependent for growth and survival in the absence of the androgen ligand (4, 6, 7). A recent study (8) has shown that an overexpression of the wild-type AR can be a molecular determinant of hormone refractoriness and may turn the best-characterized and clinically used AR antagonist bicalutamide/Casodex (9) into an agonist. Therefore, it is important to identify and develop new agents that can inhibit AR signaling in both ligand-dependent and ligand-independent manners for the chemoprevention and treatment of prostate cancer.

To this end, we have discovered recently the pyrano coumarin compound decursin as a potent anti-AR agent in the androgen-dependent LNCaP cell model (10). We have shown that decursin inhibits prostate-specific antigen (PSA) expression, induces G₀/G₁ arrest and the morphologic features of neuroendocrine differentiation (NED), inhibits AR nuclear translocation, decreases the AR protein abundance, and lacks the agonist activity of bicalutamide.

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in the absence of androgen (10). By way of background, decursin (Fig. 1A, a) was first isolated from *Angelica decursiva* (Fr. et Sav.) and later from the Korean medicinal herb *Angelica gigas* Nakai (11, 12). Other major compounds isolated from *A. gigas* Nakai include decursinol angelate (13), which is a decursin isomer (Fig. 1A, b), and decursinol (11), which comprises the pyranocoumarin core without the side chain (Fig. 1A, c). Both decursin and decursinol angelate are cytotoxic to leukemia cells, activate protein kinase C (PKC) in test tube assays (12–15) and induce reactive oxygen species (ROS) generation in cell culture (14). Daily i.p. injection of 100 mg/kg decursin or decursinol angelate has been shown to prolong the survival of sarcoma-180–bearing mice (16). These findings indicate a potential *in vivo* anticancer activity of these compounds in a broad spectrum of organs.

About possible anti–prostate cancer activities of the pyranocoumarin compounds, a recent report has shown that decursin inhibits the growth and survival of not only LNCaP cells but also DU145 and PC-3 androgen-independent prostate cancer cells, albeit requiring higher concentrations than in LNCaP cells (17). Furthermore, that work has inferred that the side chain of decursin seems to be necessary for conferring the growth-suppressing activities when compared with decursinol (17). A better understanding of the structure-activity relationship for the newly discovered anti-AR activities (10) will be crucial to provide the structural insights for the generation of more

![Figure 1. A, chemical structures of the three tested pyranocoumarin compounds from *A. gigas* Nakai. B, the differential effects of decursin and decursinol angelate (DA) versus decursinol treatment for 48 h on the secreted PSA by LNCaP cells as detected by PSA ELISA. Points, mean of three independent experiments, each with duplicate flasks; bars, SE. All decursin or decursinolangelate treatment levels versus control (*P* < 0.001). Decursin versus decursinol or decursinol angelate at each level tested (*P* < 0.01). C, the differential effects of decursin and decursinol angelate versus decursinol treatment for 48 h on the cellular PSA and AR protein abundance in LNCaP cells detected by immunoblot. Data are representative of three independent experiments. D, the differential effects of decursin and decursinol angelate versus decursinol and bicalutamide treatment for 24 h on the PSA and AR mRNA transcript abundance in LNCaP cells detected by reverse transcription-PCR (RT-PCR) and on the secreted PSA detected by ELISA (bottom) in the absence (lanes 1-9) and presence of mibolerone treatment (lanes 10-18). LNCaP cells were seeded in the phenol red–free medium supplemented with 5% charcoal-stripped serum for 2 d before treatments were initiated.](https://molcanther.aacrjournals.org/content/mct/acrrjournals.org.full)
efficacious analogues and derivatives as anti–prostate cancer agents.

Here, we compared the anti-AR signaling activities and the cell cycle arrest and apoptotic effects of these major pyranocoumarin compounds in the LNCaP cell model. We show that decursinol angelate possesses comparable potencies as decursin in all the tested activities, supporting decursin and decursinol angelate as members of a novel class of nonsteroidal anti-AR compounds. We also show that decursinol lacks the apoptotic activity and exerts a weaker anti-AR activity in the lower micromolar range and paradoxically a stimulatory activity at higher concentrations on the AR signaling and cell growth. Our data indicate that the side chain is critical for the sustained anti-AR activities and for the growth arrest and apoptotic effects. Mechanistically, we show that decursin inhibits ligand binding to AR protein, decreases AR protein abundance by increasing proteasomal degradation, and induces ROS generation that is correlated with persistent G1 arrest and apoptosis.

Materials and Methods

Preparation of Pyranocoumarin Compounds

Extraction of decursin, decursinol angelate, and decursinol from A. gigas Nakai was carried out following reported methods (18, 19). The identity of each compound after crystallization was verified by IR spectroscopy, 1H–nuclear magnetic resonance, and 13C–nuclear magnetic resonance.

