Abstract

Docetaxel is the first-line standard treatment for castration-resistant prostate cancer. However, relapse eventually occurs due to the development of resistance to docetaxel. To unravel the mechanism of acquired docetaxel resistance, we established docetaxel-resistant prostate cancer cells, TaxR, from castration-resistant C4-2B prostate cancer cells. The IC_{50} for docetaxel in TaxR cells was about 70-fold higher than parental C4-2B cells. Global gene expression analysis revealed alteration of expression of a total of 1,604 genes, with 52% being upregulated and 48% downregulated. \textit{ABCB1}, which belongs to the ATP-binding cassette (ABC) transporter family, was identified among the top upregulated genes in TaxR cells. The role of \textit{ABCB1} in the development of docetaxel resistance was examined. Knockdown of \textit{ABCB1} expression by its specific shRNA or inhibitor resensitized docetaxel-resistant TaxR cells to docetaxel treatment by enhancing apoptotic cell death. Furthermore, we identified that apigenin, a natural product of the flavone family, inhibits \textit{ABCB1} expression and resensitizes docetaxel-resistant prostate cancer cells to docetaxel treatment. Collectively, these results suggest that overexpression of \textit{ABCB1} mediates acquired docetaxel resistance and targeting \textit{ABCB1} expression could be a potential approach to resensitize docetaxel-resistant prostate cancer cells to docetaxel treatment. \textit{Mol Cancer Ther}; 12(9); 1–8. ©2013 AACR.

Introduction

Prostate cancer is the most common diagnosed cancer and second most frequent cause of cancer-related death among the men in the United States. Most of the patients with prostate cancer will initially respond to androgen deprivation therapy. However, almost all of them will relapse due to development of castration-resistant prostate cancer (CRPC; refs. 1, 2). Docetaxel is the first-line standard treatment for CRPC. Docetaxel is a cytotoxic antimitotube agent that binds to β-tubulin and prevents microtubule depolymerization, resulting in inhibition of mitotic cell division which leads to apoptotic cell death (3). However, relapse eventually occurs due to the development of resistance to docetaxel.

The molecular mechanisms of the acquired docetaxel resistance in prostate cancer cells are incompletely understood. Studies to understand the underlying mechanisms of docetaxel resistance have uncovered several potential mechanisms of docetaxel resistance in prostate cancer (4, 5). Alterations of β-tubulin isotypes, especially the increase in isotypes III and IV, has been shown to be correlated with docetaxel resistance (6). Alterations of cell survival factors that inhibit chemotherapy-induced apoptotic cell death are associated with docetaxel resistance (7). Several different groups have reported that overexpression of Bcl-2 (8) and induction of clusterin by pAkt (9, 10) are related to docetaxel resistance in prostate cancer. Aberrant activation of central transcriptional factors such as NF-κB also plays an important role in the development of resistance. Recent studies have shown that inhibition of NF-κB resensitizes docetaxel-resistant PC-3 cells to taxane-induced apoptosis (11). Furthermore, reduced intracellular drug concentration through alteration of multidrug resistance (MDR) genes is another mechanism associated with the acquired resistance to chemotherapy (12).

To further understand the molecular mechanisms of the acquired docetaxel resistance and explore potential therapeutic strategies for docetaxel-resistant CRPC, we generated docetaxel-resistant prostate cancer cells from castration-resistant C4-2B prostate cancer cells. We identified \textit{ABCB1} gene upregulation as a common mechanism involved in acquired docetaxel resistance. In addition, we show that apigenin, a natural product of the flavone...
family, inhibits ABCB1 expression and resensitizes docetaxel-resistant prostate cancer cells to docetaxel treatment by enhancing apoptotic cell death.

