Fisetin Enhances Chemotherapeutic Effect of Cabazitaxel against Human Prostate Cancer Cells

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Abstract

Although treatment of prostate cancer has improved over the past several years, taxanes, such as cabazitaxel, remain the only form of effective chemotherapy that improves survival in patients with metastatic castration–resistant prostate cancer. However, the effectiveness of this class of drugs has been associated with various side effects and drug resistance. We previously reported that fisetin, a hydroxylflavone, is a microtubule-stabilizing agent and inhibits prostate cancer cell proliferation, migration, and invasion and suggested its use as an adjuvant for treatment of prostate and other cancer types. In this study, we investigated the effect of fisetin in combination with cabazitaxel with the objective to achieve maximum therapeutic benefit, reduce dose and toxicity, and minimize or delay the induction of drug resistance and metastasis. Our data show for the first time that a combination of fisetin (20 μmol/L) enhances cabazitaxel (5 nmol/L) and synergistically reduces 22Rv1, PC-3M-luc-6, and C4-2 cell viability and metastatic properties with minimal adverse effects on normal prostate epithelial cells. In addition, the combination of fisetin with cabazitaxel was associated with inhibition of proliferation and enhancement of apoptosis. Furthermore, combination treatment resulted in the inhibition of tumor growth, invasion, and metastasis when assessed in two in vivo xenograft mouse models. These results provide evidence that fisetin may have therapeutic benefit for patients with advanced prostate cancer through enhancing the efficacy of cabazitaxel under both androgen-dependent and androgen-independent conditions. This study underscores the benefit of the combination of fisetin with cabazitaxel for the treatment of advanced and resistant prostate cancer and possibly other cancer types. Mol Cancer Ther; 15(12): 2863–74. ©2016 AACR.

Introduction

Despite advances in screening and treatment, prostate cancer remains a leading cause of death among American men (1). An estimated 180,890 new cases and 26,120 deaths are projected in 2016 (2). Androgen ablation therapy has been shown to be effective in regression of prostate cancer; however, the cancer eventually progresses to metastatic castration–resistant prostate cancer (mCRPC), for which there is no cure. A wide range of novel therapies has been introduced clinically for the treatment of mCRPC, including androgen synthesis inhibitors, immunotherapies, and microtubule-targeting agents, such as taxanes (3, 4). In 2004, two clinical studies demonstrated a survival advantage of docetaxel (Taxotere) chemotherapy in these patients (5, 6), setting a new standard of care and representing a significant milestone in the treatment of prostate cancer (7). Consequently, docetaxel is the most commonly prescribed first-line chemotherapy for mCRPC. However, many patients have limited therapeutic options once tumors become refractory to docetaxel chemotherapy. In such cases, no treatments improve survival. In 2010, the FDA approved cabazitaxel (Jevtana) as the second-line treatment for men with mCRPC, providing a new avenue for these patients during or after treatment with docetaxel chemotherapy (8). Even with cabazitaxel therapy, most patients ultimately become chemotherapy-resistant, and their treatment remains a major challenge. Thus, limited options for the management of advanced prostate cancer call for new and more effective and improved treatment approaches. Combination chemotherapy is a widely used paradigm for improving efficacy of individual drugs in the management of numerous human malignancies. It is argued that the management of cancer is more feasible and holds better promise with the use of drug combinations that can hit multiple targets (8–11). Combination chemotherapy has several advantages, such as low-dose requirement, which subsequently lessens the side effects and circumvents drug resistance and inhibits or delays metastasis (12–18). Zhang and colleagues (13) showed that genistein enhances the response to cabazitaxel treatment in mCRPC cells. Synergistic drug interactions act in concert to reduce long-term clonogenic survival and inhibit oncogenic and metastatic pathways (19–21).