Cell Culture and Treatments

The LNCaP, DU145, and PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA). The culture and treatment conditions were same as we reported recently (10).

Assays

The following assays were carried out as we reported recently (10): ELISA of PSA protein; the immunoblotting of AR and PSA; the reverse transcription-PCR of mRNA of PSA, AR, and glyceraldehyde-3-phosphate dehydrogenase; the AR nuclear translocation; and the PSA promoter transient transfection. Apoptosis was detected by an ELISA kit for the oligonucleosomal DNA fragments and by immunoblot for the cleaved poly(ADP-ribose) polymerase (PARP) as described recently (20).

Crystal Violet Staining

The long-term cell growth and NED assays were carried out in six-well plates instead of T25 flasks (10). The cell number was estimated by first fixing the cells in 1% glutaraldehyde for 15 min and then by staining with 0.02% crystal violet solution. After extensive washing with distilled water, the plates were air dried and photographed. The retained crystal violet dye was dissolved in 70% ethanol and the optical absorbance was measured at 570 nm.

In vitro AR Ligand Binding Assay

The binding assay was essentially as described by Chun et al. (21). Briefly, LNCaP cells were allowed to grow to confluence for 3 days. Cells were harvested in lysis buffer [10 mmol/L Tris (pH 7.4), 1.5 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT added immediately before use]. The cell suspension was passed through a 26-gauge needle (10–15 times) for homogenization. The homogenate was incubated on ice for 10 min and centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was collected and used as the cytosolic fraction. Total protein was estimated in the extracts and equal amounts of protein were used in the subsequent assay. The extracts were incubated with 0.5, 1, 5, and 10 nmol/L dihydrotestosterone (DHT; 3H-DHT, NET-453, specific activity of 110 Ci/mmol; Perkin-Elmer, Boston MA) and DMSO or two different levels of decursin or decursinol in a total reaction volume of 250 μL each (made up with lysis buffer). The reaction mixtures were incubated on ice throughout the assay. Dextran-coated charcoal suspension (500 μL; 0.25% charcoal and 0.025% dextran in 1× PBS) was added to each sample and incubated at 4°C with vigorous shaking for 10 min. The samples were centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant (500 μL) was added to 5 mL of scintillation fluid and counted in a liquid scintillation counter. The amount of the ligand bound to the AR in the presence or absence of decursin or decursinol was calculated as femtomole per milligram protein.

Measurement of AR Degradation

To estimate AR protein turnover, LNCaP cells were treated with 10 μg/mL cycloheximide to block new protein synthesis in the absence or presence of decursin for 3, 6, and 12 h. The AR abundance in cellular lysate was measured by Western blot. In additional experiments, an inhibitor of 26S proteasomal degradation MG-132 was used to determine the role of this intracellular degradation process in decursin-induced AR down-regulation.

Measurement of ROS

Intracellular level of ROS was examined with the aid of dihydroethidium (DHE) and 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein (DCF) diacetate (H2DCFDA) dye probes, which preferentially detect superoxide or hydrogen peroxide and hydroxyl radicals, respectively (22). In the presence of superoxide, DHE dye probe is oxidized to ethidium, which can be measured by flow cytometry using 585-nm band pass filter. Once inside the cell, H2DCFDA is cleaved by nonspecific esterases and oxidized in the presence of hydrogen peroxide to yield fluorescent DCF that can be detected using 530-nm bandpass filter (22). Briefly, the cells were seeded in T25 flasks and grown to 50% to 60% confluence. The cell cultures were treated with decursin or decursinol for the desired duration and then incubated with 2 μmol/L DHE and/or 5 μmol/L H2DCFDA for 30 min at 37°C. Subsequently, the cells were collected by gentle trypsinization, washed once with PBS, and analyzed for ethidium and DCF fluorescence using Becton Dickinson Caliber Flow Cytometer (San Jose, CA).

Statistical Analyses

Whenever appropriate and necessary, ANOVA or t tests were used to analyze the numerical data, with $P < 0.05$ considered as significantly different.
Results

Decursinol Angelate and Decursin Exert a Comparable Suppression of PSA and AR Abundance

The treatment with decursin or decursinol angelate for 48 h decreased the secreted (Fig. 1B) and cellular PSA (Fig. 1C) throughout the tested concentration range. Decursinol angelate was as efficacious as decursin, with $IC_{50}$ of ~1 and 1.3 $\mu$mol/L, respectively, for inhibiting PSA expression (Fig. 1B). Increasing the decursin and decursinol angelate concentrations $>10$ $\mu$mol/L nearly

