Preclinical Evaluation of the Supercritical Extract of Azadirachta Indica (Neem) Leaves In Vitro and In Vivo on Inhibition of Prostate Cancer Tumor Growth

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Abstract

Azadirachta indica, commonly known as neem, has gained worldwide prominence because of its medical properties, namely antitumor, antiviral, anti-inflammatory, antihyperglycemic, antifungal, and antibacterial activities. Despite these promising results, gaps remain in our understanding of the molecular mechanism of action of neem compounds and their potential for use in clinical trials. We investigated supercritical extract of neem leaves (SENL) for the following: molecular targets in vitro, in vivo efficacy to inhibit tumor growth, and bioactive compounds that exert antitumor activity. Treatment of LNCaP-luc2 prostate cancer cells with SENL suppressed dihydrotestosterone-induced androgen receptor and prostate-specific antigen levels. SENL inhibited integrin β1, calreticulin, and focal adhesion kinase activation in LNCaP-luc2 and PC3 prostate cancer cells. Oral administration of SENL significantly reduced LNCaP-luc2 xenograft tumor growth in mice with the formation of hyalinized fibrous tumor tissue, reduction in the prostate-specific antigen, and increase in AKR1C2 levels. To identify the active anticancer compounds, we fractionated SENL by high-pressure liquid chromatography and evaluated 16 peaks for cytotoxic activity. Four of the 16 peaks exhibited significant cytotoxic activity against prostate cancer cells. Mass spectrometry of the isolated peaks suggested the compounds with cytotoxic activity were nimbandiol, nimbolide, 20,30-dihydonimbolide, and 28-deoxonimbolide. Analysis of tumor tissue and plasma samples from mice treated with SENL indicated 28-deoxonimbolide and nimbolide as the bioactive compounds. Overall, our data revealed the bioactive compounds in SENL and suggested that the anticancer activity could be mediated through alteration in androgen receptor and calreticulin levels in prostate cancer. Mol Cancer Ther; 13(5); 1067–77. ©2014 AACR.

Introduction

Prostate cancer is the most frequently diagnosed malignancy among men in Western society (1). Tumor development and progression involve multiple cellular processes, including cell transformation, deregulation of programmatic cell death, proliferation, invasion, angiogenesis, and metastasis (2). Targeting a single molecule for the treatment of cancer has shown limited promise because of the diversity of deregulated pathways in cancer (3). The initial effect of the drugs currently approved by the U.S. Food and Drug Administration (e.g., abiraterone and docetaxel) for castrate-resistant disease after the failure of androgen deprivation controls disease, but many of these therapies are short lived (4, 5). This is presumably because the cancer cells develop resistance. Moreover, many therapeutics exhibit chemotherapeutic clinical effects (6). Despite the incorporation of new chemotherapies and novel hormonal regimens in prostate cancer therapeutics (7), only an incremental increase in the response rate and median overall survival for treated patients has been achieved at best, highlighting a need for continued expansion into investigating newer therapeutic approaches to improve upon the medication selection process in this tumor type. An approach to overcoming such a problem is the development of new agents that can be used in combination with existing chemotherapeutic agents yield a better result than that achieved with a single chemotherapeutic agent (8). Accumulating evidence suggests that many natural products including extracts and isolated chemicals have the potential to interact with multiple targets in the network of pathways that support numerous molecular cascades involved in controlling the progression of cancer (9). Therefore, a systemic study of natural products is needed to define their antitumor effects and understand their mechanisms of action for developing new treatments.
Leaves from *Azadirachta indica*, commonly known as neem, have been used widely in Southeast Asia for treatment of many diseases, including cancer (10–16). Although neem leaves have been used for treatment of many diseases, bioactive neem compounds and their molecular basis of action have not been well studied. In this study, we investigated the anticancer activity of supercritical extract of neem leaves (SENLE) in prostate cancer. Supercritical fluid extraction with carbon dioxide minimizes thermal and chemical degradation of compounds (17). The relatively low temperature of the process and the stability of CO₂ allow most compounds to be extracted with little damage or denaturing. The extraction process facilitates the collection of volatile oils from the processing material. Volatile oils reduce acute and chronic inflammation and, more importantly, facilitate the absorption of other bioactive compounds in humans (18). Compounds obtained by supercritical fluid extraction are very stable and can be used directly in experimental diet supplementation (19, 20).