Materials and Methods

Cell culture and reagents
DU145 cells were obtained from the American Type Culture Collection (ATCC). All experiments with cell line were conducted within 6 months of receipt from ATCC or resuscitation after cryopreservation. ATCC uses short tandem repeat (STR) profiling for testing and authentication of cell lines. C4-2B cells were kindly provided and authenticated by Dr. Leland Chung (Cedars-Sinai Medical Center, Los Angeles, CA). The cells were cultured in RPMI-1640 medium containing 10% complete FBS with 100 U/mL penicillin and 0.1 mg/mL streptomycin and maintained at 37°C in a humidified incubator with 5% CO2. C4-2B cells were incubated with gradually increasing concentrations of docetaxel. Cells that survived the maximum concentration of docetaxel were stored for further analysis and referred to as TaxR cells. Parental C4-2B cells were passaged alongside the docetaxel-treated cells as an appropriate control. Docetaxel-resistant TaxR cells were maintained in 5 nmol/L docetaxel-containing medium. Docetaxel (CAS#114977-28-5) was purchased from TSZ CHEM. Apigenin (CAS#520-36-5) and Elacridar (CAS#143664-11-3) were purchased from Sigma-Aldrich. Antibodies against ABCB1, p53, phospho-p53, cleaved PARP, and GAPDH were obtained from Santa Cruz Biotechnologies.

Plasmids and cell transfection
Lentivector-based ABCB1 shRNA constructs were obtained from Open Biosystems. TaxR cells were transiently transfected with shRNA specific against ABCB1 or shGFP as vector control using Attractene transfection reagent (QIAGEN). TaxR shABC1 and vector stable clones were selected with 2.0 mM puromycin within 3 weeks after being transfected with ABCB1 shRNA or control vector and then maintained in culture medium containing 2.0 µg/mL puromycin.

Preparation of whole-cell extracts
Cells were harvested, washed with PBS twice, and lysed in high-salt buffer [10 mmol/L HEPES (pH 7.9), 0.25 mol/L NaCl, 0.1% NP-40] supplemented with protease inhibitors (Roche). Protein concentration was determined with Coomassie Plus Protein Assay Kit (Pierce).

Western blot analysis
Equal amounts of cell protein extracts were loaded on 8% or 10% SDS-PAGE, and proteins were transferred to nitrocellulose membranes. After blocking in 5% non-fat milk in 1 × PBS/0.1% Tween-20 at room temperature for 1 hour, membranes were washed three times with 1 × PBS/0.1% Tween-20. The membranes were incubated overnight with primary antibodies at 4°C. Proteins were visualized by enhanced chemiluminescence kit (Millipore) after incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies.

Real-time quantitative reverse transcription-PCR
Total RNA was extracted with TRIzol (Invitrogen) reagent. One microgram RNA was digested using RQ1 DNase (Promega). The resulting product was reverse transcribed with random primers using Im-Prom II Reverse transcriptase (Promega). The newly synthesized cDNA was used to conduct real-time PCR. The reaction mixture contained 4 µL cDNA template and 0.5 µmol/L specific primers for ABCB1 (Forward: 5’-ATGCT CTGGC CTCT GGATG GGA-3’; Reverse: 5’-ATGCG GATCC TCTGC TTCTG CCCAC-3’), GAPDH primers were used as an internal control. The expression levels of ABCB1 were normalized to GAPDH. The experiments were repeated three times with triplicates.

Cell growth assay
C4-2B and TaxR cells were seeded in 12-well plates at a density of 1 × 105 cells per well. Cells were treated as indicated and total cell numbers were counted using Coulter cell counter.

Cell death ELISA
C4-2B and TaxR cells were seeded in 12-well plates at a density of 1 × 105 cells per well and were treated as indicated. DNA fragmentation in the cytoplasmic fraction of cell lysates was determined using Cell Death Detection ELISA Kit (Roche) according to the manufacturer’s instructions. Apoptotic cell death was measured at 405 nm.

Clonogenic ability assay
C4-2B and TaxR cells were treated with dimethyl sulfoxide or different doses of docetaxel for 6 hours. A total of 1 × 105 cells were then plated in 100 mm dish for 14 days. The cells were fixed with 4% formaldehyde for 10 minutes and then stained with 0.5% crystal violet for 30 minutes, and the numbers of colonies were counted.

cDNA microarray analysis
Twenty-four hours after plating of 1 × 105 C4-2B and TaxR cells, total RNA was isolated using TRIzol Reagent (Invitrogen) and purified with Eppendorf phase-lock-gel tube. RNA quality of all samples was tested by RNA electrophoresis to ensure RNA integrity. Samples were analyzed by the Genomics Shared Resource (UC Davis Medical Center, Sacramento, CA) using the Affymetrix Human Gene 1.0 ST array. Microarray data have been deposited at GEO with the accession number GSE47040.

