Lupeol, a Novel Androgen Receptor Inhibitor: Implications in Prostate Cancer Therapy

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Abstract

Purpose: Conventional therapies to treat prostate cancer (CaP) of androgen-dependent phenotype (ADPC) and castration-resistant phenotype (CRPC) are deficient in outcome which has necessitated a need to identify those agents that could target AR for both disease types. We provide mechanism-based evidence that lupeol (Lup-20(29)-en-3b-ol) is a potent inhibitor of androgen receptor (AR) in vitro and in vivo.

Experimental Design: Normal prostate epithelial cell (RWPE-1), LAPC4 (wild functional AR/ADPC), LNCaP (mutant functional/AR/ADPC), and C4-2b (mutant functional/AR/CRPC) cells were used to test the anti-AR activity of lupeol. Cells grown under androgen-rich environment and treated with lupeol were tested for proliferation, AR transcriptional activity, AR competitive ligand binding, AR–DNA binding, and AR–ARE/target gene binding. Furthermore, in silico molecular modeling for lupeol–AR binding was done. Athymic mice bearing C4-2b and LNCaP cell–originated tumors were treated intraperitoneally with lupeol (40 mg/kg; 3 times/wk) and tumor growth and surrogate biomarkers were evaluated. To assess bioavailability, lupeol serum levels were measured.

Results: Lupeol significantly inhibited R1881 (androgen analogue) induced (i) transcriptional activity of AR and (ii) expression of PSA. Lupeol (i) competed antagonistically with androgen for AR, (ii) blocked the binding of AR to AR-responsive genes including PSA, TIPARP, SGK, and IL-6, and (iii) inhibited the recruitment of RNA Pol II to target genes. Lupeol sensitized CRPC cells to antihormone therapy. High-performance liquid chromatography analysis showed that lupeol is bioavailable to mice. Lupeol inhibited the tumorigenicity of both ADPC and CRPC cells in animals. Serum and tumor tissues exhibited reduced PSA levels.

Conclusion: Lupeol, an effective AR inhibitor, could be developed as a potential agent to treat human CaP. Clin Cancer Res; 17(16); 5379–91. ©2011 AACR.

Introduction

According to American Cancer Society, 217,730 cases were diagnosed with prostate cancer (CaP) and 32,050 CaP patients were projected to die in the year 2010 in United States alone. Despite an initial efficacy of androgen deprivation therapy (castration), CaP progresses within a few years from androgen-dependent (ADPC) to castration-resistant (CRPC) phenotype in most of the patients. Accumulating evidences suggest that androgen receptor (AR) is a critical player in the pathogenesis of early ADPC as well as late-stage CRPC (1). Although AR activity is highly regulated by coactivator and corepressor, with many possible points for intervention, all existing approaches to inhibit AR signaling ultimately target ligand binding to AR in ADPC (1–5). This includes direct competition with competitive antagonists and reduction of systemic ligand levels with chemical castration agents (4). However, the same is not possible in CRPC. In this context, identifying effective inhibitors of AR signaling which could act independent of hormonal status are of paramount interest. This strategy may reduce the morbidity and mortality of CaP patients as well as prevent the progression of early AD tumors to become life-threatening CR tumor phenotype. Thus, agents who inhibit AR signaling in both ADPC and CRPC conditions could be extremely useful for treating CaP patients.

Lupeol [Lup-20(29)-en-3b-ol; Fig. 1A] triterpene found in fruits vegetables and medicinal plants possesses strong antioxidant, anti-inflammatory, antiarthritic, antimutagenic, and antimalarial activity in vitro and in vivo systems (ref. 6 and references therein). We recently showed that lupeol inhibits the tumorigenicity of androgen-independent but sensitive 22Rv1, CaP cells under in vitro conditions and interferes in β-catenin signaling.
Translational Relevance

This study shows that lupeol, by adopting several approaches, inhibits androgen receptor (AR) signaling in both androgen-dependent and castration-resistant prostate cancer (CaP). This study is clinically relevant because it shows that lupeol (i) sensitizes CaP cells (which generally do not respond to therapy) to become responsive to clinical therapeutic agent and (ii) reduces PSA, the clinical biomarker of CaP diagnosis and prognosis. Previously, several agents have been reported for their anti-AR activity, however most of them have not made it past to cell culture studies because of nonacceptability to humans, systemic toxicity, and nonbioavailability when tested under in vivo conditions. However, lupeol is a diet-derived agent (acceptable to humans), nontoxic, and bioavailable when tested in vivo; therefore, this study has high translational relevance. We suggest that lupeol alone or as an adjuvant has enormous translational potential to be tested in humans for CaP treatment.

Materials and Methods

The anti-AR and anti-PSA antibodies were obtained from Millipore and Santa Cruz Biotechnology, respectively. Lupeol (>99% pure) was purchased from Sigma Chemical. AR agonist R1881 (methyl trienolone) and 3[H]R1881 was procured from Perkin-Elmer.

