Serenoa repens induces growth arrest and apoptosis of human multiple myeloma cells via inactivation of STAT 3 signaling

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Abstract. Serenoa repens, a palm species native to the Southeastern United States, is one of the widely used phytotherapeutic agents in benign prostatic hyperplasia. In this study, we found for the first time that Serenoa repens induced growth arrest of a variety of human leukemia cells including U266 and RPMI 8226 multiple myeloma cells as measured by mitochondrial-dependent conversion of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. TUNEL assays showed that Serenoa repens induced apoptosis of U266 cells in a time- and dose-dependent manner. Serenoa repens also increased the expression of cleaved-PARP or p27 protein in different human leukemia cell lines. In addition, we found that Serenoa repens down-regulated basal level of phosphorylated form of signal transducer and activator of transcription 3 (STAT 3) and Interleukin-6 induced level of phosphorylated form of STAT 3 and extracellular signal-related kinase (ERK) were also reduced after Serenoa repens treatment in U266 cells. Furthermore, we found that inhibition of STAT 3 signaling by Serenoa repens or Janus family of tyrosine kinase (JAK) inhibitor of AG490 enhanced the ability of docetaxel to inhibit the growth of U266 and RPMI 8226 cells, as measured by trypan blue exclusion test. These results indicate that Serenoa repens might be useful for the treatment of individuals with multiple myeloma.

Introduction

Multiple myeloma (MM) is a malignant plasma cell disorder that accounts for ~10% of all hematologic cancers and it is the second most common hematologic malignancy in the United States (1,2). The characteristic findings in MM are lytic bone disease, renal insufficiency, anemia, hypercalcemia and immunodeficiency (3). Despite previous new insights into the pathogenesis of MM, it remains incurable with a median survival of ~3 to 4 years with conventional chemotherapies and 4 to 5 years with high-dose chemotherapy followed by autologous stem-cell transplantation (4). Therefore, novel treatment strategies for MM are urgently needed.

Signal transducers and activators of transcription (STATs) were originally discovered as latent cytoplasmic proteins with roles as signal messengers and transcription factors that participate in normal cellular responses to cytokines and growth factors (5-7). STATs are activated by phosphorylation of tyrosine and serine residues by upstream kinases (8). This signaling has been demonstrated to play a major role in tumor formation and aberrant signaling of STAT 3 is found in many types of malignancies including multiple myeloma, head and neck cancer, breast cancer and prostate cancer (9-12).

Phytotherapy have been used for many centuries for treatment of cancer (13,14). Use of these therapies has been dramatically rising in previous years in the United States (15). One of the phytotherapeutic agents frequently used by individuals with benign prostatic hyperplasia is Serenoa repens (also named Saw Palmetto) and it has also been shown to possess anti-tumor activity (16-18). For example, Serenoa repens was shown to inhibit growth and induce apoptosis of prostatic cancer cells in studies in vitro (19-21). Serenoa repens effectively induced a dose-dependent anti-proliferative effect on human breast cancer cells (22). However, the mechanism of Serenoa repens for inhibiting growth of cancer cells remains to be fully elucidated.

In this study, we found that Serenoa repens inhibited the proliferation of a variety of human leukemia cells including U266 and RPMI 8226 multiple myeloma cells. We also found that Serenoa repens inhibited basal level of phosphorylated form of STAT 3 and Interleukin-6 (IL-6) induced level of phosphorylated form of STAT 3 in U266 cells suggesting that Serenoa repens may induce growth arrest and apoptosis of human multiple myeloma cells through inactivation of STAT 3 signaling. Serenoa repens might be useful for treatment of individuals with human multiple myeloma.

Materials and methods

Cell line. Human acute leukemia cell lines (HL-60, NB4, Jurket) and multiple myeloma cell lines (U266, RPMI 8226)
were grown in RPMI-1640 supplemented with 10% fetal bovine serum (heat inactivated at 56°C for 45 min) and an appropriate amount of penicillin/streptomycin in a 37°C incubator with a humidified, 5% CO2 atmosphere.

Chemical. *Serenoa repens* was extracted from the fruit of *Serenoa repens* and one capsule of *Serenoa repens* contains 400 mg of powered herbal extracts. Stock solutions of *Serenoa repens* were prepared by exposing these herbal extracts to 70% ethanol (one capsule/1 ml of 70% ethanol).

**MTT assays.** Cells (10⁴/ml) were incubated with a variety of concentrations of *Serenoa repens* (0-2 μl/ml) for 3 days in 96-well plates (Flow Laboratories, Irvine, CA). After culture, cell numbers and viability were evaluated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma), to a colored formazan product, as previously described (23).

Trypan blue exclusion test. MM cells (2x10⁵/ml) were incubated with various concentrations of *Serenoa repens* (0-2 μl/ml) for 3 days in 96-well plates (Flow Laboratories). After culture, cell numbers and viability were evaluated by staining with trypan blue and counting using light microscopy.