Statistical analyses
All data are presented as means ± SD. Differences between multiple groups were determined using one-way ANOVA followed by the Scheffe procedure for comparison of means. P < 0.05 was considered significant.
Results

Development and characterization of a docetaxel-resistant prostate cancer cell line

We previously showed that docetaxel induces p53 phosphorylation in docetaxel-sensitive LNCaP and C4-2B cells, but fails to induce p53 phosphorylation in docetaxel-resistant DU145 cells (13). To further confirm these findings, we established a docetaxel-resistant cell line, TaxR, from C4-2B cells by culturing C4-2B cells in docetaxel in a dose-escalation manner (starting from 0.1 nmol/L). After 9-month selection, cells were able to divide freely in 5 nmol/L docetaxel. To test the effect of docetaxel treatment on parental C4-2B and TaxR cell viability, cell growth assay was conducted. Both cell lines were treated with increasing concentrations of docetaxel for 24 hours. As shown in Fig. 1A, C4-2B cells are sensitive to docetaxel treatment with an IC50 of 2 nmol/L, whereas TaxR cells are much more resistant to docetaxel with an IC50 of 140 nmol/L, about 70-fold increase over parental C4-2B cells. To determine whether docetaxel induces p53 phosphorylation in TaxR cells, TaxR cells and parental C4-2B cells were treated with increasing doses of docetaxel and cell lysates were isolated for Western blot analysis. As shown in Fig. 1B, docetaxel treatment induces p53 phosphorylation in C4-2B cells but not in TaxR cells, consistent with previous report that p53 phosphorylation is associated with docetaxel sensitivity in prostate cancer cells.

The effect of docetaxel on clonogenic ability of both C4-2B and TaxR cells was determined. The clonogenic ability of TaxR cells was significantly higher than that of parental C4-2B cells in response to docetaxel treatment (Fig. 2A and B). To test the ability of docetaxel to induce apoptotic cell death in prostate cancer cells, C4-2B and TaxR cells exposed to 5 nmol/L docetaxel for 48 hours were examined by apoptosis-specific ELISA assay as described in Materials and Methods. Docetaxel at 5 nmol/L concentration induced significant apoptotic cell death in parental C4-2B cells but had little effect on TaxR cells (Fig. 2C and D).

ABCBI is overexpressed in TaxR cells

Several mechanisms have been proposed for docetaxel resistance in prostate cancer, such as alteration in β-tubulin isotypes, reduced intracellular concentration of drug through alteration of MDR genes and alteration of cell survival factors and transcription factors (6–10). To identify genes responsible for docetaxel resistance in TaxR cells, global gene expression analysis by cDNA microarrays (~28,000 genes) was conducted using mRNA from parental C4-2B and TaxR cells. Gene expression analysis revealed that a total of 1,604 genes were altered in TaxR cells with 52% being upregulated and 48% downregulated. The 1,604 genes that are altered in TaxR cells were compared with the public database generated from docetaxel-resistant DU145DR and Rv1DR cells (14). Only 9 genes altered by docetaxel were found to be overlapping in the three-gene data sets (Fig. 3A). ABCBI, which belongs to the ATP-binding cassette (ABC) transporter family, was identified among the top upregulated genes in TaxR cells, which is also among the 9 overlapped genes in the three-gene datasets. To verify microarray analysis, total RNAs were isolated from C4-2B and TaxR cells, and ABCBI mRNA level was measured using specific primers by qRT-PCR. As shown in Fig. 3B, ABCBI mRNA was highly expressed in TaxR cells but was not detectable in parental C4-2B. ABCBI protein expression was analyzed by Western blotting in whole-cell extracts of parental C4-2B and TaxR cells. Figure 3C showed that ABCBI was overexpressed in TaxR cells but was not detectable in parental C4-2B cells. These data suggested that ABCBI is overexpressed in docetaxel-resistant TaxR cells at both mRNA and protein levels.