Our study was designed to evaluate the efficacy of SENL in prostate cancer and identify the compounds with high antitumor activity. SENL inhibits LNCaP-luc2 and PC3 prostate cancer growth. Our results demonstrated that dihydrotestosterone (DHT)-induced androgen receptor (AR) and prostate-specific antigen (PSA) levels are suppressed in LNCaP-luc2 cells with SENL treatment. Oral administration of SENL dramatically reduced LNCaP-luc2 prostate cancer xenograft tumor growth in nude mice. SENL downregulates calreticulin (CALR) levels and inhibits focal adhesion kinase (FAK) activation in LNCaP-luc2 and PC3 prostate cancer cells. We fractionated SENL by high-pressure liquid chromatography (HPLC) and tested the individual fractions for cytotoxic activity. HPLC fractions were analyzed on a mass selective detector time-of-flight (MSD-TOF) system to identify the compounds that exhibit anticancer activity. In this study, we have shown for the first time molecular targets of SENL, evaluated the efficacy to inhibit tumor growth, and identified bioactive compounds of anticancer activity.

**Materials and Methods**

**Supercritical extraction of neem leaves**

Supercritical extract of fresh neem leaves was prepared in our laboratory using the Speed SFE-2 System (Applied Separations). Fresh neem leaves were purchased from Neem Tree Farms, a US Department of Agriculture-certified organic neem farm. Supercritical fluid grade carbon dioxide was obtained from Praxair, Inc. Supercritical CO₂ is a fluid state of CO₂ that is held at or above its critical temperature (31.1°C) and critical pressure (72.9 atm/7.39 MPa). Neem leaves of the same age were washed with distilled water and air-dried, and 100 g of the pulverized leaves were used for extraction using 300-mL vessel. The following extraction parameters were used: 9,000 psi and 3°C for 1-hour static and 2-hour dynamic flow of liquid CO₂ at 3 L/min. The collection glass vial was cooled to −49°C with a dry ice and acetone bath to collect volatile oil. The process yielded approximately 5 g of the extract. An aliquot of the extract was dissolved in dimethyl sulfoxide (DMSO) plus ethanol to make a stock solution of 200 μg/μL, as described previously (21). The final concentration of DMSO in the culture medium never exceeded 0.01%. The effect of the extract on cell viability and HPLC profiles described below was assessed to standardize the method of extraction. We obtained consistent results with different lots of the extract.

**Cell line and cell culture**

LNCaP-luc2, originated from lymph node-derived LNCaP, is a luciferase-expressing, androgen-dependent prostate cancer cell line stably transfected with the firefly luciferase gene (luc2). It was purchased from Caliper Life Sciences. PC3, a bone-derived prostate cancer cell line, was purchased from the American Type Cell Collection (ATCC). LNCaP-luc2’s parental cell line was obtained from ATCC by the Caliper Life Sciences. LNCaP-luc2 and PC3 cell lines were obtained in 2012, expanded and frozen down in several aliquots. Each aliquot was thawed and used for no more than 6 months. ATCC uses Promega PowerPlex system to authenticate their cell lines. These cell lines were not re-authenticated by our laboratory. LNCaP-luc2 cells were cultured in RPMI 1640 medium, and PC3 cells were cultured in Dulbecco’s modified Eagle medium. LNCaP-luc2, originated from lymph node-derived LNCaP, is a luciferase-expressing, androgen-dependent prostate cancer cell line stably transfected with the firefly luciferase gene (luc2). It was purchased from Caliper Life Sciences. PC3, a bone-derived prostate cancer cell line, was purchased from the American Type Cell Collection (ATCC). LNCaP-luc2’s parental cell line was obtained from ATCC by the Caliper Life Sciences. LNCaP-luc2 and PC3 cell lines were obtained in 2012, expanded and frozen down in several aliquots. Each aliquot was thawed and used for no more than 6 months. ATCC uses Promega PowerPlex system to authenticate their cell lines. These cell lines were not re-authenticated by our laboratory.

**Cell growth assay in vitro and flow cytometry**

To determine cell growth, LNCaP-luc2 and PC3 cells were seeded at a density of 3 × 10^3 and 1.5 × 10^4 per well, as described (21). Cells were treated with 5 to 25 μg/mL of SENL, serial dilutions of HPLC fractions, 0.468 to 15.0 μg/mL, or the vehicle (ethanol + DMSO) as control for 24 hours. Cell medium was replenished and cell growth was determined by MTS-formazan reduction using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), as described (21, 23). To evaluate the mechanism of cell death, LNCaP-luc2 and PC3 cells were treated with IC₅₀ concentrations of SENL (12 and 15 μg/mL, respectively) and stained with annexin-V FITC and propidium iodide (PI) according to the dead cell apoptosis kit protocol (Life Technologies), then analyzed by flow-cytometry (FACSCalibur, BD) as described (24).