Figure 2. A, the concentration-dependent suppression by decursin of androgen-stimulated AR nuclear translocation. LNCaP cells were seeded in the phenol red–free medium supplemented with 5% charcoal-stripped serum for 2 d. The cells were treated with different concentration of decursin for 1 h without mibolerone (Mib) and then for 2 h with mibolerone. Nuclear (N) and cytosolic (C) extracts were prepared for immunoblot analyses of the AR protein. Full-length PARP and tubulin are immunoblotted as the nuclear and cytosolic markers, respectively. B, the effects of decursin (a) and decursinol angelate (b) versus decursinol (c and d) on the AR protein abundance in the nucleus and the cytosol of LNCaP cells in the absence and presence of androgen stimulation. The experimental procedures were identical to (A). These results are representative of two experiments. C, the differential effects of decursin and decursinol angelate versus decursinol on the basal PSA promoter transcriptional activity in the absence of mibolerone. LNCaP cells were cotransfected with the PSA promoter-luciferase and the cytomegalovirus–$\beta$-galactosidase vectors and incubated in serum-free, phenol red–free medium for 24 h. The cells were treated with the indicated pyranocoumarin for 24 h in 5% charcoal-stripped serum medium. The cell lysates were assayed for luciferase and $\beta$-galactosidase activities. The luciferase activities were normalized to the $\beta$-galactosidase activities. The luciferase activities were normalized to the $\beta$-galactosidase activities. The results were based on three experiments, each with duplicate wells per treatment condition. Decursinol effects at 50 and 100 $\mu$mol/L versus control ($P < 0.05$) versus 0.1 nmol/L mibolerone ($P < 0.001$). D, the effect of decursin and decursinol angelate versus decursinol on the androgen-stimulated PSA promoter transcriptional activity. Experimental procedures were identical to (C) with the addition of mibolerone. The results were based on three experiments, each with duplicate wells per treatment condition. Decursin, decursinol angelate, or decursinol effects versus mibolerone control ($P < 0.001$). Decursin or decursinol angelate effects versus decursin effects at each tested level ($P < 0.01$).
shut down PSA expression (Fig. 1B and C). Decursin and decursinol angelate decreased the total AR protein level with a similar efficacy and in concentration-dependent manners (Fig. 1C).

**Decursin Exerts Biphasic Effects on PSA Expression**

Decursinol decreased PSA expression in a similar manner as did decursin and decursinol angelate in the concentration range of 1 to 10 μmol/L (IC50 of ~5 μmol/L), but the slope of the response curve was more tapered than those of the decursin- and decursinol angelate–treated cells (Fig. 1B). Surprisingly, as the decursinol concentration was increased, the PSA expression reached a nadir at ~10 μmol/L and recovered in a concentration-dependent manner to near the control level with 50 or 100 μmol/L decursinol (Fig. 1B and C). Decursinol exerted a negligible effect on the AR protein abundance (Fig. 1C).

**Decursin and Decursinol Angelate versus Decursinol Affect PSA Transcript Abundance Differentially**

Our earlier work has established that decursin decreases the PSA mRNA level and the PSA promoter transcription without decreasing the AR mRNA abundance (10). To contrast the effects of decursinol from those of decursin and decursinol angelate on these processes, we compared their effects on the PSA mRNA level and PSA secretion in the absence (−) and presence (+) of 0.1 nmol/L mibolerone after 24-h exposure. In the absence of mibolerone (Fig. 1D, lanes 1-9), both decursin (Fig. 1D, lanes 2 and 3) and decursinol angelate (Fig. 1D, lanes 4 and 5) decreased the basal PSA mRNA abundance and reduced the secreted PSA protein level. On the other hand, decursinol (Fig. 1D, lanes 6 and 7) increased both variables in a concentration-dependent manner in comparison with the control cells (Fig. 1D, lane 1), and the enhancing effect was comparable or even stronger than the AR “agonist” activity of bicalutamide at the same concentrations tested (Fig. 1D, lanes 8 and 9).

In the presence of mibolerone (Fig. 1D, lanes 10-18), decursin (Fig. 1D, lanes 11 and 12) and decursinol angelate (Fig. 1D, lanes 13 and 14) effectively blocked the androgen-stimulated PSA mRNA expression and secreted PSA in comparison with the stimulated control cells (Fig. 1D, lane 10). Decursinol (Fig. 1D, lanes 15 and 16) was less inhibitory than decursin or decursinol angelate at both the PSA mRNA and the protein secretion levels. It is noteworthy that none of the three compounds decreased the AR mRNA level either in the absence or in the presence of androgen stimulation (Fig. 1D).