Cell culture and treatment

LAPC4 (wild functional AR/ADPC) cells were gifted by Dr. Robert Reiter (UCLA, Los Angeles, CA). LNCaP (mutant functional AR/ADPC); 22Rv1 (mutant functional AR/androgen-independent but responsive); C4-2b cells (mutant functional AR/CRPC) and PC-3 and DU-145 (lack of endogenous AR) were grown under standard cell culture conditions. We suggest that lupeol is a potent inhibitor of AR and could be developed as therapeutic agent to treat both ADPC and CRPC conditions, therefore, could have significant clinical relevance.

Cell viability assay

This was done as described earlier (7). For combination set of experiments, cells were treated with either agonistic androgen analogue R1881 (1 nmol/L), or antagonist bicalutamide (10 μmol/L), and/or combination (R1881 + lupeol) for 48 hours. After incubation for specified times at 37°C, MTT assay was done as described previously (11). For sensitization studies, hormone-refractory C4-2b cells were treated with lupeol for 24 hours. After 24 hours, cells were incubated with bicalutamide (10 μmol/L) for further 24 hours. Cells were assessed for viability.

3[H]thymidine incorporation, colony formation studies, and immunoblot assay

This was done by the methods as described earlier (11).

AR transcriptional activity reporter assay

LAPC4 and LNCaP cells were transfected with plasmids ARE-Luc (200 ng/well; Cignal Reporter Assay Kit; SA Biosciences) as per vendor’s protocol. After 24 hours, transfected cells were treated with either R1881 (1 nmol/L), or bicalutamide (10 μmol/L), or lupeol (10–50 μmol/L) and/or combinations (lupeol + R1881) for 48 hours. Luciferase activities were measured by using Dual-Luciferase Assay Kit (Promega).

PSA expression levels in CaP cells

This was done by using a standard real-time PCR assay. Primers used to detect human transcripts of PSA were sense 5’-GTGCTGTGCGCTCTCGT-3’; antisense, 5’-CAGCAAGATGCAGCGCTTTTGT-3’.

Lupeol binding with AR in silico

Lupeol was docked with AutoDock4 after fitting in the active region of the AR (2PNU.pdb) by using the modeling programs Sybyl (Tripos Corp.). Autodock scoring was done based upon the estimated free energy of binding and consists of the summation of the final intermolecular energy of docking, total internal energy, and the torsional free energy of the ligand, minus the systems unbound energy.

AR ligand binding in CaP cells

This assay was done by using the method as described by Jones and colleagues (4). Briefly, LAPC4 and LNCaP cells were treated with 1 nmol/L 3[H]R1881 in the absence or presence of 0.1- to 100-fold molar excess of unlabeled competitor ligands for 90 minutes at 37°C. Bound ligands were extracted in ethanol for 30 minutes at room temperature and detected by using a scintillation counter.

AR–DNA binding assay

This was done by employing electrophoretic mobility shift assay (EMSA) as described earlier (12). The sequence for AR oligonucleotide was sense 5’-TGCAAGACGCAAGTGCCTAGC-3’; antisense strand 5’-GCTAGCATTGCTGTCTGCA-3’.

in CaP cells (7, 8). It is notable that AR forms complex with β-catenin and A/β-catenin complex in nucleus activates transcriptional activation of several proliferation-associated genes (9, 10). Keeping in view the reports that (i) lupeol interferes with signaling molecules, which either are regulated by AR or cross-talk with AR, and (ii) the structural similarly of lupeol with androgen, we tested lupeol for its efficacy on AR signaling. Here, we report the mechanism-based anti-AR activity of lupeol in both ADPC and CRPC cells under in vitro and in vivo conditions. We suggest that lupeol is a potent inhibitor of AR and could be developed as therapeutic agent to treat both ADPC and CRPC conditions, therefore, could have significant clinical relevance.

Materials and Methods

The anti-AR and anti-PSA antibodies were obtained from Millipore and Santa Cruz Biotechnology, respectively. Lupeol (>99% pure) was purchased from Sigma Chemical. AR agonist R1881 (methyl trienolone) and 3[H]R1881 was procured from Perkin-Elmer.