**PSA and DHT determination**

LNCaP-luc2 cells were treated with SENL (12 μg/mL) in the presence or absence of 10 nmol/L DHT (Sigma-Aldrich). PSA assay was performed using the supernatants collected from LNCaP-luc2 cells after 24 hours of treatment. PSA secretion was determined by ELISA PSA [Human] ELISA Kit; Abnova. DHT measurements were performed in the LNCaP-luc2 cells after 8 and 24 hours of SENL treatment. Total proteins were extracted using RIPA buffer as described (21). Deuterated stable isotope (d₄-DHT) was added to the protein extract as internal standard. DHT levels were measured by conventional...
liquid chromatography on a multiplexed LC System and analyzed on a tandem mass spectrometer equipped with an electrospray ion as described (25).

**Western blot analysis and immunofluorescence**

For Western blot analysis, LNCaP-luc2 and PC3 prostate cancer cells were treated with SENL (12 and 15 μg/mL, respectively) for 8 and 24 hours in the presence and absence of 10 nmol/L DHT or 50 μmol/L MG132 (S2619; Selleckchem). Total proteins were extracted using RIPA buffer, immunoblotting was performed, as described in ref. 26. The primary antibodies for androgen receptor (SC-816; Santa Cruz Biotechnology), phospho-AR (S81) (07-135; Millipore), (phospho-FAK (Y-397) (ab4803; Abcam), FAK (ab131435; Abcam), integrin β1 (4706; Cell Signaling), CALR (2891; Cell Signaling), Rab5 (2143; Cell Signaling), and glycerinaldehyde-3-phosphate dehydrogenase (GAPDH; sc-137179; Santa Cruz Biotechnology, Inc.) were purchased from companies. For immunofluorescence, LNCaP-luc2 and PC3 cells were grown on coverslips at a density of 4 × 10^4 and 3 × 10^4 cell per well, respectively. After treatment of cells with SENL for 24 hours, fixation, permeabilization, and immunofluorescence of the cells were performed, as described (27). Primary antibodies FAK rabbit polyclonal antibody (ab131435; Abcam) and integrin β1 mouse monoclonal antibody (610467; BD Biosciences) and secondary antibodies red, Cy5-labeled goat antirabbit IgG (Alexa Fluor 594; Life Technologies Corp.) and green, FITC-labeled goat antimouse IgG (Alexa Fluor 488; Life Technologies Corp.) were used for immunostaining. Nuclei were stained with 4’,6’-diamidino-2-phenylindole (DAPI), and confocal images were taken on a laser scanning microscope (LSM 780; Carl Zeiss Microscopy GmbH) at ×100 magnification.

**Xenograft animal model**

All the procedures involving animals were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee. Male 5-week-old athymic nu/nu mice (Charles River Laboratories) were implanted subcutaneously in the flank with LNCaP-luc2 cells (2 × 10^6 cells/100 μL/mouse) suspended in 50% basement membrane matrix (Matrigel; BD Biosciences) in RPMI medium. Tumor dimensions were measured 2 to 3 times per week, and volume was calculated as length × width × height × 0.52. Once tumors reached 30 mm³, the mice were randomly assigned to 3 groups of 8 each. Group 1 received vehicle (olive oil; Sigma-O1514); group 2 received SENL, 100 mg/kg body weight; and group 3 SENL, 200 mg/kg body weight. Animals in each group received the same volume of (vehicle or SENL in 100 μL of olive oil or 100 μL of olive oil orally by gavage, 6 days per week for 9 weeks. Tumor growth, tumor imaging, and body weights were determined, as described (21). At the end of the study, 2 hours before the mice were euthanized, they were given either vehicle or SENL according to their groups, and blood was collected from each mouse via retro-orbital plexus into heparinized tubes; plasma was separated and stored at −80°C until used for analysis. The mice were sacrificed by CO₂ inhalation; xenograft tumor tissues and the organs, including heart, lungs, liver, kidneys, and spleen, were excised for fixation and hematoxylin and eosin staining or stored frozen at −80°C for analysis of SENL compounds.

We performed scoring of 2 sections of tumor tissues from each of 8 mice in both untreated control and SENL treated group for tumor fibrosis, apoptosis, necrosis, and mitotic activity on a scale of 0 to 3 (0, negative; 1, mild; 2, moderate; and 3, strong). Immunohistochemistry staining was performed using primary antibodies AKR1C2 (ab170613; Abcam) and PSA (NCL-PSA-431; Leica Biosystems) as described (28). Brown color was considered positive staining for antibodies, and nuclear staining was denoted by blue color.