**All Three Pyranocoumarins Inhibit the Androgen-Stimulated AR Nuclear Translocation and Transactivation, whereas Decursinol Increases the Basal Nuclear AR Abundance and Transactivation**

Translocation of AR to the nucleus after androgen binding in the cytosol is crucial for the transcriptional activation of the AR-regulated genes, such as PSA (3, 4, 23). We have shown that decursin potently inhibits the androgen-stimulated AR nuclear translocation (10), as was confirmed in Fig. 2A in a concentration-dependent manner and was very rapid, which is effective with 1-h pretreatment. When each compound was tested at 20 μmol/L, there was no discernible difference in the nuclear AR abundance in the absence of mibolerone (Fig. 2B, decursin, a5 versus a1; decursinol angelate, b5 versus b1; and decursinol, c5 versus c1). For cells treated with 100 μmol/L decursinol, the nuclear AR level was slightly increased compared with the basal control (Fig. 2B, d5 versus d1).

All three compounds inhibited the mibolerone-stimulated AR translocation to the nucleus (Fig. 2B). Decursin and decursinol angelate had a similar inhibitory efficacy (Fig. 2B, a6 versus a2 and b6 versus b2), but decursinol (Fig. 2B, c6 versus c2) was less inhibitory at the same treatment concentration of 20 μmol/L. Increasing the decursinol concentration to 100 μmol/L further suppressed the nuclear AR abundance (Fig. 2B, d6 versus d2).

Next, we assessed the effect of the three compounds on the basal and the androgen-stimulated AR transactivation of a transiently transfected PSA promoter-luciferase plasmid as described previously (10). Decursin and decursinol angelate did not stimulate PSA transcription in the absence of mibolerone (Fig. 2C, columns 2 and 3, columns 4 and 5 versus column 1). In contrast, decursinol increased the basal PSA transcription by ~6- and 11-fold at 50 and 100 μmol/L, respectively (Fig. 2C, columns 7 and 8 versus column 1), being comparable to the action of 0.05 nmol/L mibolerone (Fig. 2C, column 10).

In the presence of mibolerone, decursin and decursinol angelate inhibited the PSA promoter transcription with the same potency, shutting down the transcription completely at 50 μmol/L (Fig. 2D, columns 3-6 versus column 2). Decursinol also inhibited the mibolerone-stimulated PSA promoter transcription in a concentration-dependent manner, without any indication of a biphasic recovery (Fig. 2D, columns 7-9 versus column 2). However, decursinol was much less potent than decursin or decursinol angelate at each tested concentration (Fig. 2D, column 7 versus column 3 or 5; column 8 versus column 4 or 6) and did not completely shut down the PSA promoter transcription even at 100 μmol/L (Fig. 2D, column 9 versus column 1).

**Decursin Inhibits DHT Binding to AR More Potently than Decursinol**

Docking simulation has suggested that decursin and decursinol may enter the AR ligand binding pocket. To test the possibility of interference with ligand binding, a competition assay was done between 3H-labeled DHT and decursin or decursinol using cytosolic AR extract prepared from LNCaP cells. As shown in Fig. 3A, 5 μmol/L decursin decreased binding by 50%, whereas 50 μmol/L nearly blocked DHT binding. Decursinol also inhibited DHT binding but was less effective with 50% inhibition achieved at 10 μmol/L and increasing to 100 μmol/L did not increase the inhibition (Fig. 3B). The results therefore were consistent with a more potent inhibition of DHT binding to AR by decursinol than decursinol in the mol cancer ther 2007:6(3). March 2007
concentration range that inhibited AR translocation and PSA transcription.

Decursin Increases Proteosomal Degradation of AR

Because decursin did not decrease AR mRNA level (Fig. 1D) yet decreased AR protein abundance (Fig. 1C), we tested AR protein degradation by estimating the half-life after blocking new protein synthesis with cycloheximide (Fig. 3C). The data showed increased degradation of AR by decursin treatment as early as 3 h. Because AR is degraded by 26S proteasome (24), we included the proteasomal inhibitor MG-132 (24), we included the proteasomal inhibitor MG-132 in the absence of androgen and observed a complete block of AR down-regulation by decursin (Fig. 3D, lane 4 versus lane 3). This inhibitor partially reversed AR degradation induced by decursin in the presence of androgen (Fig. 3D, lane 8 versus lane 7). The abundance changes of cyclin D1, a protein also well known to be degraded through the 26S proteasomal pathway (25), verified the specificity of MG-132. Therefore, these data supported an induction of proteasomal degradation of AR by decursin as one mechanism of down-regulating AR protein abundance.