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Figure 1. Effect of lupeol on growth, proliferation, and AR transactivation in CaP cells grown in an androgen-rich environment. A, effect of lupeol on cell growth. As detailed in Materials and Methods, LNCaP, LAPC4, 22Rv1, C4-2b, and normal prostate cells (RWPE1) were treated with lupeol for 48 hours, and the viability of cells was determined by the MTT assay. The values are represented as percent viable cells where vehicle (V; alcohol + DMSO)-treated cells were regarded as 100% viable. Each bar in the histogram represents mean \( \pm \) SE of 3 independent experiments, * represents \( P < 0.05 \), compared with the vehicular control group. B, histogram represents the effect of lupeol on R1881-induced cell growth in ADPC cells (LNCaP and LAPC4) as described under Materials and Methods. Each bar in the histogram represents mean \( \pm \) SE of 3 independent experiments. V, vehicle control (alcohol + DMSO)-treated cells were regarded 100% viable. Each bar in the histogram represents mean \( \pm \) SE of 3 independent experiments, ** represents \( P < 0.05 \), compared with control. C, histogram shows cell proliferation as measure in terms of \(^{3}H\) thymidine uptake in LNCaP and LAPC4 cells. Each bar in the histogram represents mean \( \pm \) SE of 3 independent experiments, * represents \( P < 0.05 \), compared with control. D, histogram shows the number of colonies formed by LNCaP and LAPC4 cells. Cells seeded in agarose and incubated at 37°C, were treated with lupeol as described under Materials and Methods. Each bar in the histogram represents mean \( \pm \) SE of 3 independent experiments, * indicates \( P < 0.05 \) compared with R1881 treated cells (1 nmol/L); ** represents \( P < 0.05 \), compared with control. E, histogram shows that the growth suppression of LNCaP cells by lupeol was partially reversed by increasing the R1881 in LNCaP (AR positive) cells but not in PC-3 (AR negative). LNCaP and PC-3 cells in RPMI-1640 medium supplemented with CS-FBS were treated with dimethyl sulfoxide (DMSO) vehicle, lupeol at 25 and 50 \( \mu \)mol/L in the presence of 1.0 to 1.5 nmol/L R1881 for 48 hours. Each bar in the histogram represents mean \( \pm \) SE of 3 independent experiments. V, vehicle control (alcohol + DMSO); F, LNCaP and LAPC4 cells were transiently transfected with AR promoter reporter along with Renilla luciferase and treated with vehicle (alcohol + DMSO) and lupeol (5–50 \( \mu \)mol/L) for 48 hours with or without R1881 (1 nmol/L). Each bar in the histogram represents mean \( \pm \) SE of 3 independent experiments, * represents \( P < 0.05 \), compared with R1881 treated cells (1 nmol/L); ** represents \( P < 0.05 \), compared with control.
AR binding to target genes
This was done by employing a quantitative ChIP assay as described by Im and colleagues. (13). The following primers were used: PSA (ARE III), sense, 5'-TTGTTCCATGTGAGAAAC-3'; antisense, 5'-TGTT- CTCCTGCAAGTTCATACTTGC-3'; SKG-1 (ARB-6.35), sense, 5'-AGGTGTTTCAAGGACAGC-3'; antisense 5'-TGAG- GAGTTACAACCGGAG-3'; TIPARP (ARB-3.23), sense, 5'-CCCTCTGCTGCACTCCTGAG-3'; anti-sense 5'-AGG- GTGTTTCTGAGAGTG-3'; IL-6 (ARB-1.23), sense, 5'-TGCCCTCTTATTAGTTTGCG-3'; antisense, 5'-AAT- CTCTCTTCTGTGCTGTC-3'; HSP70 (promoter) sense 5'-TTCTGAGGTCTAGGCGAGG-3'; antisense, 5'-CCCT- TCGAGCCAATCCAGG-3'. The primers used for the detection of the PSA promoter (TATA site) were; sense, 5'-GTTGCTCCACCCCTACAGCAG-3'; antisense, 5'-CCCTA- TAAAAGCTCAATTTCCCA-3'.

Tumor studies in xenograft mouse models
Tumor studies were conducted in athymic nude mice and 2 cohorts of animals were created. A total of $3 \times 10^6$ cells were injected subcutaneously in the right flanks of each mouse. Each cohort received a specific cell type either LNCaP or C4-2b. One week postimplantation, 20 mice (with visible tumors) in each cohort were randomly divided into 2 groups, with 10 animals in each group. The first group of animals received intraperitoneal (i.p.) administration of corn oil (100 μL) and served as control. The second group of animals received i.p. administration of lupeol (40 mg/kg in 100 μL of corn oil) 3 times/wk. Body weights and tumor volumes were recorded as described earlier (7, 11). All animals of group 1 and group 2 were sacrificed when tumors crossed a preset endpoint volume of 1,000 mm$^3$.

PSA levels in serum of athymic nude mice and cell culture medium
This was done by using PSA Kit (Anogen) as per vendor's protocol.

Lupeol pharmacokinetic study in mice
We measured lupeol serum levels by employing 2 protocols as per the method of Udeani and colleagues. (14). Under first protocol, mice received one time administration of lupeol (200 mg/kg; $n = 7$, each time point). Under second protocol, we measured serum lupeol levels in mice receiving lupeol (40 mg/kg) for 8 weeks. Under this protocol, blood samples were collected after 24 hours of last i.p. administration and serum samples were prepared ($n = 7$, each time point). This was done in mice under tumor protocol.