**Flow cytometry assay.** The DNA content of cultured cells was analyzed by flow cytometry assay. Apoptotic cells have a lower DNA content than normal cells and appear as a pre-G₁ peak on a DNA cell-cycle histogram and cells were harvested to quantitate the amount of apoptosis present after various treatments. In brief, cells were trypsinized and washed with cold phosphate-buffered saline (PBS). Cells were then fixed with 70% ethanol and stored overnight at -20°C. Propidium iodide (10 μg/ml; Sigma) was added to stain cells in the culture showing that apoptosis of MM cells in response to *Serenoa repens* treatment. The cells with DNA content smaller than G₀/G₁ were considered to be apoptotic cells. *Serenoa repens* showed dose-dependent apoptotic in U266 and RPMI 8226 cells, respectively (p<0.05) (Fig. 1B and C). A TUNEL assay was utilized to further test the pro-apoptotic effect of *Serenoa repens*. After 24 h culture, 0.5 and 1.0 μl/ml of *Serenoa repens* induced a mean 9.0±0.7 and 18.4±3.2% of U266 cells to become apoptotic and which increased to a mean of 30.0±3.9 and 45.3±5.0% of U266 cells to become apoptotic, respectively, after another 24 h culture showing that *Serenoa repens* induced apoptosis of these cells in a dose- and time-dependent manner (p<0.05) (Fig. 1D).

**Assessment of apoptosis.** Apoptotic cell death was examined by terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) method using the In Situ Cell Death Detection kit (Roche Molecular Biochemicals, Germany), as previously described (24). For quantification three different fields were counted under the microscope and at least 300 cells were counted in each field. All experiments were performed at least twice.

Western blotting. Lysates were made by standard methods, as previously described (25). Equal amounts of proteins were resolved by 9-15% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to an immobilized polyvinylidene difluoride membrane (Amersham Corp., Arlington Heights, IL), and probed sequentially with antibodies. Anti-PARP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 (sc-819, Santa Cruz Biotechnology), anti-p27kip1 (sc-527, Santa Cruz Biotechnology), anti-STAT 3 (Santa Cruz Biotechnology), anti-p-STAT 3 (Cell Signaling, Beverly, MA), anti-ERK (Santa Cruz Biotechnology), anti-p-ERK (Cell Signaling), anti-β-actin antibodies (Santa Cruz Biotechnology) were used. The blots were developed using the enhanced chemiluminescence kit (Amersham Corp.).

**Statistical analysis.** Statistical analysis was performed to assess the difference between two groups under multiple conditions by one-way analysis of variance (ANOVA) using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA).

**Results**

Effect of *Serenoa repens* on proliferation of MM cells. To explore the anti-proliferative effects of *Serenoa repens* on MM cells, U266 and RPMI 8226 MM cell lines were cultured in the presence of various concentrations of *Serenoa repens* (0-2 μl/ml). *Serenoa repens* effectively inhibited the growth of U266 and RPMI 8226 cells with the effective dose that inhibited 50% growth (ED₅₀) of ~0.7 and 1.2 μl/ml, respectively as measured by MTT assay on the third day of culture (Fig. 1A).

*Serenoa repens* induced apoptosis of MM cells. Next, flow cytometry was performed to analyze changes in cell death of MM cells in response to *Serenoa repens* treatment. The cells with DNA content smaller than G₀/G₁ were considered to be apoptotic cells. *Serenoa repens* showed dose-dependent apoptotic in U266 and RPMI 8226 cells, respectively (p<0.05) (Fig. 1B and C). A TUNEL assay was utilized to further test the pro-apoptotic effect of *Serenoa repens*. After 24 h culture, 0.5 and 1.0 μl/ml of *Serenoa repens* induced a mean 9.0±0.7 and 18.4±3.2% of U266 cells to become apoptotic and which increased to a mean of 30.0±3.9 and 45.3±5.0% of U266 cells to become apoptotic, respectively, after another 24 h culture showing that *Serenoa repens* induced apoptosis of these cells in a dose- and time-dependent manner (p<0.05) (Fig. 1D).