For analysis of SENL compounds in plasma and tissues, we initially performed spike and recovery experiments using human plasma samples to obtain maximum recovery rates for selection of a suitable method for extraction. Extraction of spiked SENL from human plasma treated with methanol and chloroform in 1:3:1 ratio yielded maximum recovery. Plasma and tumor tissue samples collected from SENL-treated mice were analyzed for bioavailability of SENL compounds, and the profiles were compared with the vehicle-treated group as a control. Mouse plasma or tumor tissues were treated with methanol and chloroform in 1:3:1 ratio for deproteinization; after incubation at room temperature for 5 minutes, the mixture was centrifuged at 3,500 rpm for 15 minutes. The extraction procedure was performed in glass tubes. The supernatant was evaporated using a centrifugal evaporator at 4°C, reconstituted in methanol, and analyzed by HPLC and electrospray ionization mass spectrometry (ESI-MS), as described below. HPLC profiles indicate that the variations in the extraction recoveries for mouse plasma are within ±10% compared with the human plasma.

**HPLC analysis**

Because SENL contains a mixture of compounds, active anticancer compounds present in the SENL were separated by HPLC to evaluate the effect on growth inhibitory activity (Shimadzu LC-10AT vp Pump). Separation was performed on a core-shell silica column (250 × 4.6 mm, particle size 5 μm, pore size 100 Å) (Kinetex C18; Phenomenex). Column temperature was maintained at 40°C. The autosampler injected 20 μL of sample. Mobile phase A was 100% water and phase B was 100% methanol. A flow rate of 1.0 mL/min, starting with a 50-minute linear gradient from 50% to 100% B, 50 to 56 minutes with 100% B, and 56 to 58 minutes to 10% B, and a total run time of 60 minutes, was used with UV detection at 254 nm. Methanol and water used for analysis were HPLC grade obtained from Sigma-Aldrich.

**ESI-MS analysis**

The fractions collected from HPLC were separated on a reversed-phase analytical column (2.1 × 30 mm, 3.5 μm;
Zorbax Eclipse 300SB-C18) on a MSD-TOF instrument (Agilent Technologies) in a positive-mode ESI, as described in ref. 21. The instrument was operated with mobile phase A as water and phase B as methanol, with a gradient 50% B to 100% B over 10 minutes, 100% B for 5 minutes and 5 minutes to equilibrate, and a flow rate of 300 μL/min.

The scan range for acquisition was 300 to 1,000 counts versus mass-to-charge (m/z) range; scan rate: 1 spectra/s; gas temperature 325°C; gas flow 7 L/min; Nebulizer: 40 psi; Capillary voltage: 3,800 V; Fragmentor: 140 V; Skimmer: 65 V; Octopole: 750 V. Nimboide, purchased from BioVision Inc, and 2,3-dehydroxorisol, purchased from Sigma-Aldrich, were used as standards.

Statistical analysis

Data are presented as the mean ± SD for the indicated number of separate experiments. Statistical significance was tested by using the Student t test, one-way ANOVA, Fisher exact test, and Kruskal–Wallis nonparametric ANOVA based on ranks with a Dunn multiple comparison test used to compare the different experimental groups. P value less than 0.05 was considered significant. Fifty percent inhibition concentration (IC50) values were calculated by probit regression.

Results

SENL suppresses cell growth, induces apoptosis, and inhibits DHT-induced androgen receptor and PSA levels in prostate cancer cells

To assess anticancer activity, we investigated the cell death effects induced by SENL in LNCaP-luc2 and PC3 prostate cancer cell lines. The viability of cells treated with SENL was measured by MTS assay (23). Exposure of cells to SENL for 24 hours exhibited a dose-dependent inhibition of LNCaP-luc2 and PC3 cell growth. LNCaP-luc2 cells exhibited IC50 of 15 μg/mL, whereas PC3 cells had IC50 of 15 μg/mL (Fig. 1A). To determine the mechanism of death, apoptosis was evaluated with annexin V/propidium iodide staining and flow cytometry. SENL induces apoptosis of LNCaP-luc2 and PC3 cells (Fig. 1B). We next investigated the effects of SENL on the expression levels of PSA and DHT levels in LNCaP-luc2 cells. PSA levels in the cell supernatants were analyzed by ELISA (21), and intracellular DHT levels were analyzed by liquid chromatography/mass spectrometry (LC/MS; ref. 25). Treatment with DHT induced PSA expression, whereas SENL significantly reduced DHT-induced PSA and intracellular DHT levels in LNCaP-luc2 cells (Fig. 1C and D). To ascertain if the effect was mediated by androgen receptor regulation, Western blot analysis was performed to monitor the expression levels of androgen receptor after SENL treatment. DHT-induced androgen receptor Ser-81 phosphorylation and total androgen receptor expression was significantly inhibited in LNCaP-luc2 cells after SENL treatment (Fig. 2A). Furthermore, to delineate the mechanism of androgen receptor degradation, LNCaP-luc2 cells were treated with proteasome inhibitor MG132 and SENL. Immunoblotting suggests that SENL suppresses androgen receptor levels in the presence of MG132 (Fig. 2B).