Statistical analyses
These were done as described earlier (7, 11). A value of $P < 0.05$ was considered to be statistically significant.

Results
Lupeol inhibits growth of CaP cells under androgen-rich environment
First we tested the efficacy of lupeol against the growth and viability of ADPC (LAPC4, LNCaP), androgen independent but responsive and CRPC cells (22Rv1 and C4-2b) grown under standard culture conditions. The choice of these human CaP cells was also based on the fact that these cells show differential AR status and functionality. LAPC4 cells are androgen dependent and express functional wild AR. LNCaP cells are androgen dependent and express functional T877A mutated AR; 22Rv1 cells are androgen independent but responsive, which express functional H874Y mutated AR. C4-2b cells are castration resistant and express functional T877A mutated AR. Therefore, these 4 cell lines constitute a panel of diverse cellular models for CaP (5). Lupeol (10–50 μmol/L) treatment for 48 hours resulted in a dose-dependent growth inhibition of ADPC cells viz., LAPC4 and LNCaP cells with an IC$_{50}$ of 15.9 and 17.3 μmol/L, respectively (Fig. 1A). Lupeol also inhibited the growth of 22Rv1 with an IC$_{50}$ of 19.1 μmol/L (Fig. 1A). Furthermore, lupeol inhibited the growth of C4-2b cells with an IC$_{50}$ of 25 μmol/L (Fig. 1A). These data suggest that lupeol has the potential to inhibit the growth of CaP cells of both ADPC and CRPC phenotype. Androgens by activating AR are known to drive the growth of CaP cells (1, 15). Next, we asked whether lupeol inhibits the androgen-induced growth of CaP cells. For this purpose, LAPC4 and LNCaP, grown under the influence of androgen were tested for their growth and proliferation and simultaneously challenged by lupeol. An androgen analogue, R1881, was used to stimulate the growth and proliferation of ADPC cells. We employed bicalutamide, an androgen antagonist (used at clinics to treat ADPC), as a reference control for our studies. R1881 (1 nmol/L) treatment for 48 hours was observed to cause a significant increase in the growth and rate of proliferation of LAPC4 and LNCaP cells (Fig. 1B and C). However, R1881-stimulated growth of ADPC cells was observed to be significantly reduced in presence of lupeol (31–50 μmol/L; Fig. 1B). As assessed by $[^3]$H[thymidine uptake assay, the rate of proliferation of R1881-stimulated LAPC4 and LNCaP cells was significantly reduced by lupeol (Fig. 1C). Similar data were observed in 22Rv1 (data not shown).

We asked whether the long-term treatment of lupeol could inhibit proliferation of ADPC cells in an androgen-rich environment. For this purpose, CaP cell colonies were established in soft agar. The daily addition of androgen analogue R1881 (1 nmol/L) in fresh media was observed to induce the clonogenic potential of LNCaP and LAPC4 cells (Fig. 1D). However, lupeol treatment (added every 3rd day for 14 days) in a parallel set of experiments was observed to significantly reduce the influence of R1881 (Fig. 1D). Because both bicalutamide and lupeol decreased R1881-stimulated growth, we speculated that bicalutamide and lupeol might have AR as a common molecular target.
Lupeol, a Novel Androgen Receptor Inhibitor

We further asked whether the growth inhibitory effect of lupeol is due to the antiandrogenic activity of lupeol. For this purpose, LNCaP (AR positive) and PC-3 (AR negative) cells were treated with lupeol in the presence of 1.0 to 1.5 nmol/L R1881 (Fig. 1E). Interestingly, the growth suppression of LNCaP cells by lupeol was partially reversed by increasing the R1881 concentration from 1.0 to 1.5 nmol/L at 25 μmol/L but not 50 μmol/L (Fig. 1E). In contrast, this R1881 rescue of the growth suppression by lupeol was not observed in the AR-negative PC-3 cells (Fig. 1E). These results indicate that the growth inhibitory effect of lupeol in LNCaP cells is, at least in part, attributable to its antiandrogenic activity. Similar data were observed in LAPC4 and Du145 cells (data not shown).