Downregulation of ABCBI reverses docetaxel resistance

Having identified that ABCBI is overexpressed in docetaxel-resistant TaxR cells, we next tested whether overexpression of ABCBI leads to docetaxel resistance in TaxR cells. As shown in Fig. 4A, inhibition of ABCBI expression by ABCBI shRNA resensitized TaxR cells to docetaxel treatment. Figure 4B confirmed that ABCBI protein expression was knocked down by ABCBI shRNA. This observation was confirmed in another docetaxel-resistant DU145-R cell line, in which knockdown of ABCBI expression by ABCBI shRNA reversed docetaxel resistance (Supplementary Fig. S1). To further confirm that downregulation of ABCBI could restore sensitivity to docetaxel, we established stable transfectant TaxR cells expressing ABCBI shRNA and measured clonogenic ability. As shown in Fig. 4C, inhibition of ABCBI expression by ABCBI shRNA reversed resistance of TaxR cells to docetaxel treatment, as clonogenic ability of parental C4-2B cells was significantly higher than that of parental TaxR cells with ABCBI shRNA transfectant. These data suggested that ABCBI is overexpressed in docetaxel-resistant TaxR cells at both mRNA and protein levels.

Figure 1. TaxR cells are more resistant to docetaxel than parental C4-2B cells. Parental C4-2B cells and TaxR cells were treated with indicated concentrations of docetaxel in media containing complete FBS for 24 hours. A, cell growth assay. Results are expressed as the percentage of treated cells relative to untreated cells. B, docetaxel induces phospho-p53 expression in C4-2B cells but not in TaxR cells. Whole-cell lysates were collected and subjected to Western blot analysis using the indicated antibodies.

Figure 2. Docetaxel induces apoptotic cell death in C4-2B cells but not in TaxR cells. A, cell growth assay. Results are expressed as the percentage of treated cells relative to untreated cells. B, docetaxel induces phospho-p53 expression in C4-2B cells but not in TaxR cells. Whole-cell lysates were collected and subjected to Western blot analysis using the indicated antibodies.

Figure 3. Overexpression of ABCBI in TaxR cells. A, gene expression analysis by cDNA microarrays. B, ABCBI mRNA level was measured using specific primers by qRT-PCR. C, ABCBI protein expression was analyzed by Western blotting in whole-cell extracts of parental C4-2B and TaxR cells.

Figure 4. Inhibition of ABCBI expression by ABCBI shRNA resensitized TaxR cells to docetaxel treatment. A, inhibition of ABCBI expression by ABCBI shRNA resensitized TaxR cells to docetaxel treatment. B, ABCBI protein expression was analyzed by Western blotting in whole-cell extracts of parental C4-2B and TaxR cells. C, inhibition of ABCBI expression by ABCBI shRNA resensitized TaxR cells to docetaxel treatment.
ABCB1 shRNA. Two independent clones (clones No. 2 and No. 30) with ABCB1 downregulation were selected for further analysis (Fig. 4C). As shown in Fig. 4D, downregulation of ABCB1 increased the sensitivity of TaxR cells to docetaxel treatment. The IC50 of docetaxel was reduced from 140 nmol/L (vector control of parental TaxR cells) to about 20 nmol/L in both clone No. 2 and No. 30. Furthermore, we used ABCB1 inhibitor, Elacridar, to test whether inhibition of ABCB1 activity reverses docetaxel resistance in TaxR cells. As shown in Fig. 4E, treatment with 0.5 μmol/L Elacridar for 24 hours resensitized TaxR cells to docetaxel treatment, leading to both growth inhibition (Fig. 4E) and induction of apoptosis (Fig. 4F). Collectively, these data suggested that while ABCB1 overexpression enhances docetaxel resistance, inhibition of ABCB1 expression resensitizes prostate cancer cells to docetaxel treatment.