Fisetin, a dietary tetrahydroxylflavone that belongs to the flavonoid group of polyphenols, is present in many vegetables and fruits and has been found to inhibit multiple oncogenic pathways both in vitro and in vivo in many different types of cancer (22–24). The development of new agents, such as fisetin, could provide more effective therapeutic options for prostate cancer patients. We previously reported that fisetin is a microtubule-stabilizing agent...
that significantly inhibits prostate cancer cell proliferation, migration, and invasion (25). Similarly, Haddad and colleagues (26) observed a decrease in proliferation with concomitant induction of apoptosis in prostate cancer cells upon fisetin treatment. We further observed that fisetin inhibited mTOR complexes 1 and 2 and suppressed Cap-dependent translation (27). In separate studies from our laboratory, we observed that fisetin acts as a dual inhibitor of PI3K/Akt and mTOR pathways in non–small cell lung cancer cells (28). This appears to be an exciting observation as both Akt and mTOR pathways are among the major signaling networks that have been implicated in advanced cancer. Using in silico modeling, we showed that fisetin interacts with mTOR at two sites, thereby explaining its inhibitory effect on cellular growth and proliferation (28). NudC, a protein associated with the microtubule motor dynein/dynactin complex that regulates microtubule dynamics, was inhibited by fisetin treatment (25). We have also shown that fisetin treatment in athymic nude mice implanted with androgen receptor (AR)–positive CWR22Rv1 human prostate cancer cells, inhibited tumor growth, and reduced serum PSA levels. Taking advantage of this finding, we showed that fisetin acts as an inhibitor of AR signaling and suggested that it could be a useful chemopreventive and chemotherapeutic agent against prostate cancer (22). A recent study showed that the combination of paclitaxel and fisetin induces mitotic catastrophe and autophagic cell death in the in vitro model of A549 non–small cell lung cancer cells (29). We hypothesized that lower dose of fisetin will enhance the efficacy of cabazitaxel against advanced and metastatic human prostate cancer cells. Many anti-tubulin agents are substrates of P-glycoprotein (P-gp), a broad-spectrum ATP-dependent efflux pump that reduces the efficacy of anticancer drugs. As a result, higher doses of these drugs are required to achieve adequate intracellular concentration in multidrug-resistant cancer cells.

The overall goal of this study was to determine whether fisetin would increase the chemosensitivity of prostate cancer cells to cabazitaxel for the purpose of reducing cabazitaxel dosage and toxicity and increasing its effectiveness in overcoming drug resistance. Combined fisetin and cabazitaxel treatment synergistically inhibited the growth of 22Rv1 cells, decreased the expression of proliferative markers (PCNA and Ki67) and the antiapoptotic markers (Bcl-2) and induction of the apoptotic marker (Bax). In addition, fisetin and cabazitaxel treatment inhibited metastases of PC-3M-luciferase androgen-independent cells.

Materials and Methods

Materials

Thiazolyl blue tetrazolium blue (MTT) was purchased from Sigma-Aldrich. Bax, Mcl-1, Bcl-2, Ki67, and PCNA antibodies and Annexin V-FITC Staining Kit were obtained from Cell Signaling Technology. Anti-keratin and anti-rabbit secondary antibodies and horse radish peroxidase (HRP) conjugate was obtained from Amersham Life Science Inc. Fisetin was purchased from Sigma Chemical Co. Cabazitaxel was purchased from LC Laboratories. BCA Protein Assay Kit was obtained from Pierce.

Cell culture and treatment

Human prostate cancer PrEC, 22Rv1, and C4-2 cells were obtained from ATCC in August 2012 and authenticated using a multiplex PCR Amplification Kit (PowerPlex 16 HS System, Promega). PC-3M-luc-6 cells were obtained from PerkinElmer in 2013, used within 6 months, and not authenticated further. NCI/ADR-RES cells were obtained from the NCI (Bethesda, MD) in 2012 and were not authenticated further. The cells were cultured in RPMI1640, F12K, or HBSS medium supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were maintained under standard cell culture conditions at 37°C and 5% CO2, in a humid environment. Fisetin and cabazitaxel were dissolved in DMSO (stock concentration, 10 mmol/L). Cells (60%–70% monolayer confluent) were treated with fisetin and after 48 hours treated with cabazitaxel in complete growth medium.

Cell viability

Cells were seeded (6 × 105 cells/2 mL) in 12-well plates (24 hours) and treated with fisetin (0–40 μmol/L) for 48 hours or cabazitaxel (0–40 mmol/L) for 24 hours. For the combination treatment, cells were incubated with fisetin for 48 hours and then with cabazitaxel for an additional 24 hours. Cell viability was determined by MTT according to the manufacturer’s protocol. Briefly, after treatment, cells were then treated with media containing MTT solution (1 mg/mL) for 3 to 4 hours. Afterward, the MTT solution was removed, and the purple crystalline precipitate internalized by the cells was dissolved in DMSO. Finally, plates were placed in a plate reader to measure absorbance at 570 nm.

Colonony formation assay

Cells were seeded (500–1,000 cells/5 mL) in petri dish plates for 24 hours. Cells were treated with fisetin and after 48 hours with cabazitaxel and allowed to form colonies in 5 to 10 days. Colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v), and photographs were taken at 40× magnification.

Western blotting

For immunoblotting, 30 to 40 μg protein was resolved over 8% to 12% polyacrylamide gels and transferred to nitrocellulose membrane. The blot was incubated in blocking buffer (5% nonfat milk, 1% Tween 20 in 20 mmol/L TBS, pH 7.6) for 1 hour at room temperature. The membrane was incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer followed by incubation with anti-mouse or anti-rabbit secondary antibody HRP conjugate and detected by chemiluminescence and autoradiography using Bio-Rad Gel-Doc.