Lupeol inhibits activity of transcriptional factor AR

The growth of LNCaP and LAPC4 cells depends on functional AR signaling. Activated AR acts as a transcriptional factor and binds to DNA at specific androgen response elements (ARE) or AR-binding sites (ARB) on target genes and induce the activation of several genes involved in cell proliferation (1, 16, 17). Because lupeol was observed to inhibit the growth of both ADPC and CRPC cells, we asked whether lupeol targets AR signaling in CaP cells. For this purpose, ADPC cells were transfected with ARE reporter construct and grown in presence of R1881 (1 nmol/L) and lupeol (5–50 μmol/L) for 48 hours. As assessed from luciferase-based reporter assay, R1881 treatment caused a 3.5- to 5-fold induction in the transcriptional activity of AR in both LAPC4 and LNCaP cells (Fig. 1F). However, lupeol treatment was observed to significantly inhibit the R1881-induced transcriptional activity of AR in ADPC cells (Fig. 1F). In this set of experiments, bicalutamide was used as a reference control. These data suggest that lupeol has the potential to interfere with the activation of AR in CaP cells. Similar data were observed in 22Rv1 cells (data not shown).

Lupeol decreases the mRNA and protein expression of AR target gene PSA in ADPC and CRPC cells

The expression of PSA is regulated by AR (18, 19). It has been shown that the serum PSA levels correlate directly with advancing clinical and pathologic disease stages of CaP (20, 21). In humans, PSA has been extensively used as the most reliable biomarker to screen CaP and a surrogate marker to monitor response to antiandrogen therapy in humans (20, 21). Because lupeol was observed to inhibit the AR transcriptional activation, we next determined the effect of lupeol on transcriptional and translational levels of PSA in all CaP cells irrespective of androgen sensitivity status. Lupeol treatment for 48 hours was found to decrease the mRNA expression levels of PSA in a dose-dependent manner in LNCaP, LAPC4, 22Rv1, and C4-2b cells (Fig. 2A and B). These data were further validated by conducting immunoblot analysis of LNCaP, 22Rv1, and C4-2b cells for PSA protein (Fig. 2C). We next determined the effect of lupeol treatment on PSA expression in ADPC cells grown under the influence of R1881. As assessed by real time PCR technique, R1881 treatment was found to cause a sharp increase in the mRNA expression levels of PSA in LNCaP and LAPC4 cells (Fig. 2D). In a sharp contrast, lupeol treatment caused a decrease in the R1881-induced mRNA levels of PSA in both cells (Fig. 2D). By employing immunoblot analysis, we next observed that lupeol reduced protein levels of PSA in androgen-stimulated cells (Fig. 2E). These data were observed to conform to the mRNA levels in these cells (Fig. 2E). A similar effect was also observed with C4-2b cells, except bicalutamide did not show any observable effect at 10 μmol/L concentration (data not shown).

We further determined the effect of lupeol on secreted levels of PSA (secreted by CaP cells in culture media) in LNCaP and LAPC4 cells. Lupeol treatment was observed to decrease the secreted levels of PSA in a concentration-dependent manner (Fig. 2F). The growth of LNCaP and LAPC4 cells depends on functional AR signaling, thus, the observed inhibitory effects of lupeol on cell growth are largely because of the suppression of AR transactivation and thereby decrease in target gene such as PSA expression and secretion.

Inhibition of AR signaling by lupeol: mechanism of action

AR plays a critical role in the development of CaP, and it has been shown that an increased AR signaling correlates with increase in CaP growth (1). Because lupeol reduced the expression level of AR target gene PSA and also decreased the transcriptional activation of AR, we hypothesized that this observed effect might be due to (i) competition of lupeol with androgen for AR in ADPC cells and (ii) decreased availability/recruitment of AR to ARE-binding sites on target genes such as PSA in both ADPC and CRPC cell types. To test our hypothesis, we conduct following studies.

Lupeol docks with AR at ligand-binding site. The in silico molecular modeling showed that lupeol binds with the AR with a binding energy of E = −10.66 Kcal/mole approximately 56 nmol/L (Fig. 3C). Lupeol interacted with the AR through 17 putative amino acid residues and 2 hydrogen bonds (Fig. 3A and B). Lupeol is surrounded by a hydrophobic pocket in the core of the AR and consists of several hydrophobic side chains and 6 methionines (742, 745, 749, 780, 787, and 895) on 5 different helices. The hydrophilic ends of the molecules have hydrophilic interactions with Trp 752 at one end and Asn 705 at the other end (Fig. 3B).

Lupeol binds with AR more efficiently than other natural agents. We selected 2 natural agents (EGCG, Fisetin) which previously were reported to inhibit AR activity to be compared with lupeol for its docking efficiency with AR in silico. It is noteworthy that EGCG and Fisetin do not fit the AR ligand–binding pocket as well as the other pockets despite having more hydrogen bond acceptors and donors. However, lupeol molecule despite the lack of H bond acceptors and having only one donor, docks with AR more...
efficiently (than EGCG and Fisetin), with a high free energy of binding. Lupeol was observed to bind with AR with a binding energy of $E = -10.66$ Kcal/mole approximately 56 nmol/L (Fig. 3C). The AR-binding efficiency of lupeol is more than EGCG ($E = -8.5$ Kcal/mole) and Fisetin ($E = -6$ Kcal/mole).
Lupeol competes with androgen for AR in ADPC cells. To confirm whether lupeol binds to AR, LAPC4 and LNCaP cells were incubated with 1 nmol/L [3H]R1881 and unlabeled inhibitors, R1881, bicalutamide, and lupeol. Unlabeled R1881 and the bicalutamide effectively competed [3H]R1881 binding in ADPC LNCaP and LAPC4 cells.
Lupeol at 25 to 50 μmol/L concentrations competed with labeled R1881 for ligand-binding domain of AR. Taken together, these data indicate lupeol acts as a competitive antagonist for AR at its effective concentrations (Fig. 3D and E).