Effect of *Serenoa repens* on expression of apoptotic-related protein in MM cells. Furthermore, the proapoptotic effect of *Serenoa repens* was explored by examining for the cleavage of poly (ADP-ribose) polymerase (PARP) which is thought to be a late event in apoptosis. As shown in Fig. 1E, U266 and RPMI 8226 cells were exposed to *Serenoa repens* (1.0 μl/ml, 24 h) and Western blot analysis of cellular lysates showed cleaved of PARP band in RPMI 8226 cells (Fig. 1E). Other apoptotic-related proteins such as Mcl-1 and p27kip1 were also detected in these two cell lines. For example, exposure of U266 cells to *Serenoa repens* (1 μl/ml, 24 h) decreased the level of Mcl-1 by 60.0% compared with that in control cells. Whereas, *Serenoa repens* (1 μl/ml, 24 h) did not modulate level of Mcl-1 in RPMI 8226 cells (Fig. 1E). Level of the p27kip1 protein was negligible in RPMI 8226 cells and exposure of U266 cells to *Serenoa repens* (1 μl/ml, 24 h) resulted in a dramatic increased on p27kip1 protein level (Fig. 1E). These results indicate that *Serenoa repens* induces growth arrest and apoptosis of human MM Cells.

Effect of *Serenoa repens* on proliferation and apoptosis of human leukemia cells. The effects of *Serenoa repens* on other malignant hematopoietic cells apart from its role in
the proliferation and apoptosis of human MM cells were explored in the human acute myelogenous leukemia (HL-60, NB4) and acute lymphoblastic T-cell leukemia cells (Jurkat) in this study. As shown in Fig. 2A, *Serenoa repens* effectively inhibited the growth of NB4 and Jurkat cells with the effective dose that inhibited 50% growth (ED50) of ~1.3 and 0.8 μl/ml, respectively as measured by MTT assay on the third day of culture (Fig. 2A). *Serenoa repens* partly inhibited the growth of HL-60 cells although the inhibiting effect was weaker than the other two cell lines (Fig. 2A).

Next, apoptotic-related proteins such as PARP and p27<sup>kip1</sup> were detected in these cells. As shown in Fig. 2B, the
cleaved bands of PARP were increased by 5.0- and 5.6-fold, respectively, in Jurkat and NB4 cells after Serenoa repens (1.0 μl/ml, 24 h) treatment. Exposure of Jurkat and NB4 cells to Serenoa repens (1 μl/ml, 24 h) resulted in a dramatic increase of p27kip1 protein level (Fig. 2B). On the other hand, no matter the level of cleaved PARP, p27kip1 was increased in HL-60 cells (Fig. 2B). These findings were consistent with the resultant proliferation as determined by MTT assay showing that Serenoa repens has an effect on proliferation and apoptosis of human acute leukemia cells (Fig. 2A).

Effect of Serenoa repens on IL-6-stimulated phosphorylation of STAT 3 and ERK in MM cells. A recent study showed that Serenoa repens induced growth arrest and apoptosis of prostate cancer cells via inactivation of STAT 3 signaling (25). We next explored the effect of Serenoa repens on STAT 3 signaling in U266 cells. U266 cells were exposed to Serenoa repens (1.0 μl/ml, 24 h) and then harvested for Western blot analysis. As shown in Fig. 3A, U266 cells constitutively expressed the phosphorylated form of STAT 3 and the expression of phosphorylated form of STAT 3 was reduced by 80.0% after Serenoa repens treatment (Fig. 3A). U266 cells were serum-starved for 24 h and exposure of these cells to IL-6 (20 ng/ml, 30 min) dramatically increased the level of the phosphorylated form of STAT 3 by 20.0-fold, and pretreatment (3 h) of these cells with 1.0 μl/ml of Serenoa repens for 3 h almost completely blocked the IL-6-induced phosphorylation of STAT 3 by 85.0% (Fig. 3B). In addition, we explored the effect of Serenoa repens on phosphorylation of ERK mediated by IL-6 in U266 myeloma cells. Levels of the phosphorylated form of ERK 1/2 were negligible in control U266 cells which were serum-starved for 24 h. Addition of IL-6 (20 ng/ml, 30 min) to these cells induced phosphorylation of ERK and pretreatment of these cells with 1.0 μl/ml of Serenoa repens for 3 h almost completely blocked the IL-6-induced phosphorylation of ERK1/2 (Fig. 3B). These data indicate that Serenoa repens may reduce the expression of phosphorylation of STAT 3 or ERK mediated by IL-6 in MM cells.
Combination effects of Janus family of tyrosine kinase (JAK) inhibitor AG490 and Serenoa repens on growth of MM cells.

To study the role of STAT 3 signaling in survival of MM cells, we blocked this pathway using a Janus family of tyrosine kinase (JAK) inhibitor AG490. As shown in Fig. 4A, the expression of phosphorylated form of STAT 3 was effectively reduced after AG490 (20-40 nM, 48 h) treatment. AG490 significantly inhibited the proliferation of U266 and RPMI 8226 cells in a dose-dependent manner with ED$_{50}$s of ~28.45 and 13.06 nM, respectively as measured by MTT assay (Fig. 4B). We next studied the interaction of AG490 and Serenoa repens on growth of MM cells. As shown in Fig. 4C, U266 and RPMI 8226 cells were pre-incubated with AG490 (20 nM) for 3 h and then exposed to Serenoa repens (0.5 μl/ml) for 2 days and cell numbers and their viability were evaluated at the indicated time point by trypan blue exclusion test. Results represent the mean ± SD of three experiments performed in triplicate. The statistical significance was determined by one-way analysis of variance.