Apoptosis assessment by Annexin V-FITC staining

The Annexin V-FITC Staining Kit was used to identify apoptotic cells. Cells were treated with fisetin for 48 hours and then with cabazitaxel for 24 hours, washed, centrifuged, and the cell pellet suspended in Annexin V-FITC binding buffer. Cell suspensions were then incubated with Annexin V-FITC conjugate and propidium iodide solution. Data were collected on a Becton Dickinson FACScalibur and analyzed in FlowJo, Version 9.7 (FlowJo, LLC).

In vivo tumor xenograft study

Two xenograft mouse models were used to assess tumor growth, invasion, and metastasis in vivo. In the first model, a total of 24 athymic nude male mice 6 to 8 weeks old were injected subcutaneously with 1 × 106 22Rv1 cells. In the second model, 24 athymic nude male mice 6 to 8 weeks old were injected subcutaneously with 3 × 106 PC-3M-luc-6 cells. Two weeks later, tumor-
bearing mice were randomly divided into four groups \((n = 6)\) and treated intraperitoneally with (i) fisetin \((20 \text{ mg/kg}; \text{three times/week})\); (ii) cabazitaxel \((3 \text{ mg/kg}; \text{once/week})\); (iii) the combination of fisetin \((20 \text{ mg/kg}; \text{three times/week})\) and cabazitaxel \((3 \text{ mg/kg}; \text{once/week})\); or (iv) vehicle (control).

**Immunohistochemical analysis**
Sections (5-mm thick) were cut from paraffin-embedded tumor tissues. Immunostaining was performed using specific antibodies with appropriate dilutions. The slides were developed in diaminobenzidine and counterstained with a hematoxylin stain. The stained slides were dehydrated and mounted in Permount and visualized on Nikon Eclipse IT system (Nikon Instruments, Inc.). Images were captured with an attached camera linked to a computer.

**Bioluminescent imaging**
In-vivo bioluminescent imaging was performed with an IVIS Imaging System (Xenogen). Animals were placed onto the warmed stage inside the camera box and received continuous exposure to 1% to 2% isoflurane to sustain sedation during imaging. Imaging times ranged from 1 second to 2 minutes, depending on the bioluminescence, and 3 to 5 mice were imaged at a time. Regions of interest (ROI) from displayed images were drawn around the tumor sites and quantified as photons/second using the Living Image software (Xenogen). Background in vivo bioluminescence was measured as approximately \(1 - 2 \times 10^7\) photons/s for similarly sized ROIs at non-tumor sites of mice.

**Ex vivo imaging**
\(\beta\)-Luciferin \((150 \text{ mg/kg})\) was injected into the mice immediately prior to necropsy. Animals were humanely sacrificed and tissues of interest were removed, placed into separate petri dish culture plate, and imaged for 1 to 2 minutes. Tissues were subsequently fixed in 10% formalin (Sigma) and prepared for standard histopathology evaluation.

**Statistical analysis**
Results were analyzed using a two-way ANOVA to assess statistical significance and \(P\) values <0.05 were considered significant.

**Synergistic quantification of drug combination**
Drug interactions were analyzed by the combination index (CI) method developed by Chou (30) using the CompuSyn software (Biosoft). CI <0.9 indicates synergism, 1.1 additive, and >1.1 antagonism.

**Results**
Effect of treatment with fisetin, cabazitaxel, and their combination on cell viability and colony formation in prostate cancer cells
To determine whether fisetin enhances cabazitaxel sensitivity in prostate cancer cells in vitro, cell viability was determined using normal human prostatic epithelial cells (PrEC) and several prostate cancer cell lines, including 22Rv1, PC3M-luc-6, and C4-2 cells. As shown in Fig. 1A, fisetin (20 \(\mu\text{mol/L}\)), cabazitaxel

![Figure 1](https://example.com/figure1.png)

**Figure 1.**
Effect of fisetin, cabazitaxel, and their combination in vitro. Representative histogram images of prostate cancer cells showing cell viability assessed by MTT assay with fisetin \((20 \mu\text{mol/L})\), cabazitaxel \((5 \text{ nmol/L})\), and the combination (fisetin 20 \(\mu\text{mol/L}\) and cabazitaxel 5 \(\text{nmol/L}\)). A–D, PrEC cells (A), 22Rv1 cells (B), PC-3M-luc-6 cells (C), and C4-2 cells (D). E, Representative photograph showing the effect of fisetin \((20 \text{ \(\mu\text{mol/L}\})\) and cabazitaxel \((5 \text{ \(\text{nmol/L}\})\) treatment on the growth of PC-3M-luc-6 cells investigated by monolayer colony formation assay. Data are shown as mean ± SD of at least three independent experiments performed in triplicate. **+++**, \(P \leq 0.002\); NS, not significant.
Table 1. CI to assess the degree of drug combination using the Compusyn software.