Decursin and Decursinol Angelate Inhibit, Whereas Decursinol Stimulates, Cell Growth in the Absence of Androgen

Because the short-term studies described above have revealed significant differential effects between decursinol versus decursin and decursinol angelate on AR protein abundance, ligand binding, and the ligand-independent (absence of mibolerone) and the androgen-stimulated AR transactivation activities, we further compared their effects on the long-term growth of LNCaP cells. Sparsely seeded LNCaP cells were treated with each compound for 10 days in the absence of mibolerone or with the addition of 0.1 nmol/L mibolerone for the last 7 days. As shown in Fig. 4A, deprivation of androgen in LNCaP cells (Fig. 4A, −Mib versus +Mib) leads to the appearance of morphology features resembling NED ("neuron-like" spikes). Decursinol at 20 μmol/L or higher increased the cell number and decreased the morphologic NED in the absence of androgen (Fig. 4A, −Mib). Crystal violet staining quantitation showed that decursinol at the concentrations of 20, 50, and 100 μmol/L increased the cell growth by 56%, 97%, and 132%, respectively, over the basal control cells (Fig. 4E, a, columns 4-6 versus column 1). At these concentrations, decursinol stimulated PSA secretion by 2.3-, 5.0-, and 11.0-folds, respectively (Fig. 4E, b, columns 4-6 versus column 1). In the presence of mibolerone, decursinol modestly suppressed the cell growth at concentrations ≤20 μmol/L and derepressed the growth inhibition at higher concentrations (Fig. 4A, +Mib; Fig. 4E, c, columns 2-6 versus column 1). The secreted PSA mirrored the cell number changes with a biphasic response pattern (Fig. 4E, d, columns 2-6 versus column 1).

In contrast, decursin and decursinol angelate each decreased the cell number and intensified the morphologic NED in the absence of androgen (Fig. 4B and C, −Mib). Both compounds exerted a profound inhibition of the androgen-stimulated cell growth and increased NED (Fig. 4B and C, +Mib; Fig. 4E, e and d). It is remarkable that the lowest concentration tested (5 μmol/L) inhibited cell number by >82% (Fig. 4E, e, column 7 versus column 1). Furthermore, the potency of decursin or decursinol angelate to suppress the androgen-stimulated cell growth and PSA secretion was greater than an equal concentration of bicalutamide (Fig. 4D and E, c and d, columns 7-9, columns 10-12 versus columns 13-15). In the absence of androgen, bicalutamide stimulated the cell growth by 44% (Fig. 4E, a, column 15 versus column 1) and the secreted PSA by 2.3-fold at 20 μmol/L (Fig. 4E, b, column 15 versus column 1). These results indicate that decursin and decursinol angelate exert a long-lasting inhibition on the ligand-dependent and ligand-independent AR signaling to PSA and cell growth. On the other hand, in the absence of androgen, decursinol possesses an AR agonist activity that is comparable with bicalutamide.
Decursinol Does Not Stimulate Growth of Prostate Cancer Cells Lacking AR

To support a requirement of AR in LNCaP cells to mediate the growth-stimulatory effect of high levels of decursinol, we tested its effects on the clonal growth of the AR-negative DU145 and PC-3 cells in serum-free medium. As shown in Fig. 4F, exposure to decursinol in the tested range for 7 days did not stimulate the growth of either cell line. Instead, a trend for mild inhibition of cell growth was observed, consistent with reported data observed with DU145 cells in serum-rich complete medium (17). Decursin treatment under the same culture conditions decreased the cell number in a concentration-dependent manner, producing 58% and 37% inhibition at 5 μmol/L in the PC-3 cells and the DU145 cells, respectively. These results further emphasized the importance of the side chain present in decursin or decursinol angelate for the AR-independent growth suppression activities.

Decursinol Lacks the Apoptotic Activity of Decursin and Decursinol Angelate

Our earlier work has shown that decursin induces a potent G1 arrest at ≥10 μmol/L and apoptosis at ≥40 μmol/L in LNCaP cells (10). Cell cycle analyses showed that, like decursin, decursinol angelate induced a potent G1 arrest in

Figure 4. The effects of decursinol (A), decursin (B), decursinol angelate (C), or bicalutamide (D) on the morphology of sparsely seeded LNCaP in the absence of mibolerone (−Mib) and in the presence of 0.1 nmol/L mibolerone (+Mib). LNCaP cells were seeded into six-well plates with the phenol red–free medium supplemented with 5% charcoal-stripped serum along with one of the compounds. Mibolerone was added to the +Mib wells 3 d after seeding. The cells were photographed at 10 d after seeding under phase-contrast magnification. E, quantitation of secreted PSA in conditioned medium (b and d) and cell number (a and c). The cells were fixed and stained with crystal violet. Relative cell number was estimated by the absorbance reading of the dissolved dye in duplicate wells for each datum point (a and c). The results are expressed as percentage of the +Mib control cells based on the average of duplicate wells with variation within 5% of each average. F, the lack of stimulation by decursinol on the growth of the AR-negative prostate cancer cell lines. The sparsely seeded cells were exposed to decursinol (DL) and to decursin (D) for 7 d before the staining with crystal violet. Points, average of duplicate wells, with variation within 5% of each average.
a concentration-dependent manner with an exposure concentration ≤50 μmol/L (Fig. 5A). Above this concentration, both decursin and decursinolangelate caused a slight rebound of cells distributed in S and G2, which was accompanied by an increased apoptosis as indicated by Death ELISA for the released oligonucleosomes and by increased cleavage of PARP (Fig. 5B).