**Lupeol inhibits AR–DNA binding in CaP cells.** Activated AR is reported to act as a transcriptional factor by binding to ARE sites on AR-responsive genes. As assessed by an EMSA, lupeol treatment was observed to significantly reduce the AR–DNA binding efficiency in LNCaP cells (Fig. 3F). Similar data were observed in LAPC4, C4-2b, and 22Rv1 cells (data not shown).

**Lupeol inhibits AR occupancy on AR-binding sites on AR-responsive genes.** Because gene transcription positively correlates with promoter activity, we tested the effects of lupeol on AR occupancy on AREs on androgen-responsive gene PSA or ARBs located on AR target genes SGK-1 (serum/glucocorticoid-induced protein kinase-1), TIPARP (TCDD-inducible poly(ADP-ribose) polymerase), and interleukin 6 (IL 6) by employing a ChIP assay. The androgen unresponsive HSP70 promoter was used as a negative control (data not shown). R1881 was observed to stimulate AR occupancy of binding sites near androgen-induced genes (ARE III and ARBs) in LNCaP cells (Fig. 4A). Lupeol

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effect of lupeol (A and B) on AR occupancy on AR-binding sites in AR-responsive genes and (C and D) occupancy of RNA Pol II enzyme on promoter site of PSA. A, histogram shows the effect of lupeol on AR occupancy in AR-responsive genes PSA, SGK-1, TIPARP, and IL6 in LNCaP cells. Cells were treated with the R1881, bicalutamide, and lupeol for 48 hours and were assayed for AR occupancy by employing ChIP assay as described in Materials and Methods. B, histogram shows the effect of lupeol on AR occupancy in AR-responsive gene PSA in C4-2b cells as assessed by ChIP assay. Cells were treated with lupeol for 48 hours, followed by ChIP assay. C and D, histogram shows the effect of lupeol on the RNA Pol II occupancy on promoter site of PSA in LNCaP and C4-2b cells. Each bar in the histogram represents mean ± SE of 3 independent experiments. * represents P < 0.05 of 3 independent experiments, compared with R1881 treated cells (1 nmol/L). ** represents P < 0.05, compared with control.
treatment was observed to decrease the AR occupancy of ARE and ARBs on the AR-responsive genes in LNCaP cells (Fig. 4A). It is noteworthy that bicalutamide treatment was observed to modestly recruit AR to ARE and ARBs in LNCaP cells (Fig. 4A). This observation is in accordance with previous published reports (4, 22, 23).

We next determined the potential of lupeol on inhibiting the recruitment of AR to ARE sites on PSA gene in CRPC cell C4-2b. Lupeol treatment significantly reduced the AR occupancy on ARE sequences of C4-2b cells (Fig. 4B). This is to be noted that C4-2b cells express constitutively activated AR that is highly active in androgen-free environment, thus mimicking a condition that is observed in CRPC patients (24). These data provide strong evidence that lupeol has the potential to decrease the AR transcriptional activation in ADPC and CRPC cells by blocking AR occupancy on ARE sites in target genes (Fig. 4A and B). We also observed that lupeol inhibits the AR occupancy at ARE III region of PSA gene in the presence of R1881.

Lupeol inhibits RNA Pol II recruitment to PSA promoter sites. RNA polymerase II (RNA Pol II) recruitment is essential for the expression of PSA gene (4 and references therein). We determined the effect of lupeol treatment on RNA Pol II recruitment at PSA promoter in LNCaP and C4-2b cells by employing a ChIP assay. R1881 treatment resulted in accumulation of RNA Pol II at the start site of LNCaP cells, whereas treatment with lupeol reduced RNA Pol II occupancy (Fig. 4C). Interestingly, lupeol was observed to inhibit RNA Pol II recruitment on promoter of PSA gene in CRPC cells C4-2b, both in presence and absence of androgen (Fig. 4D).