<table>
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<tr>
<th>Cell lines</th>
<th>Treatment</th>
<th>$D_{50}$</th>
<th>Parameters</th>
<th>CI value</th>
<th>DRI value</th>
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<tr>
<td>22Rv1</td>
<td>Fisetin</td>
<td>27.0305</td>
<td>$-0.7615 \pm 0.1735$</td>
<td>$-0.9753$</td>
<td>8.34449</td>
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<td></td>
<td>Cabazitaxel</td>
<td>7.71559</td>
<td>$-2.193 \pm 0.18013$</td>
<td>$-0.9964$</td>
<td>2.96809</td>
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<tr>
<td></td>
<td>Combination (4,000:1)</td>
<td></td>
<td></td>
<td>0.45676</td>
<td></td>
</tr>
<tr>
<td>PC-3M-luc-6</td>
<td>Fisetin</td>
<td>0.24692</td>
<td>$-0.353 \pm 0.01560$</td>
<td>$-0.9990$</td>
<td>1.35079</td>
</tr>
<tr>
<td></td>
<td>Cabazitaxel</td>
<td>0.02675</td>
<td>$-0.1611$</td>
<td>$-1.0000$</td>
<td>158.195</td>
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<tr>
<td></td>
<td>Combination (4,000:1)</td>
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<td></td>
<td>0.74663</td>
<td>1.39825</td>
</tr>
<tr>
<td>C4-2</td>
<td>Fisetin</td>
<td>3.6264</td>
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<td>$-0.9784$</td>
<td>141.146</td>
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<td>$-0.2726 \pm 0.10660$</td>
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<td></td>
<td>0.72226</td>
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NOTE: Fisetin (20 μmol/L), cabazitaxel (5 nmol/L), and the combination (fisetin 20 μmol/L and cabazitaxel 5 nmol/L) synergistically inhibit cell viability in 22Rv1, PC-3M, and C4-2 cell lines. Slope ($m$), correlation coefficients ($r$), and median-effect dosages ($D_{50}$) from median-effect plots, CIs and dose reduction indexes (DRI) for fisetin alone, cabazitaxel alone, and fisetin and cabazitaxel combination treatment.

(5 nmol/L), and their combination had minimal effects on the viability of normal PrECs. In contrast, we observed 45%, 49%, and 74% decreased cell viability with fisetin and 32%, 11%, and 38% decreased cell viability with cabazitaxel in 22Rv1, PC-3M-luc-6, and C4-2 cells, respectively. Fisetin and cabazitaxel in combination reduced viability by approximately 79%, 53%, and 78%, respectively (Fig. 1B–D).

A clonogenic assay was used for studying the effectiveness of fisetin, cabazitaxel, and their combination on the survival and proliferation of prostate cancer PC-3M-luc-6 cells. Combined treatment with fisetin (20 μmol/L) and cabazitaxel (5 nmol/L) significantly suppressed colony formation compared with treatment with either agent alone (Fig. 1E). Isobologram analysis suggested that the interaction between fisetin (20 μmol/L) and cabazitaxel (5 nmol/L) was highly cell dependent. For example, the synergism (CI < 1) was the most significant in 22Rv1 (CI = 0.45), whereas in PC-3M-luc-6 (CI = 0.75) and in C4-2 cells (CI = 0.72) as shown in Supplementary Fig. S1 and Table 1. These observations demonstrate that fisetin synergistically and significantly enhanced the sensitivity of prostate cancer cells to cabazitaxel treatment and inhibited cell viability and the long-term clonogenic growth of prostate cancer cells.

Effect of treatment with fisetin, cabazitaxel, and their combination on cellular proliferation and apoptosis in prostate cancer 22Rv1 cells

To investigate whether apoptosis is induced by the combination of fisetin and cabazitaxel, we examined the activation of PARP, a key effector molecule of apoptosis. Our results showed that fisetin and cabazitaxel, alone increased cleavage of PARP, whereas the combination significantly ($P \leq 0.0001$) increased the expression of cleavage of PARP compared with the control, or each agent alone (Fig. 2A). Furthermore, to understand the molecular basis of the effects of the combination, we studied several molecules involved during the initiation and execution of apoptosis. Levels of proapoptotic Bax were increased by fisetin and cabazitaxel, and this increase was augmented by the combination treatment (Fig. 2B). At the same time, levels of antiapoptotic Mcl-1 decreased significantly ($P = 0.0005$). Modulation in the expression of Bax and Mcl-1 by the combination resulted in an increased Bax:Mcl-1 ratio in a way that favored apoptosis ($P = 0.001$, Fig. 2B). These data indicate that the combination was significantly effective than fisetin and cabazitaxel alone in the induction of apoptosis.

Overexpression of proliferating cell nuclear antigen (PCNA) and Ki67 are considered prognostic biomarkers for various types of cancers, including prostate cancer. Thus, to determine the potency and putative antiproliferative effect of fisetin, cabazitaxel, and the combination treatment in vitro, we used PCNA protein expression as a marker. The level of PCNA expression was inhibited in the combination treatment compared with the effects of each agent alone (Fig. 2C).