Decursin caused a minor G1 arrest at an exposure concentration of 10 μmol/L (Fig. 5B). Higher decursin concentrations reversed this G1 arrest effect. Decursinol did not induce apoptosis in the tested concentration range (Fig. 5B) or even as high as 200 μmol/L (data not shown). These data indicate that the side chain of decursin and decursinol angelate is crucial for their cell cycle arrest and apoptotic activities.

**ROS Generation Is Induced by Decursin but Not by Decursinol**

Because it has been shown recently that treatment of leukemia cells with decursin or decursinolangelate increases ROS (14), we tested the production of DCF-detectable ROS (hydroperoxy radicals) and DHE-detectable ROS (superoxide) in LNCaP cells after decursin and decursinol treatment. As shown in Fig. 5C, decursin induced a rapid and time-dependent increase in DCF-detectable ROS, whereas decursinol lacked such an effect. In the LNCaP cells, decursin treatment did not induce DHE-detectable ROS (data not shown). Pretreatment for 24 h with N-acetylcysteine (5 mmol/L, pH adjusted to neutral), which exerts antioxidant activity by increasing intracellular reduced glutathione synthesis through enhancing cysteine availability (26), resulted in a minor reduction (~20–30%) of decursin-inducible ROS and no protection against apoptosis (data not shown). The data support an association of the rapid generation of ROS by decursin with its potent cell cycle arrest and apoptotic activities.

**Effects of Decursin on Select Cell Cycle Proteins**

To determine potential molecular mediators of the cell cycle arrest and apoptotic effects, we analyzed the abundance of key cell cycle regulatory proteins (Fig. 6A) and caspase cleavage patterns (Fig. 6B) in LNCaP cells after 24-h treatment with decursin. We observed concentration-dependent decreases of cyclin-dependent kinase (CDK) 4, CDK2, and the early G1 cyclin D1 with a concomitant increase of CDK inhibitor p27kip1 (Fig. 6A). There was a minimal effect on another CDK inhibitor p21cip1 or on CDK6 or on the S-phase cyclin E. Such data were consistent with arresting cells in the G1-S boundary and agreed well with published data in DU145 cells, except for p21cip1, which was strongly induced by decursin in this p53 mutant cell line (17).

In terms of apoptotic execution and signaling, we observed increased abundance of the cleaved products for procaspase-3 (17 of 19 kDa) and procaspase-8 (41 of 43 kDa intermediates) at the 100 μmol/L concentration, corresponding to the sharp increase of PARP cleavage (Fig. 6B). Procaspase-9 cleavage was not obvious (data not shown). There was no change in the abundance of the mitochondria death mediator protein, Bax. A pancaspase inhibitor completely blocked the PARP cleavage (Fig. 6C, top) and apoptotic DNA fragmentation (Fig. 6C, bottom) induced by decursin. In addition, an inhibitor of caspase-8

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**Figure 5.** Effects of decursin, decursinolangelate, and decursinol on the cell cycle distribution (A) and apoptosis (B) as detected by ELISA for DNA fragmentation and by immunoblot of the PARP cleavage in LNCaP cells after 48 h treatment. Asynchronous LNCaP cells were treated in the complete medium. Immunoblot data are representative of at least two independent experiments. C, the differential effects of decursin versus decursinol (50 μmol/L) on DCF-detectable ROS generation in LNCaP cells at 2- and 6-h treatment. Data are representative of at least three independent experiments. Columns, mean; bars, SD. Decursin effect versus control or decursinol (P < 0.01) at both time points. DHE-detectable ROS (superoxide) was not induced by decursin or decursinol (data not shown).
or caspase-3 exerted a similar level of protection against apoptosis and was more protective than the caspase-9 inhibitor. Furthermore, blocking of cell death by the caspase inhibitors did not restore AR or PSA expression (Fig. 6C, top). These results indicate that the apoptosis induced by decursin is mediated by caspases and with a greater involvement of the death receptor/caspase-8 activation cascade than the mitochondrial/caspase-9 activation cascade (27–29). The results also indicate that the down-regulation of AR protein abundance by decursin or decursinol angelate is not a consequence of apoptosis.