Effect of lupeol on tumorigenicity of ADPC and CRPC cells implanted in athymic nude mouse model

We next determined whether the antiandrogen activities of lupeol (observed in vitro) would translate into an in vivo xenografts model. Lupeol treatment did not cause any loss in the body weight, food intake, or exhibited apparent signs of toxicity in animals. Implantation of ADPC (LNCaP) and CRPC (C4-2b) cells onto nude mice produced visible tumors in mice with a mean latent period of 12 days. The average volume of tumors in control mice increased as a function of time and crossed a preset endpoint of 1,000 mm³ tumor volume), even at the end of 12th week (Fig. 5B and D). Tumors from 4 animals from each control and treated group were excised at the 56th day posttreatment when 100% control (corn oil treated) animals crossed the preset endpoint (1,000 mm³ tumor volume). From these data, we conclude that lupeol is an effective agent that has the potential to inhibit the tumorigenicity of CaP cells in vivo. At the conclusion of the study on day 56, the total circulating serum PSA levels (secreted by the implanted tumor cells) were measured. At 56th day postimplantation, PSA levels were observed between 11.95 to 12.79 ng/mL in control animals with LNCaP tumors and C4-2b tumors, respectively. However, Lupeol-treated counterpart animals exhibited reduced serum PSA levels in a range of 4.25 to 7.09 ng/mL. These data were further validated by conducting immunoblot analysis of tumor tissues for PSA protein (Fig. 5G). Tumor tissues of animals receiving lupeol treatment exhibited reduced serum PSA levels as compared with control (Fig. 5G).

Lupeol is stable in serum and bioavailable to animals

Lupeol was spiked with serum by employing a standard protocol followed by a high-performance liquid chromatography (HPLC) analysis. Lupeol standard exhibited a retention time (RT) of 13.4 minutes (Fig. 6A). Lupeol was detected at a similar RT in serum, suggesting the stability and detection of lupeol in vivo (Fig. 6B). Next, we measured serum lupeol levels in mice receiving one time i.p. administration of lupeol (200 mg/kg). We observed that lupeol serum levels were 3.08 and 5.22 µmol/L at 4 and 8 hours postadministration, respectively (Fig. 6C). Under second protocol, we measured lupeol serum levels in mice implanted with tumors and receiving lupeol (40 mg/kg) for 8 weeks. Serum lupeol levels were 10 to 20 µmol/L in mice receiving lupeol for 4 to 8 weeks (Fig. 6D). We speculate that lupeol levels build up in the mice (receiving repeated treatments of lupeol) reaching to the physiologic concentrations which are required to slow down the growth of tumor cells.

Lupeol sensitizes CRPC cells to antiandrogen therapy

We asked whether lupeol could be used as an adjuvant to sensitize highly aggressive CRPC cells for bicalutamide treatment. For this purpose, C4-2b cells were used as a model because these cells do not require androgens for growth and are derived from androgen-sensitive LNCaP cells (25–28). C4-2b cells treated with bicalutamide alone exhibited no significant decrease in the cell growth (Fig. 6E). However, lupeol treated C4-2b cells when subsequently were treated with bicalutamide, a significant decrease in the cell growth was observed, suggesting that lupeol sensitized hormone-refractory CaP cells for...
antihormone therapy (Fig. 6E). One important finding in our study was that pretreatment of C4-2b cells with lupeol and then bicalutamide was observed to cause a decrease in the expression levels of PSA in C4-2b cells (Fig. 6F).

Discussion

Androgen deprivation has been used as a standard therapy for treating CaP in humans; however, the use of antiandrogens to treat CaP of all types is limited. Reports suggest that antiandrogen agents decrease the tumoral androgen concentrations and cause death of normal prostate epithelial cells, thus exposing surviving CaP cells to a relative abundance of androgen (1, 29). In addition, prolonged treatment with bicalutamide is reported to induce AR (26, 27). The environment within a prostate tumor is heterogeneous and most of the agents target a specific cell type (such as ADPC), although allowing other cell types to grow within a tumor (30). Agents which exhibit the potential to combat AR under both situations (androgen and nonandrogen environment) are rare, however are desired. The outcome of this study is significant because it shows that lupeol while sparing normal cells preferentially inhibits the growth of tumors from LNCaP and C4-2b cell-derived tumors excised at the 56th day posttreatment as determined by immunoblot analysis. Equal loading was confirmed by stripping the membrane and reprobing them for β-actin. The immunoblot data shown here are representative of 3 samples from each group. The details are described under Materials and Methods.
strengthened by our data showing that lupeol effectively competes with androgen for the LBD region of AR in ADPC cells. Studies have shown that AR point mutations (21%–44% of metastatic tumors) which are predominantly localized to the LBD region cause an induction in AR transactivation (31–34). It is noteworthy that lupeol inhibits ligand-binding potential of AR in CaP cells having wild-type AR (represented by LAPC4) and mutant AR (represented by LNCaP).