Effect of treatment with fisetin, cabazitaxel, and their combination on the growth of 22Rv1 tumors in athymic nude mice and on overall survival

We next evaluated the effect of fisetin, cabazitaxel, and the combination on tumor growth in a xenograft mouse model. We observed that treatment with fisetin alone resulted in 22% inhibition of tumor growth; cabazitaxel treatment alone resulted in 31% inhibition, whereas fisetin and cabazitaxel in combination resulted in 53% inhibition of tumor growth compared with the control group (Fig. 3A).

The outcome of any chemotherapeutic agent is evaluated on the basis of its ability to increase overall survival. We, therefore, evaluated the effect of the combination on overall survival in mice over the course of treatment. Compared with the expected median survival of 55 days in the combination treatment group, cabazitaxel and fisetin alone resulted in median life expectancy of 33 and 31 days, respectively (Fig. 3B). This significant increase in overall survival clearly suggests that the combination possesses a high potential for prostate cancer survival.

To explore cellular mechanisms that could account for the tumor inhibition by the combination, we assessed tumor cell proliferation using PCNA and Ki67 expression and apoptosis using Bax and Bcl-2. Cabazitaxel alone decreased PCNA and Ki67 by 47%, increased Bax by 75%, and decreased Bcl-2 by 55% in the 22Rv1 xenografts (Fig. 3C). The combination reduced PCNA and Ki67 expression by 75% and Bcl-2 by 88%, whereas it increased Bax expression by 80% (Fig. 3C). Furthermore, these findings suggest that fisetin and cabazitaxel inhibit tumor growth through a combination of decreasing proliferation and inducing apoptosis. Taken together, in vivo studies indicate that combined treatment with low dose of fisetin and cabazitaxel is more effective in suppressing tumor growth than either agent alone.

Effect of treatment with fisetin, cabazitaxel, and their combination on the growth of metastatic PC-3M-luc-C6 tumors in athymic nude mice

To precisely monitor the effects of fisetin, cabazitaxel, and the combination on tumor growth in live mice, cells were implanted subcutaneously in 24 mice.
Three days after PC-3M cell implantation, mice were randomly divided into four groups. Treatments were given up to 7 weeks and stopped 24 hours before euthanizing the animals. No apparent toxicity was observed in any of the combination-treated mice as observed using the body weight (data not shown). Combination treatment with fisetin and cabazitaxel significantly ($P=0.0001$) inhibited tumor growth compared with control and individual treatment groups (Fig. 4A and B). We observed only a 9-fold increase in tumor volume in the combination group over 7 weeks, whereas fisetin or cabazitaxel alone resulted in 22- and 20-fold increase, respectively, and tumor volume in the control group increased by 32-fold. In addition, we also observed that treatment with fisetin and cabazitaxel alone resulted in 18% and 29% inhibition in tumor growth, respectively. However, combination of fisetin and cabazitaxel resulted in 81% inhibition in tumor growth. Overall, the effects of the combined treatments were highly significant than control or each agent alone (Fig. 4C).

To determine the effects of fisetin, cabazitaxel alone, and the combination treatment on prostate cancer metastasis, we excised distant organs (lymph nodes, liver, kidneys, lungs, and heart) from each group and subjected them to ex vivo imaging. Mice from the control group showed high incidence of metastasis into distant organs, which was reduced in fisetin, cabazitaxel alone, and in the combination-treated mice. As shown in Supplementary Fig. S2, increased bioluminescence signal intensity was observed in the lymph nodes, liver, kidneys, lungs, and heart (Supplementary Fig. S2A) of control mice, which was significantly decreased in the excised liver ($P=0.05$) from the combination-treated mice (Supplementary Fig. S2B), but not significantly decreased in lymph nodes and lungs.

**Figure 2.**
Effect of treatment with fisetin, cabazitaxel, and their combination on markers of proliferation and apoptosis. **A–C,** Representative blots showing the effect of fisetin (20 µmol/L), cabazitaxel (5 nmol/L), and the combination (fisetin 20 µmol/L and cabazitaxel 5 nmol/L) treatment on cleavage of PARP (C. PARP), BAX, Mcl-1, and PCNA proteins, respectively, in 22Rv1 cells. Data are shown as representative of three independent experiments. GAPDH served as a loading control. Intensities of the bands were measured using densitometry and values first normalized to respective GAPDH and then reported below each gel as relative to control. Data are shown as mean ± SD of at least three independent experiments performed in triplicate; *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$. 
Activation of matrix metalloproteinase (MMP), urokinase plasminogen activator (uPA), and angiogenic factors (VEGF and CD31) is shown to promote prostate cancer metastasis into distant organs (31–33). A possibility was explored whether fisetin, cabazitaxel, alone and in combination inhibit the expression of MMP2, MMP9, and CD31 in PC-3M-luc-6 cells. Western blot analysis indicated a significant decrease in MMP2 and MMP9 expression in PC-3M-luc-6 cells with combination treatment (Fig. 5A and B). Similarly, combination treatment resulted in decreased expression of CD31 (Fig. 5C). We further examined the effect of combination treatment on other metastatic markers, such as uPA, and observed a decrease in expression in the combination-treated PC-3M-luc-6 cells (Fig. 5D).