Apigenin downregulates ABCB1 expression and reverses docetaxel resistance

As downregulation of ABCB1 reverses docetaxel resistance, we attempted to search for agents that can inhibit docetaxel treatment, leading to both growth inhibition (Fig. 4E) and induction of apoptosis (Fig. 4F). Collectively, these data suggested that while ABCB1 overexpression enhances docetaxel resistance, inhibition of ABCB1 expression resensitizes prostate cancer cells to docetaxel treatment.

Figure 2. Effects of docetaxel on clonogenic ability and apoptotic cell death in TaxR cells and C4-2B cells. A, effects of docetaxel on clonogenic ability. C4-2B and TaxR cells were treated with docetaxel as indicated. After 6 hours of treatment, 1,000 cells were plated in 100 mm dish in media containing complete FBS. Visible clones were formed after 3 weeks. B, numbers of colonies were counted. C, effects of docetaxel on apoptotic cell death. C4-2B and TaxR cells were treated with 5 nmol/L docetaxel for 48 hours. Mono- and oligonucleosomes of cytoplasmic fraction were analyzed by cell death detection ELISA. D, photographs of C4-2B and TaxR cells treated with 5 nmol/L docetaxel were taken.

Figure 3. ABCB1 is overexpressed in TaxR cells. A, analysis of gene expression data sets. Genes with at least 1.2-fold increase (↑) and decrease (↓) in expression on comparison of parental C4-2B and TaxR cells are listed. B, real-time PCR analysis of ABCB1 mRNA in C4-2B and TaxR cells. C, ABCB1 protein expression. ABCB1 expression was determined by Western blot analysis using antibody specific against ABCB1.
ABCB1 expression and therefore increase docetaxel sensitivity. Apigenin (4',5,7-trihydroxyflavone), a natural product belonging to the flavone family, has the ability to modulate multidrug resistance genes and induce apoptosis in prostate cancer cells (15). We tested whether apigenin inhibits ABCB1 expression in docetaxel-resistant TaxR cells. TaxR cells were treated with increasing concentrations of apigenin. Total proteins were extracted after 48 hours of exposure and assessed by Western blot analysis. As shown in Fig. 5A, apigenin downregulates ABCB1 protein expression in a dose-dependent manner.

To examine whether apigenin could reverse docetaxel resistance in TaxR cells, TaxR cells were treated with 20 nmol/L docetaxel alone or in the combination with 10 µmol/L apigenin. As shown in Fig. 5B, docetaxel alone at 20 nmol/L had little effect on cell growth, apigenin alone at the concentration of 10 µmol/L reduced TaxR cell growth by about 10% to 20%, whereas the combination of 20 nmol/L docetaxel and 10 µmol/L apigenin reduced growth of TaxR cells by about 50%. Cell death ELISA showed that the combination of apigenin and docetaxel induced apoptotic cell death (Fig. 5C and D). The combination treatment also induced cleavage of PARP, a marker of apoptotic cell death, as shown by Western blot analysis.
These results show that apigenin restores docetaxel sensitivity of TaxR cells, suggesting that treatment with a combination of apigenin and docetaxel could be a potential strategy to overcome docetaxel resistance in castration-resistant prostate cancer.

Discussion

Docetaxel is used as first-line treatment for patients with castration-resistant prostate cancer that failed bicalutamide therapy. However, relapse eventually occurs due to the development of resistance to docetaxel. In this study, we generated a docetaxel-resistant prostate cancer cell subline, TaxR, from parental castration-resistant C4-2B cells. We identified ABCB1, which belongs to the ATP-binding cassette (ABC) transporter family, as the top upregulated gene in TaxR cells through global gene expression analysis and expression validation. Knockdown of ABCB1 expression by its specific shRNA or the inhibitor, Elacridar, resensitized docetaxel-resistant TaxR cells to docetaxel. In addition, we show that apigenin, a natural product of the flavone family, inhibits ABCB1 expression and resensitizes docetaxel-resistant TaxR cells to docetaxel treatment.