SENL inhibits the formation of focal adhesions in LNCaP-luc2 and PC3 prostate cancer cells

In our previous study (23), we showed inhibition of migration, and angiogenesis of human umbilical vein endothelial cells after treatment with ethanol extraction of neem leaves. FAK, which is involved in endothelial cell proliferation, migration, and survival, is upregulated in many cancers (29). To determine whether growth inhibition of prostate cancer cells is mediated through FAK modulation, we assessed FAK activation signaling in prostate cancer cells after SENL treatment. LNCaP-luc2 and PC3 cells were examined for integrin β1 and FAK activation (30). Western blot analysis showed that the levels of integrin β1 and phosphorylation of FAK at Y-397 were reduced with SENL treatment, suggesting that FAK signaling was inhibited (Fig. 2C). Immunofluorescence for integrin β1 and FAK further demonstrated the suppression of formation of the focal adhesions after SENL treatment in LNCaP-luc2 and PC3 cells (Fig. 3). Because integrin and FAK changes may be affected by alterations in the calcium-signaling protein CALR and membrane traffic-regulating protein Rab5, we examined the SENL effects on these 2 proteins. SENL downregulated CALR levels in both LNCaP-luc2 and PC3 cells, whereas modest inhibition of Rab5 was observed in LNCaP-luc2 compared with PC3 cells (Fig. 2C).

SENL induces hyalinization of tumor tissue and inhibits the growth of human LNCaP-luc2 prostate cancer xenografts in nude mice with reduction in the PSA, and increase in the AKR1C2 levels

We next evaluated whether administration of SENL via gavage exhibited anticancer activity in vivo in a xenograft tumor model. Male nude mice bearing LNCaP-luc2 xenografts were treated with SENL or vehicle control. At the start of the treatments, mean LNCaP-luc2 tumor volumes were similar in the 3 groups. Treatment with SENL significantly suppressed tumor growth. By 9 weeks, mean tumor volume in the SENL-treated group (200 mg/kg body weight) was 189 mm3 compared with 712 mm3 in the control group (P ≤ 0.05; Fig. 4A and B). At the end of the study, a significant decrease was found in tumor weights with all SENL treatments (Fig. 4C). There was no significant change in body weight in any of the groups after treatment, which suggests that oral administration of SENL causes no major toxic effects in mice (Fig. 4D).

Histologic examination revealed hyalinization and apoptosis of the tumor tissues in 7 of 8 mice treated with SENL (200 mg/kg body weight; Fig. 5). The group mean score for fibrosis, apoptosis, necrosis, and mitotic activity in SENL-treated xenograft tumor tissues was 3, 2, 0, and 1, respectively, whereas in the vehicle control group was 0, 1,
3, and 3 respectively (0 = none, 1 = mild, 2 = moderate, and 3 = strong). More fibrosis and apoptotic activity shows more hyalinized connective tissue and regression of tumor in the SENL-treated mice. High mitotic activity and necrosis indicates more proliferative tumors in control mice. Immunohistochemistry analysis of SENL-treated mice tumor tissues revealed reduction in the PSA and increase in the AKR1C2 levels (Fig. 5). These data demonstrate that oral administration of SENL caused regression of the tumor tissue and inhibited tumor growth by promoting hyalinization and apoptosis. There was no significant change in the histology of the heart, lungs, liver, kidneys, and spleen after 9 weeks of SENL treatment compared with the control group, which indicates that SENL has no adverse effects on these vital organs.

Separation of SENL compounds by HPLC and evaluation of cytotoxic activity on prostate cancer cells in vitro

Chromatographic separation of compounds in SENL was performed using HPLC column. A methanol gradient was used for fractionation. The HPLC profile of the SENL is illustrated in Supplementary Fig. S1. UV absorption of the compounds at 254 nm showed a total of 16 peaks. Fractions 1 to 11 eluted with the 70% to 80% methanol gradient at retention time of 22 to 34 minutes were colorless, whereas fractions 12 to 16 eluted with the 100% methanol at 50 to 56 minutes were yellow. The fractions were concentrated at 4 °C using a centrifugal evaporator, and equal weight fractions were reconstituted in ethanol. Serial dilutions of the fractions 0.468 to 15 μg/mL were used to determine the
efficacy to inhibit cell growth. Cell viability was measured by MTS assay after treatment of PC3 prostate cancer cells for 24 hours with the isolated fractions. Vehicle-treated cells were included as controls. Although fractions 2 to 11 exhibited some reduction in the viability of cells, fractions 2, 3, and 5 exhibited a stronger dose-dependent inhibition of cell viability. The IC50 concentration to inhibit cell growth for fractions 2, 3, and 5 were less than 1.85 μg/mL.