Inhibition of STAT 3 signaling by Serenoa repens or AG490 sensitized MM cells to anti-proliferative effect of docetaxel.

Docetaxel has activity against multiple malignancies including MM (26). Therefore, we explored whether inhibition of STAT 3 signaling by either Serenoa repens or AG490 sensitized MM cells to docetaxel-mediated growth inhibition. The U266 and RPMI 8226 cells were pre-incubated with Serenoa repens (0.5 μl/ml) for 3 h and exposed to docetaxel (10$^{-9}$ M). After 48 h, cell numbers were assessed by trypan blue exclusion test (Fig. 5A). Either Serenoa repens (0.5 μl/ml) or docetaxel (10$^{-9}$ M) alone inhibited the growth of U266 cells by 79.7 or 90.1%, respectively. The combination of Serenoa repens and docetaxel inhibited the growth of these cells by 51.6% compared to control cells (Fig. 5A, left panel). Statistical analysis with ANOVA showed significant difference between either drug alone and combination of both (p<0.01). Similarly, Serenoa repens enhanced anti-proliferative activity of docetaxel against RPMI 8226 cells (Fig. 5A, right panel). We next studied the interaction of AG490 (JAK inhibitor) and docetaxel (Fig. 5B). For example, RPMI 8226 cells were pre-incubated with AG490 (20 nM) for 3 h and exposed to docetaxel (10$^{-9}$ M, 48 h). Either AG490 or docetaxel alone inhibited the growth of RPMI 8226 cells by 34.8 or 84.6%, respectively. When cells were exposed to the combination of both, the cell growth was inhibited by 23.9% compared to control cells (p<0.001) (Fig. 5B, right panel). The enhanced anti-proliferative effect was also observed when U266 cells were treated in combination with AG490 (20 nM) and docetaxel (10$^{-9}$ M) (Fig. 5B, left panel). These data indicate that both Serenoa repens and AG490 sensitize MM cells to anti-proliferative effect of docetaxel through inhibition of STAT 3 signaling.

Discussion

Phyotherapy (including herbal) have been used for many centuries for treatment of cancer. For example, Chinese herbal mixture denoted PC-SPES was shown to inhibit the growth of different kinds of cancer cells such as non-small cell lung
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**Figure 5. Inhibition of STAT 3 signaling by Serenoa repens or AG490-sensitized MM cells to anti-proliferative effect of docetaxel.**

(A) Trypan blue exclusion test. U266 and RPMI 8226 cells (10^5/ml) were plated in 96-well plates and cultured either with or without Serenoa repens (0.5 μl/ml) for 3 h and then cells were exposed to docetaxel (10^{-6} M) for 2 days. Cell numbers and their viability were evaluated at the indicated time point by trypan blue exclusion test. Results represent the mean ± SD of three experiments performed in triplicate. (B) Trypan blue exclusion test. U266 and RPMI 8226 cells (10^5/ml) were plated in 96-well plates and cultured either with or without AG490 (20 nM) for 3 h. Then cells were exposed to docetaxel (10^{-6} M) for 2 days. Cell numbers and their viability were evaluated at the indicated time point by trypan blue exclusion test. Results represent the mean ± SD of three experiments performed in triplicate. The statistical significance was determined by one-way analysis of variance.

**Data Analysis:**

- **Statistical Significance:**
  - *p < 0.01

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**Discussion:**

- **Saw Palmetto**
  - Induces apoptosis of MM cells via STAT 3 signaling.
  - Synergistic effect with docetaxel.

- **Rabdosia rubescens**
  - Inhibits growth of MM cells.

- **Cell Lines:**
  - Multiple myeloma (MM)
  - Prostate cancer

- **Agents:**
  - Docetaxel
  - R115777
  - AG490

- **Pathways:**
  - STAT 3 signaling

- **Conclusion:**
  - Serenoa repens may be a novel therapeutic agent for MM.
  - Synergistic effect with docetaxel.

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**References:**

- **Cancer Cells and Prostate Cancer Cells**
  - Rabdosia rubescens

- **Herbal Agents**
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**Summary:**

- **Serenoa repens** induces apoptosis and growth arrest in MM cells.
  - Synergy with docetaxel.

- **Therapeutic Potential:**
  - Additional research needed for combination therapy.

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**Key Points:**

- **Cell Lines:** Multiple myeloma, prostate cancer
- **Agents:** Docetaxel, R115777, AG490
- **Pathways:** STAT 3 signaling
- **Conclusion:** Serenoa repens as a potential therapeutic agent in MM and prostate cancer.
References