Several docetaxel-resistant prostate cancer cell lines have been generated including PC-3, DU145, and CWR22Rv1 (11, 14, 16). Using these docetaxel-resistant cell models, several genes have been identified to be associated with docetaxel resistance. Overexpression of Notch and Hedgehog signaling has been found to be linked to docetaxel resistance in DU145 and CWR22Rv1 cells (14). Downregulation of CDH1 and IFIH1 has been identified in docetaxel-resistant PC-3 and DU145 cells and verified in prostate tumors from docetaxel-resistant patients (16). We generated the TaxR docetaxel-resistant cell line from C4-2B cells cultured in media containing docetaxel for a long period of time. The cells are about 70-fold more resistant to docetaxel compared with parental C4-2B cells, with an IC50 of 140 nmol/L docetaxel. Interestingly, in addition to docetaxel, TaxR cells are resistant to paclitaxel, but not to doxorubicin (Fig. 6). This may be due to different mechanisms of action between taxane (docetaxel and paclitaxel) family (microtubule stabilization) and doxorubicin (DNA intercalation and topoisomerase II inhibition). Using global gene expression analysis, we identified ABCB1 as a top upregulated gene in TaxR cells compared with parental C4-2B cells, with an IC50 of 140 nmol/L docetaxel. Using Western blot analysis using specific antibodies as indicated (right), D, photographs of TaxR cells treated as indicated were taken.
The results show that resensitization of TaxR cells to docetaxel treatment by downregulation of ABCB1 may have potential clinical implications. ABCB1 (P-glycoprotein, or MDR1) belongs to the ATP-binding cassette (ABC) transporters that use the energy of ATP hydrolysis to transport substrates including alkaloids, anthracyclines and taxanes across cell membranes and diminish efficacy of the drug (17). Early studies showed that increased expression of ABCB1 confers resistance to chemotherapeutic agents including docetaxel (11, 18, 19). In addition, ABCB1 expression is directly correlated with prostate tumor grade and stage (20). It is possible that while patients respond initially to docetaxel treatment, ABCB1 expression is induced by docetaxel and subsequently transports docetaxel across cell membranes, thus diminishing efficacy and resulting in development of resistance. Together with the results showing that ABCB1 is overexpressed in docetaxel-resistant TaxR cells and downregulation of ABCB1 expression increases docetaxel sensitivity, induction of expression of ABCB1 by docetaxel may be one of the key mechanisms that are responsible for the acquired docetaxel resistance, and targeting ABCB1 expression could be a valid strategy to augment docetaxel efficacy and enhance the duration of treatment.

Overcoming docetaxel resistance presents a huge challenge to the treatment and management of docetaxel-resistant CRPC. On the basis of our finding that downregulation of ABCB1 re-sensitizes docetaxel-resistant prostate cancer to docetaxel treatment, we attempted to identify inhibitors of ABCB1 and tested their ability to overcome docetaxel resistance. Toward this goal, we have identified apigenin, a natural product belonging to the flavone family, as an inhibitor of ABCB1. Apigenin, 4′, 5, 7-trihydroxyflavone, has gained particular interest in recent years because of its low cytotoxicity and significant effects on cancer cells versus normal cells (21, 22). The antitumor effects of apigenin have been identified in a wide variety of malignant cells including prostate (15, 23, 24), melanoma (25, 26), breast (27, 28), leukemia (29), lung (30), and colon (31). Apigenin acts on a broad range of molecular signaling including suppression of the PI3K-Akt pathway in breast cancer (27, 28), induction of G2–M arrest by modulating cyclin-CDK regulators and MAPK activation (32), and alteration of the expression levels of proapoptotic factors like Bax/Bcl2, which leads to caspase activation and PARP cleavage (23). More recently, apigenin has been shown to be able to sensitize cancer cells to taxane-induced apoptotic death by promoting the accumulation of ROS in a caspase-2-dependent manner (33). Our findings showing that apigenin inhibits ABCB1 expression and overcomes docetaxel resistance provide additional evidence that apigenin may play a role in the management of CRPC after failure of docetaxel therapy.

Collectively, these results suggest that overexpression of ABCB1 may be a potential mechanism that is responsible for docetaxel resistance in prostate cancer and that targeting ABCB1 expression may be an attractive approach to resensitize docetaxel-resistant prostate cancer cells to docetaxel.

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

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Inhibition of ABCB1 Expression Overcomes Acquired Docetaxel Resistance in Prostate Cancer

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