Discussion

The data presented above, together with our previously published data (10), support decursin and decursinol angelate as members of a novel class of nonsteroidal compounds with potent and long-lasting activities against AR signaling in the LNCaP model. The novelty is best shown by a comparison with the clinically used anti-androgen drug bicalutamide (9). Decursin and decursinol angelate strongly inhibited the PSA mRNA abundance and secretion in the presence of androgen (ligand dependent) as well as in the absence of androgen (ligand independent; Fig. 1D). In contrast, bicalutamide exerted a weak stimulatory effect in the absence of mibolerone on the PSA mRNA and protein abundance (Fig. 1D) and on the long-term cell growth (Fig. 4). Decursin and decursinol angelate potently suppressed the long-term growth in the absence and the presence of mibolerone (Fig. 4), lacking any AR agonist activity. Further distinguishing them from the action of bicalutamide, which binds AR and stimulates nuclear translocation (9), both decursin and decursinol angelate rapidly inhibit AR nuclear translocation (Fig. 2A and B).

Extending on our published work (10), the current study has revealed significant structure-activity relationships governing the anti-AR activities and the cell cycle arrest and apoptotic effects of these compounds as well. All three compounds seemed to share a common target to exert the inhibitory effect on the androgen ligand-dependent AR signaling in the lower micromolar concentration range (≤10 μmol/L). This was supported by the concentration-dependent inhibition of the PSA expression and secretion (Fig. 1B and C), and the androgen-stimulated AR nuclear translocation (Fig. 2A) and PSA promoter transcription (Fig. 2D). The structure required for this activity was the pyranocoumarin core as represented by decursin. The ligand binding assay results (Fig. 3A and B) supported computer simulation that all three compounds may dock into the androgen binding pocket (ligand binding domain) of AR. In particular, decursin was more potent than decursinol to inhibit DHT binding to AR (Fig. 3A versus B), consistent with the moderate difference in their potency to affect AR nuclear translocation (Fig. 2B) and PSA transcription (Fig. 2D). The data provide a plausible explanation of the common AR inhibitory effect among the three compounds in the lower micromolar range through their competition for the binding with androgen to the AR.

In contrast to the persistent inhibitory actions of decursin and decursinol angelate, decursinol imparted a stimulatory effect on the AR signaling and cell growth at higher concentrations, which was best manifested in the absence of androgen. Decursinol increased both the PSA mRNA and secretion (Fig. 1D) and on the long-term cell growth (Fig. 4). Decursin and decursinol angelate potently suppressed the long-term growth in the absence and the presence of mibolerone (Fig. 4), lacking any AR agonist activity. Further distinguishing them from the action of bicalutamide, which binds AR and stimulates nuclear translocation (9), both decursin and decursinol angelate rapidly inhibit AR nuclear translocation (Fig. 2A and B).

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cancer cells lacking AR, such as DU145 and PC-3 cells, were not stimulated to grow by the high concentrations of decursinol (Fig. 4F). We believe that the AR agonist effect of decursinol counteracts its high-affinity inhibitory activity, resulting in the biphasic response patterns observed for both the PSA expression (Fig. 1B) and the long-term cell growth in the presence of androgen.

Our data support that the side chain of decursin and decursinolangelate nullifies the AR agonist action of decursinol and are crucial for apoptotic induction. These data agreed with an earlier report of the requirement of the side chain for growth suppression and apoptotic induction in the DU145 cells by decursin (17). How the side chain structure contributes to the anti-AR and proapoptotic effects remains to be elucidated. A reduction of the AR protein abundance was a mechanism shared by decursin and decursinolangelate, which was not observed for decursinol treatment (Fig. 1C). We have provided evidence of increased degradation through the 26S proteasomal pathway as a likely mechanism to account for the rapid down-regulation of AR protein abundance by decursin (Fig. 3C and D). An increased generation of ROS has been reported in the decursin- and decursinolangelate–treated leukemia cells (14). We also observed that decursin, but not decursinol, induced DCF-detectable ROS in LNCaP cells (Fig. 5C). Our attempt with N-acetylcysteine failed to establish a causal relationship of ROS with apoptosis, reflecting either an extent of ROS production too overwhelming for the reduced glutathione to mitigate (26) or the ROS produced are not targetable by reduced glutathione. More specific genetic approaches with introduction of catalase or other antioxidant enzymes into the cells may be necessary to crucially test the role of ROS in the anti-AR and apoptotic actions. Synthetic decursin derivatives with different side chains have been prepared that may provide further structure-activity insights into the role of ROS in the anti-AR and proapoptotic activities.