Effect of treatment with fisetin, cabazitaxel, and their combination on tumor growth and metastasis in mice implanted with 22Rv1 cells. A, Line graph showing tumor volume growth determined by weekly measurements. Each value in the graph is the mean ± SE from 6 mice. ***, P < 0.0005 was considered as significant. B, Line graph showing survival analysis. C, At the end of the study, tumors were harvested from mice and subjected to IHC for proliferation (anti-Ki67) and (anti-PCNA) apoptosis (anti-BAX and anti-Bcl-2). Left, representative photomicrographs of Ki67, PCNA, BAX, and Bcl-2-stained tumor section (40×); right, quantitation of Ki67, PCNA, BAX, and Bcl-2; ***, P ≤ 0.0001.
combination had a greater effect on these parameters than either agent alone.

The MMPs are an important component of cell invasion capable of degrading a range of extracellular matrix proteins, allowing cancer cells to immigrate and invade. Accordingly, we examined proteins that enhance the metastatic process. Fisetin and cabazitaxel alone decreased the expression of MMP-9 protein by 45% and 50%, respectively (Fig. 6), whereas the combination treatment decreased the expression of MMP-9 protein by 90%. These findings suggest that the combination inhibited tumor growth in a multipronged manner through decreasing proliferation, inducing apoptosis, and blocking tumor angiogenesis and metastasis.

Effect of treatment with fisetin, cabazitaxel, and their combination on cellular proliferation and apoptosis in drug-resistant NCI/ADR-RES cells

One of the widely investigated mechanisms of taxane resistance is increased efflux of drug via the P-gp pump. Cancer cells with multidrug resistance (MDR1) gene expression reduce the intracellular concentration of taxanes by increasing drug efflux through P-gp. We examined the susceptibility to fisetin and cabazitaxel treatment in a P-gp–overexpressing NCI/ADR-RES cell line. We previously reported (25) that treatment of NCI/ADR-RES cells with fisetin results in decreased cell viability and colony formation. We investigated whether fisetin could sensitize this resistant cancer cell line to cabazitaxel treatment. As shown in Fig. 7A, combined fisetin and cabazitaxel treatment significantly inhibited colony formation of these cells as compared with either agent alone.

We compared the induction of apoptosis by fisetin (20 μmol/L), cabazitaxel (5 nmol/L), and the two drugs in the combination in NCI/ADR-RES cells. Cells treated with fisetin and then cabazitaxel were more sensitive and underwent apoptosis (42.5%) more than cells that were treated with cabazitaxel (3.37%) or fisetin (14%) alone (Fig. 7B); these results support the suggestion that fisetin is a key contributor to the cytotoxic effects of cabazitaxel treatment in NCI/ADR-RES cells. Fisetin decreased the expression of P-gp, which would suggest decreased ability of these cells to actively efflux drugs. These findings highlight the novel effect of fisetin as a microtubule-stabilizing agent and provide evidence that fisetin could be further developed to sensitize resistant cancer cells and used in combination with cabazitaxel for therapy of prostate cancer and potentially other cancer types. Regulation of P-gp is critical to the maintenance of intracellular concentration of taxanes and...
regulating drug efflux. We asked whether fisetin treatment downregulated P-gp expression in response to cabazitaxel treatment. Marked downregulation of P-gp protein is shown in Fig. 7C in response to the combination treatment of the NCI/ADR-RES cells. These data support the conclusion that fisetin treatment renders NCI/ADR-RES cells more sensitive to cabazitaxel treatment.

**Discussion**

Taxanes have proven to be one of the best antitumor agents in the clinic. Their enhanced clinical activity against a variety of human malignancies has encouraged scientists to continue investigation of this class of drugs. However, their inherent shortcomings in terms of both toxicity and resistance quickly become apparent. Continued investigation into the development and discovery of new drugs and exploring new treatment strategies that reduce side effects and circumvent drug resistance may provide more effective therapeutic options for cancer patients. Considering these facts, we believe that natural agents from dietary sources, such as fisetin, could offer a safe and effective alternative to existing repertoire of microtubule-targeting agents in combination with standard of care.