Figure 2. Suppression of AR, CALR, integrin-β1 expression, and FAK activation in prostate cancer cells. A, LNCaP-luc2 cells were treated with SENL (12 μg/mL) in the presence and absence of 10 nmol/L DHT for 8 and 24 hours, and AR protein levels were measured with specific antibodies by Western blot analysis. B, LNCaP-luc2 cells treated with SENL (12 μg/mL) in the presence and absence of 50 μmol/L MG132 and SENL for 8 hours. AR protein levels were measured with specific antibodies by Western blot analysis. All experiments were performed in triplicate; vehicle-treated cells were used as control. GAPDH was the loading control and the representative blot is shown.

Figure 3. Inhibition of FAK and integrin β1 expression in prostate cancer cells. LNCaP-luc2 and PC3 cells were treated with SENL for 24 hours, and immunofluorescence was performed for FAK and integrin β1 localization. Cy5-labeled secondary antibody was used for FAK staining, FITC-labeled secondary antibody was used for integrin β1 costaining, and the nuclei were stained with DAPI. Confocal images were taken on LSM 780 at ×100 magnification. A and B, LNCaP-luc2 and PC3 cells treated with the vehicle control show extensions of the focal adhesions and integrin β1 expression at the focal adhesions. C and D, treatment with SENL caused cell rounding, with significant reduction in FAK and integrin β1 expression. Arrows. FAK expression. Scale bar, 10 μm.
Fractions 6 to 11 exhibited an IC₅₀ concentration in the range of 7.5 to 15.0 mg/mL. Our results revealed fractions 2, 3, and 5 were the most active fractions to inhibit prostate cancer cell growth (Supplementary Fig. S2).

**Identification of the compounds in SENL by ESI-MS analysis**

ESI-MS analysis was performed on the described HPLC fractions to identify potential active anticancer compounds.

Figure 4. SENL inhibited the growth of LNCaP-luc2 prostate cancer xenografts in nude mice. A, bioluminescence imaging of mice implanted with LNCaP-luc2 tumors. Group 1 animals received vehicle control (olive oil) orally. Group 2 and 3 animals were administered SENL, 100 or 200 mg/kg body weight (bw), 6 days a week. A representative image of the mice from each group at the end of 9 weeks of treatment is shown. B, tumor volume changes of mice treated by oral gavage with vehicle, and SENL, 100 or SENL 200 mg/kg body weight, for 9 weeks. C, tumor weight of the mice after 9 weeks of treatment. D, body weight of mice over the treatment period. Data represent mean ± SD of tumor volume, tumor weight, and body weight changes of 8 mice per group. *P < 0.05.

Figure 5. Histologic changes of LNCaP-luc2 tumor tissues of mice treated with SENL, 200 mg/kg body weight. At the end of 9 weeks, xenograft tumor tissues were collected and stained with hematoxylin and eosin. Two sections of tumor tissue from each of 8 mice in a group were examined for histologic changes. A, tumor tissue of vehicle-treated mouse showing mitotic figures (thick arrows), and apoptosis (thin arrows). B, tumor tissue of a SENL-treated mice shows nests of tumor cells separated by hyalinization. C and D, immunohistochemistry analysis for PSA expression in the tumor tissues of vehicle- and SENL-treated mice. E and F, immunohistochemistry analysis for AKR1C2 expression in the tumor tissues of vehicle- and SENL-treated mice. Arrows, positive staining for PSA and AKR1C2 expression. Original magnification, ×400.
in the SENL. The major compounds identified by mass spectrometric analysis are shown in Table 1. Mass spectrometric analysis of SENL fractions 2, 3, 4, 5, 6, 7, 9, 11, 12, and 14 obtained from HPLC and the standards nimbinol and 2,3'-dehydronimbolide are shown in Supplementary Fig. S3 to S6. We did not perform mass spectra for fractions 1, 8, 10, 13, 15, and 16 because they have not shown significant cytotoxic activity. The structures of the compounds identified in the mass spectra are presented in Supplementary Fig. S7.