In an effort to shed light on potential molecular targets or mediators of decursin, in addition to AR, for the cell cycle arrest and apoptotic actions, we observed in LNCaP cells decreased abundance of cyclin D1, CDK4, and CDK2 and increased expression of p27kip1 in a decursin concentration-dependent manner (Fig. 6A). These results agreed well with data reported for DU145 cells (17) except the lack of p21cip1 induction in LNCaP cells. It is noteworthy that these changes occurred at doses of decursin higher than those for suppressing PSA or AR abundance (Fig. 6A), consistent with “off-target” actions that could be important to account for the cell cycle arrest and especially apoptotic activities of decursin and decursinolangelate in androgen-independent prostate cancer cells (17) as well as in non-androgen–regulated cells, such as leukemia and sarcoma (12–16). Our data support decursin induction of caspase-mediated apoptosis in LNCaP cells (Fig. 6B and C), consistent with the caspase-mediated action for apoptosis in DU145 cells (17). Extending beyond the caspase data of DU145 cells, our results with LNCaP cells suggest the caspase-8 activation cascade playing a more prominent role for decursin-induced apoptosis than the mitochondria/caspase-9 cascade (27–29). Efforts are under way to elucidate the detailed caspase activation pathways in LNCaP and other prostate cancer cells to account for the difference in their sensitivity to decursin (17). In further support of “off-target” actions, we observed that blocking apoptosis with caspase inhibitors did not restore AR or PSA abundance, which are targeted by decursin with concentrations one order of magnitude lower than required for the apoptotic activity.

We should note that the best-studied biochemical activity of decursin and decursinolangelate is as a direct activator of PKC in test tube assays, which was first reported a decade ago (12, 13). Decursin displayed growth suppression (“cytotoxic” activity against various human leukemia and other cancer cell lines with ED50 in the 5 to 16 μg/mL (15–48 μmol/L) range, which was one order of magnitude higher than that required for PSA suppression in the present study (Fig. 1B). In recently published articles by the same group, decursin or decursinolangelate was shown to antagonize, rather than mimic or enhance, the tumor-promoting PKC activator phorbol 12-myristate 13-acetate–induced or phorbol-12,13-dibutyrate–induced leukemia differentiation (14, 15). The authors reported that decursin inhibited several differentiation markers induced by these PKC activators (14, 15) and induced ROS (14) and cytotoxicity (14, 15). Decursin inhibited the binding of phorbol-12,13-dibutyrate to PKC in a competitive manner (15). In K562 cells, decursin induced a more rapid down-regulation of PKCα and PKCβII isoforms than that induced by phorbol-12,13-dibutyrate. Unlike phorbol-12,13-dibutyrate, decursin promoted the translocation of PKCα and PKCβII to the nuclear membrane, rather than to the cell membrane (15), and was not affected by the presence of phorbol-12,13-dibutyrate (15). These results indicate that in spite of being PKC activators in the test tube assays, decursin and decursinolangelate exert distinct and “antagonistic” actions on megakaryocytic differentiation and PKC modulation in K562 leukemia cells than the canonical PKC activators. In terms of the effects of PKC activator 12-O-tetradecanoylphorbol-13-acetate or PMA in LNCaP cells, nanomolar levels have been shown to induce apoptosis and to suppress PSA expression (30–32). The PKC general inhibitor staurosporine has been shown to reverse the PSA-suppressing action of 12-O-tetradecanoylphorbol-13-acetate (30, 31). Bryostatin-1, which also activates PKC, effectively antagonizes the apoptotic effect of PMA (33). Therefore, it will be of great interest for us to investigate whether the PKC pathway is involved in the anti-AR activity of decursin or decursinolangelate in prostate cancer cells in the future.

In conclusion, the current study together with data reported in our earlier publication (10) supports decursin and decursinolangelate as members of a novel class of compounds with potent and long-lasting inhibitory activities against AR signaling in both ligand-dependent and ligand-independent manners. They do not possess the weak agonist activity of bicalutamide in the LNCaP model.
system and are more potent than bicalutamide at inhibiting cell growth and survival. The side chain is crucial for the poten
t and persistent anti-AR activities, for the ROS generation, and for the cell cycle arrest and apoptotic effects. Besides a rapid block of AR nuclear translocation, we have identi
cied the following additional mechanisms to account for the specific anti-AR actions: an inhibition of binding of
DHT to AR and an increased proteasomal degradation of AR
protein abundance. Further investigations of the biochemi
cal and molecular pathways underlying these mechanisms and the in vivo efficacy of this class of agents in relevant
preclinical animal models are warranted for an eventual
clinical translation into improved patient care and prostate
cancer risk reduction in men.

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