In line with this, our study was designed to investigate the effect of a combination of fisetin and cabazitaxel to achieve maximum therapeutic benefit with the intention that this approach would eventually reduce drug dose and toxicity and minimize or delay the induction of drug resistance. In this study, we demonstrate the effect of fisetin alone and in combination with cabazitaxel in cell culture and in vivo in a subcutaneous xenograft mouse model. Combination-mediated antitumor activity was associated with both inhibition of proliferation and promotion of apoptosis. Furthermore, combination resulted in the inhibition of tumor invasion and angiogenesis.

Figure 5.
Effect of treatment with fisetin, cabazitaxel, and their combination on metastasis and angiogenesis in a subcutaneous PC3M-luc-6 xenograft model. Representative blots showing the effect of fisetin and cabazitaxel treatment on metastasis. **A**-**D**, Anti-MMP-2 (**A**); anti-MMP-9 and angiogenesis (**B**); anti-CD31 activity (**C**), and anti-uPA (**D**). Data are shown as representative of three independent experiments. GAPDH served as a loading control. Bands were measured using densitometry and values first normalized to respective GAPDH bands and then reported below each gel as relative to control. *, *P* ≤ 0.05; ***, *P* ≤ 0.001 were considered as significant.

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invasion and metastasis. These results provide evidence that (i) fisetin may have therapeutic benefit for patients with prostate cancer through enhancing the efficacy of cabazitaxel; (ii) fisetin enhances the efficacy of cabazitaxel-induced apoptosis in androgen-dependent and androgen-independent prostate cancer cells; and (iii) several potential cellular and molecular mechanisms inhibit the proliferation of tumor, block tumor angiogenesis, coupled with induction of apoptosis.

The addition of fisetin to cabazitaxel resulted in enhanced anticancer activity in both 22Rv1 and C4-2 cells and the metastatic PC-3M-luc-6 cells. Taxanes are known to mediate their anticancer activity through two key mechanisms: (i) the inhibition of microtubule depolymerization and (ii) the attenuation of the antiapoptotic activity of BCL-2 and BCL-XL (34). Cabazitaxel-mediated inhibition of microtubule depolymerization induces cell-cycle arrest at G2–M phase. We reported that fisetin is a microtubule-stabilizing agent that binds to β-tubulin and significantly affects microtubule dynamics (25). In addition, we reported that fisetin inhibits cellular proliferation, migration, and invasion of prostate cancer cells (25). This suggested that the combination of two agents may complement each other and result in enhanced anticancer activity. We concluded from our docking analysis that fisetin localizes within the taxol-binding pocket (25). It remains unclear whether fisetin competes with other taxanes binding to microtubules. Further elucidation of these differences will validate these findings. Although both agents enhanced the polymerization of purified tubulin, their mechanisms of action differ. Fisetin acts as a potent antiproliferative agent, whereas cabazitaxel is a potent apoptotic agent. A combination of both agents induced an enhanced effect. This implies that fisetin or cabazitaxel has additional targets that are independent of tubulin binding. This finding is in agreement with a previous study that observed synergistic interaction between taxol and discodermolide in four human carcinoma cell lines. However, in that study, both agents were microtubule-stabilizing agents, formed polymers that are stable to cold and calcium, and

Figure 6.
Effect of treatment with fisetin, cabazitaxel, and their combination on proliferation, apoptosis, and tumor angiogenesis in a subcutaneous PC3M-luc-6 xenograft model. At the end of the study, tumors were harvested from mice, subjected to IHC for proliferation (anti-Ki67 and anti-PCNA), metastasis (anti-MMP-9), and angiogenesis (anti-CD31 and anti-VEGF). Left, representative photomicrographs of Ki67, PCNA, MMP-9, CD31, and VEGF-stained tumor section (40×). Right, quantitation of Ki67, PCNA, MMP-9, CD31, and VEGF; *** P < 0.001.
caused cell-cycle arrest. In addition, discodermolide competitively inhibits the binding of paclitaxel to tubulin polymers (35).

Tumor growth is composed of a balance between cell proliferation and cell apoptosis. These activities are regulated by many factors, such as the BCL-2 family, Bax, and PARP. It is well known that fisetin interacts with many different signaling cascades to regulate critical cellular processes (24, 36). Fisetin has been reported to induce cell-cycle arrest (37), induce apoptosis via inhibiting antiapoptotic proteins, and impair phosphorylation of MEK and AKT in various cancer cells (23). Several preclinical and clinical trials have reported on the promising combined treatment of microtubule-stabilizing agents with mTOR inhibitors and reported enhanced activity linked to increased tumor cell apoptosis (38–41). A study reported that the addition of a LY294002, a PI3K inhibitor, to tamoxifen and everolimus, an mTOR inhibitor, improved the antitumor effect compared with tamoxifen alone or the other two agents in combination. The triple treatment had the greatest efficacy in inhibiting MCF-7 tumor growth and angiogenesis (42). Likewise, fisetin is known as a dual inhibitor of both PI3K/Akt pathways (28) and also an inhibitor of the mTOR pathway (22, 37). Our results suggest that the anticancer effects of the combination treatment in prostate cancer involve multiple mechanisms that culminate in an overall inhibition of tumor growth through both inhibition of proliferation and induction of apoptosis. In terms of affecting apoptosis, our results showed that fisetin and cabazitaxel combination is associated with an increased proapoptotic/antiapoptotic ratio that favors apoptosis.