Table 1. Fractionation of SENL was performed using a Kinetex C18 column on a Shimadzu HPLC system

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Major compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Nimbandiol and nimbolide</td>
</tr>
<tr>
<td>3</td>
<td>2',3'-Dihydrornimbolide</td>
</tr>
<tr>
<td>4</td>
<td>Nimbolide, 2',3'-dihydrornimbolide, and desacetyl/nimbin</td>
</tr>
<tr>
<td>5</td>
<td>Desacetyl/nimbin and 28-deoxonimbolide</td>
</tr>
<tr>
<td>6</td>
<td>28-Deoxonimbolide and desacetyl/Salannin</td>
</tr>
<tr>
<td>7</td>
<td>Desacetyl/Salannin and salannin</td>
</tr>
</tbody>
</table>

NOTE: Major compounds identified by mass-spectrometric analysis in each HPLC fraction are shown.

Bioavailability of SENL compounds in plasma and tumor tissues of mice

We examined the availability of SENL compounds in the plasma and xenograft prostate tumor tissues of the treated mice. The extraction recoveries for SENL with methanol and chloroform were 82% to 92%. The bioavailability of SENL compounds was assessed by ESI-MS analysis of plasma and tumor tissues of SENL treated (200 mg/kg body weight) and vehicle-treated mice. Plasma and tumor tissues pooled from 4 mice of each group were extracted with methanol and chloroform. HPLC was performed as described above with the plasma and tumor tissue extracts; fraction 1 and fraction 2 was collected from 20 to 25 minutes and 25.01 to 30 minutes, respectively. Both the fractions from plasma and tissues were analyzed for identification of the compounds by mass spectrometry in duplicate. Tumor tissues and plasma of mice treated with SENL suggested the presence of both 28-deoxnimbinolide and the sodium salt of nimbinolide (Supplementary Fig. S8). Plasma and tumor tissues of vehicle treated group and nimbolide are the most active bioavailable compounds in the SENL. The major compounds identified by mass spectrometric data for nimbolide and 28-deoxonimbolide.

Discussion

Natural products are an important source of potential cancer chemotherapeutic and antimetastatic agents (31). In our initial study, we evaluated the antitumor activity and of ethanol extracts of neem leaves in prostate cancer models both in vitro and in vivo (21, 23). To obtain higher extraction efficiencies of bioactive compounds, we adopted a supercritical extraction method (17). The IC_{50} concentration of ethanol extracts of neem leaves to inhibit PC3 cells is 25 μg/mL, whereas for SENL is 15 μg/mL. Compared with the ethanol extract of neem leaves, the current method of supercritical extraction showed improved extraction efficacy. The research described in this communication is novel for the following reasons: first, it focuses on a molecular mechanism for anticancer activity of the SENL. Second, it utilizes a well-established prostate cancer model for demonstration of the in vivo efficacy. Third, it reveals several important active anticancer compounds in the SENL, some of which can be detected in plasma and tissues after treatment.

Our data show that androgen-dependent LNCaP-luc2 cells were slightly more sensitive to SENL treatment compared with androgen-independent PC3 cells (Fig. 1A and B). This could be because of the differential expression of genes in the cell lines (32). DHT is the most effective androgen to activate androgen receptor functions (33). SENL treatment significantly decreased the expression of DHT-induced PSA and intracellular DHT levels in LNCaP cells (Fig. 1C and D). To address the role of SENL in suppression of PSA and DHT levels, we analyzed the modulation of DHT-induced androgen receptor expression levels. SENL significantly inhibited DHT-induced androgen receptor Ser 81 phosphorylation and total androgen receptor levels in LNCaP-luc2 cells (Fig. 2A). To delineate the mechanism for suppression of androgen receptor levels through proteasome degradation, we analyzed the expression of androgen receptor in the presence of proteasome inhibitor MG132 (26). SENL treatment reduced androgen receptor levels in the presence of MG132 (Fig. 2B), which indicates that proteasome degradation pathway may not play a major role in the SENL effects on the androgen receptor.

We showed previously that ethanol extracts from neem leaves induces AKR1C2 in prostate cancer cells and suppresses DHT levels in xenograft prostate tumor tissues of mice (21). AKR1C2 plays a prominent role in DHT catabolism (34). We speculate that increase in the AKR1C2 levels with SENL treatment induces degradation of DHT. Reduction in the DHT levels affects ligand induced stability of the androgen receptor levels. The effects of SENL on the inhibition of androgen-dependent LNCaP-luc2 cell growth could be partially mediated by downregulation of the androgen-regulated pathways; however, the inhibition of androgen receptor--negative PC3 cell growth indicates that androgen receptor--independent mechanisms may be affected by SENL treatment.