The primary regulator of PSA expression is AR, which induces PSA expression through AREs containing enhancer elements located in the proximal 6 kb of the PSA promoter (35, 36). PSA is also found in CaP cells which express AR but are nonresponsive to androgen (ref. 1 and references therein). This is phenomenon resonates in CaP patients who initially respond to hormone ablation therapy (marked by reduced PSA levels), however comeback to clinics with CRPC disease with elevated PSA levels (1, 37). It is noteworthy that lupeol treatment causes a decrease in the recruitment of AR to the different ARBs on different AR-responsive genes in ADPC and CRPC cells. Our data are further strengthened by the observation that lupeol treatment decreased PSA expression in cells and tumor xenografts representing both ADPC and CRPC phenotype. We also confirmed whether the observed reduction in cell-associated PSA was because of increased secretion of PSA into the culture media. Lupeol was observed to decrease the PSA secretion from LNCaP and LAPC4 cell into the culture media. These data suggest that lupeol decrease both intracellular as well as secretory PSA levels. The observations that lupeol treatment reduced PSA levels in both ADPC and CRPC cells, within tumor tissues and in serum, carry a high

Figure 6. Lupeol bioavailability studies. A and B, the graph represents the detection of lupeol in serum plasma as assessed by HPLC as described under Materials and Methods. The peaks in the graphs represent the retention times at which lupeol was detected in serum plasma. C, histogram represents serum lupeol levels in mice receiving one time administration of lupeol (200 mg/kg), serum collected at 4 and 8 hours. D, histogram represents serum lupeol levels in mice receiving lupeol (40 mg/kg) up to 8 weeks. Lupeol levels were detected by HPLC as described under Materials and Methods. E, histogram represents the effect of lupeol and bicalutamide treatment, alone and in combination (Lupeol + bicalutamide) on cell viability of C4-2b cells. Cells were treated with specified concentrations of lupeol (30–50 μmol/L) and bicalutamide (10 μmol/L). Cells were treated with lupeol for 24 hours and were then exposed to bicalutamide for 24 hours. After bicalutamide treatment, cell viability was assessed by MTT assay. The values are represented as percent viable cells where vehicle (alcohol + DMSO)-treated cells were regarded as 100% viable. Data represents mean ± SE of 3 independent experiments. * represents P < 0.05, compared with control. F, histogram represents mRNA expression of PSA in Lupeol-sensitized C4-2b cells (exposed to bicalutamide treatment). Each bar in the histogram represents mean ± SE of 3 independent experiments. * represents P < 0.05, compared with control.

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clinical significance. Coactivators enable AR to become transcriptionally active and facilitate RNA polymerase II (RNA Pol II) recruitment to a distant enhancer element of the AR-target genes (1, 31–36). One of the important finding of this study is that lupeol significantly inhibited the recruitment of RNA Pol II to the PSA promoter in both ADPC and CRPC cells. In addition to androgens, several molecules are known to enhance the AR activation in CaP cells (9, 10). β-Catenin has emerged as an important player in CaP pathogenesis and has been reported to activate AR by physically binding with it (9, 10). Song and colleagues showed that β-catenin enhances androgen-induced AR signaling in CaP cells, and lupeol has been shown to be an effective β-catenin inhibitor in various tumor cells, including CaP cells (8, 37). Keeping in view these studies and our current data, we suggest that lupeol inhibits AR at multiple levels and reduction of β-catenin levels by lupeol, in part might be playing a role in the AR inhibitory activity.

There are several reports which show the in vitro efficacy of agents in targeting CaP cells (1, 38). However, most of these agents have not made it past to cell culture studies because of several reasons, such as the systemic toxicity associated with these agents, and the most importantly, nonbioavailability of agents when tested under in vivo conditions (38). Our data are significant because lupeol was found to be bioavailable and build up (during repeated administrations) in the serum to the levels equaling effective concentrations. Given this line of reasoning, future studies to compare lupeol with antiandrogens should be done by using equivalent doses to determine whether there is any benefit to increasing the dose of lupeol. Because lupeol was observed to be nontoxic and physiologically available, we suggest that the dose of lupeol could be tailored (as per the requirements) if the current study advances to the translational level.

In a recent review, Attard and colleagues (39) suggested that a significant proportion of CaP remains dependent on the AR signaling. Keeping in view, the published studies which suggest the important of AR for both ADPC and CRPC, our data are highly significant as they provide evidences that lupeol is a novel agent that can disrupt AR signaling in both ADPC as well as CRPC cells. Collectively, these data show that lupeol by adopting several approaches inhibits AR signaling in CaP cells irrespective of AR and androgen sensitive status and shows the potential of lupeol as a more effective disruptor of AR signaling than known antiandrogens (e.g., bicalutamide). Further studies to compare lupeol with FDA approved antiandrogens to validate our data in relevant animal models (representing CaP development and progression) are warranted. We suggest that lupeol alone or as an adjuvant has enormous translational potential to be tested in humans for CaP treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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