Multiple signaling pathways provide cross-talk between the epithelial and the stromal compartments to enhance tumor growth, including AR signaling. Disrupting this "two-compartment" cross-talk has led to the development of drugs that target tumor stromal elements in addition to cancer cells. Impairment of microtubule function leads to decreased AR nuclear translocation (43). This mechanism is independent of taxane-induced mitotic arrest and could provide an alternative mechanism of drug action that could explain its clinical activity. Our laboratory also reported fisetin inhibits AR signaling in androgen-dependent prostate cancer cells (22). The strong in vivo treatment responses observed in our study demonstrate that the combination inhibited AR signaling, that is known to promote tumor growth. Chien and colleagues reported that fisetin has inhibitory effects on the adhesion, migration, and invasion ability of highly metastatic PC-3 cells. Further, results from this study demonstrated that fisetin inhibited MMP-2 and MMP-9 expression and activity through suppressing PI3K/Akt and JNK signaling pathways. This suggested that fisetin can serve as a potential candidate for treating cancer metastasis (44).

Figure 7.
Effect of treatment with fisetin, cabazitaxel, and their combination on colony formation, apoptosis, and P-gp in drug-resistant NCI/ADR-RES cells. A, Representative photographs showing the effect of fisetin treatment on NCI/ADR-RES cell growth investigated by monolayer colony formation assay. B, The induction of apoptosis in NCI/ADR-RES cells was assessed by flow cytometry using a FITC-conjugated Annexin V (x-axis) marker coupled with propidium iodide (PI, y-axis) exclusion; bottom, quantitation of apoptosis; *** P < 0.001. C, Representative blots showing the effect of fisetin and cabazitaxel treatment on P-gp activity. Data are shown as representative of three independent experiments. GAPDH served as a loading control. Bands were measured using densitometry and values first normalized to respective GAPDH bands and then reported below each gel as relative to control. Data are shown as representative of three independent experiments for each assay.
Tumor-induced angiogenesis is a major contributor to tumor growth. Therefore, blocking angiogenesis is an archetypal stromal-targeting strategy that has proven to be successful in treating a variety of different metastatic tumor types (45). The study by Broggini-Tenzer and colleagues demonstrated that specific downregulation of proangiogenic VEGF signaling by mTOR inhibition or VEGF neutralization with clinically approved agents strongly re sensitized patupilone-resistant lung adenocarcinoma cell–derived tumors to patupilone (14). Likewise, taxane-resistant colon carcinomas, which are MDR overexpressing, were re sensitized upon VEGF deprivation (46). Similarly, fisetin has been reported to inhibit the angiogenesis of many tumors (47). Our results suggest that fisetin and cabazitaxel treatment reduce microvessel density through downregulation of VEGF and CD31 activity. We speculate that fisetin could have inhibited angiogenesis through decreasing NF-κB signaling, which is known to contribute to angiogenesis. This is consistent with previous results from our laboratory that showed that fisetin induced apoptosis and decreased invasion of chemoresistant AsPC-1 pancreatic cancer cells through suppression of DR3-mediated NF-κB activation (48). In addition, our laboratory also provided evidence that fisetin can induce apoptosis and suppress the growth of colon cancer cells by inhibition of COX2 and Wnt/EGFR/NF-κB signaling pathways (49). Another study reported that fisetin was potent in suppressing TNF-induced NF-κB activation. The expression of NF-κB–regulated gene products involved in antiapoptosis (Bcl-2, Bcl-xl, XIAP, and Survivin), proliferation (cyclin D1, c-Myc, and COX-2), invasion (MMP-9), and angiogenesis (VEGF) were also downregulated by fisetin (50). Our observations support that the combination treatment affects tumor vasculature. Therefore, the suppression of tumor growth and metastasis occurred not only through the inhibition of proliferative activity of tumor cells but also through inhibition of microvessel density. These experiments have several limitations. We used subcutaneous, not orthotopic, injection to model soft tissue growth. Although orthotopic injection is ideal, it is challenging to model, as mice have four biologically different and anatomically separated lobes of prostate. It is not clearly defined how well any of them recapitulates the human prostate.

In summary, this research identified that fisetin enhanced cabazitaxel-mediated cytotoxicity, suggesting that fisetin could be developed as a safe and effective agent to improve cabazitaxel chemotherapy in prostate cancer and potentially other cancer types.

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

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