Many studies have reported FAK as a positive regulator of tumor progression (35, 36). FAK is the primary enzyme involved in the engagement of integrins and assembly of focal adhesion (29). Phosphorylation of FAK at Y-397 has been shown to be elevated in tumor metastasis (37). We...
demonstrated that activation of FAK at Y-397 and integrin β1 levels were reduced in SENL-treated cells (Fig. 2C). Further evidence of inactivation of FAK by SENL is demonstrated by our immunofluorescence staining, which reveals inhibition in the formation of focal adhesions in LNCaP-luc2 and PC3 cells (Fig. 3). These data indicate that SENL may contribute to antimigration, anti-invasion, and growth inhibitory effects by suppressing integrin and FAK signaling.

CALR is a multicompartmental protein, which regulates many important cellular responses (38, 39). Elevated CALR expression promotes the migration and invasion of cancer cells (40). SENL treatment decreased CALR expression in LNCaP-luc2 and PC3 cells (Fig. 2C). We speculate that suppression of CALR expression with SENL partially contributes to the inhibition of focal adhesions in prostate cancer cells. The formation of endoplasmic reticulum tubules in vitro requires a Rab family GTPase, Rab-5 (41). SENL did not substantially alter Rab-5 in LNCaP-luc2 cells, whereas it decreased Rab-5 significantly in PC3 cells (Fig. 2C). Although the exact mechanism of SENL on inhibition of prostate cancer cells is not completely understood, it seems to entail FAK inactivation, androgen receptor downregulation, and DHT degradation pathways. We postulate that SENL effects are mediated through CALR for the regulation of FAK required for tumor cell migration and metastasis.

We also demonstrated that oral administration of SENL suppressed xenograft tumor growth. There were no significant changes in the body weight in the SENL-treated groups compared with the control group, which confirms that SENL at both 100 and 200 mg/kg body weight has no adverse physiologic effects (Fig. 4). The most significant histologic change observed after SENL treatment in mice was the formation of hyalinized tumor tissue in more than 80% of the mice (200 mg/kg body weight; Fig. 5). In our previous study, we observed a similar change in the C4-2B and PC3 xenograft tumor tissues of mice-treated peritoneally with ethanol extract of neem leaves (23). Fibrous tissue formation is an indicator of decreased tumor invasiveness and improved tumor regression (42, 43). This histologic feature of hyalinization confirms the tumor regression with SENL treatment. We have shown that SENL treatment of prostate cancer cells in vitro reduces PSA (Fig. 1C) and increases AKR1C2 levels (21). Immunohistochemistry analysis of SENL treated mouse tumor tissues revealed reduction in the PSA and increase in AKR1C2 levels, which further supports our in vitro findings (Fig. 5).

Mass spectrometric analysis of tumor tissues and plasma suggests 28-deoxynimbolide and nimbolide as the bioavailable compound after SENL treatment (Supplementary Fig. S8). We anticipate that SENL can mediate antitumor activity in vivo by modulating multiple pathways in tumor development and progression. We speculate that neem, with its low risk of toxicity, can be used safely for prostate cancer prevention and treatment trials.

Terpenoids constitute the largest class of natural compounds for drug discovery (44–46). More than 70 terpenoids have been identified in the neem plant (47, 48). To our knowledge, nimbolide is the only neem terpenoid reported to have anticancer activity (49, 50). In addition to nimbolide, our data reveal that the anticancer activity of other terpenoids from neem leaves, which includes nimbadiol, Z,Z′-dihydrornimbolide, and 28-deoxynimbolide in prostate cancer cells (Table 1 and Supplementary Fig. S2). Therefore, these compounds can be further exploited to study their anticancer efficacy. In summary, we have shown that terpenoids in SENL are active compounds for inhibition of cancer growth. Anticancer activities of SENL are mediated through regulation of CALR, androgen receptor, and FAK levels. We also demonstrated the in vivo therapeutic potential of SENL in a preclinical prostate cancer model. Further studies are required to elucidate the mechanism of action of isolated neem compounds on inhibition of tumor progression individually and in combinations.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: M. Kohli, J.C. Cheville, C.Y.F. Young, K.V. Donkena
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Q. Wu, H.R. Bergen III, K.V. Donkena
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Wu, H.R. Bergen III, K.V. Donkena
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Study supervision: K.V. Donkena

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# Molecular Cancer Therapeutics

## Preclinical Evaluation of the Supercritical Extract of *Azadirachta Indica* (Neem) Leaves *In Vitro* and *In Vivo* on Inhibition of Prostate Cancer Tumor Growth


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