Gene expression profiling revealed novel molecular targets of docetaxel and estramustine combination treatment in prostate cancer cells

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Abstract

Both docetaxel and estramustine are antimicrotubule agents with antitumor activity in various cancers including prostate cancer. Clinical trials for docetaxel and estramustine combination treatment have suggested improved antitumor activity in hormone-refractory prostate cancer. However, the molecular mechanisms involved in the combination treatment with docetaxel and estramustine have not been fully elucidated. In order to establish such molecular mechanisms in both hormone insensitive (PC-3) and sensitive (LNCaP) prostate cancer cells, gene expression profiles of docetaxel- and estramustine-treated prostate cancer cells were obtained by using Affymetrix Human Genome U133A Array. Total RNA from PC-3 and LNCaP cells untreated and treated with 2 nmol/L docetaxel, 4 μmol/L estramustine, or 1 nmol/L docetaxel plus 2 μmol/L estramustine for 6, 36, and 72 hours was subjected to microarray analysis. Real-time PCR and Western blot analysis were conducted to confirm the microarray data. Clustering analysis based on biological function showed that docetaxel and estramustine combination treatment down-regulated some genes that are known to regulate cell proliferation, transcription, translation, and oncogenesis. In contrast, docetaxel and estramustine combination treatment up-regulated some genes related to induction of apoptosis, cell cycle arrest, and tumor suppression. Docetaxel and estramustine also showed differential effects on gene expression between mono- and combination treatment. Combination treatment with docetaxel and estramustine caused alternations of a large number of genes, many of which may contribute to the molecular mechanisms by which docetaxel and estramustine inhibit the growth of prostate cancer cells. These results provide novel molecular targets of docetaxel and estramustine combination treatment in prostate cancer cells. This information could be utilized for further mechanistic research and for devising optimized therapeutic strategies against prostate cancer. [Mol Cancer Ther 2005;4(3):389–98]

Introduction

Although prostate cancer mortality has been declining in recent years, it is still the second leading cause of cancer death in men in the United States, with an estimated 230,110 new cases and 29,500 deaths in 2004 (1). Metastatic stage disease is the terminal step in the natural history of prostate cancer. Despite an initial response to androgen deprivation, virtually all patients with metastatic prostate cancer will progress and die of hormone-refractory disease (2). For cytotoxic chemotherapy, taxanes and estramustine have been increasingly used in patients with androgen-independent, metastatic, or symptomatic prostate cancer (3–5).

Docetaxel, a member of the taxane family, is semisynthesized from an inactive taxoid precursor extracted from the needles of the European yew, *Taxus baccata*. Docetaxel has shown clinical activity in a wide spectrum of solid tumors including breast, lung, ovarian, prostate cancers, etc. (3, 6). A clinical trial has found that docetaxel and estramustine combination treatment in patients with androgen-independent prostate cancer is associated with improvement in survival (7). The known basic cellular target of docetaxel is the microtubule. Docetaxel binds to tubulin and deranges the equilibrium between microtubule assembly and disassembly during mitosis (8). Stabilization of microtubules by docetaxel impairs mitosis and cell proliferation in tumors (8). Docetaxel also induces apoptosis with down-regulation of bcl_{XL} and bcl-2 and up-regulation of $p21^{WAF1}$ and p53 (9, 10). We have previously reported that docetaxel down-regulates some genes for cell proliferation, mitotic spindle formation, transcription factors, and oncogenesis and up-regulates some genes related to induction of apoptosis and cell cycle arrest in prostate cancer cells, suggesting pleiotropic effects of docetaxel on prostate cancer cells (11).

Estramustine is a synthetic fusion of nitrogen mustard and estradiol moiety. It has been shown to interact with tubulin and/or microtubule-associated proteins, causing tubule depolymerization and mitotic arrest (12). It has also been shown that estramustine may directly interact with cell membrane components and induce alteration in cell

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size and shape (13, 14). The induction of apoptosis by estramustine has been reported with the alteration of Akt, Bak, and caspase pathways (15, 16). We have previously reported that estramustine regulated the expression of genes, which are important in the regulation of cell cycle, apoptosis, iron homeostasis, cytoskeleton, and cell signaling transduction, suggesting its effects on cell survival and physiologic behaviors (17).

A clinical study found that estramustine potentiated taxane in prostate and refractory breast cancers (18). Combination treatments with docetaxel and estramustine have shown improved antitumor activity and survival in hormone-refractory prostate cancer (7, 19, 20). However, the molecular mechanisms in support of the combination treatment with docetaxel and estramustine have not been elucidated. In this study, we utilized a high-throughput gene chip, which contains 22,215 known genes, to determine the alternation of gene expression profiles of hormone insensitive (PC-3) and sensitive (LNCaP) prostate cancer cells exposed to both docetaxel and estramustine.

Table 1. The primers used for real-time reverse transcription-PCR and the comparison of fold changes tested by reverse transcription-PCR and Western blot analysis

Genes	Primer sequence	PCR product (bp)	Fold change tested by reverse transcription-PCR	Fold change tested by Western blot
Cathepsin C	cgg ctt ccc ata cct tat cct tca ttt tgc atg gag	104	−2.8 (PC-3/D+E)	−5.5 (PC-3/D+E)
Cathepsin K	aat cca tcc tgc tct tcc cca act ccc ttc caa agt	68	4.2 (PC-3/D+E)	3.6 (PC−3/D+E)
Annexin A2	ggc aaa ggg tag aag agc tca tag aga tcc cga gca	78	3.8 (LN/D+E)	2.3 (LN/D+E)
ATF5	ctc ctc ctt ctc cac ctc aa gcc gac ttg ttc tgg tct ct	103	−2.78 (PC-3/D+E)	—
Cyclin A2	aat cag ttt ctt acc caa tac ctg atg gca aat act tga	127	−2.7 (LN/D+E)	−3.2 (LN/D+E)
Fas/Apo-1	caa aag tgt taa tgc cca agt gca gtc tgg ttc atc cc	187	6.2 (LN/D)	—
FOXM1	gcc aca ctt agc gag acc c atc aca agc att tcc gag aca	189	−5.5 (LN/D+E)	—
GADD45	cgc ctg tga gtg agt gc ctt atc cat cct ttc ggt ctt	154	1.9 (LN/D+E)	—
IGFBP2	atg ggc gag ggc act t cag ctc ctt cat acc cga ctt	189	−2.8 (LN/D)	—
uPA	ggg agc aga gac act aac gac t ctc aca gcc cac aca aca ag	108	1.7 (PC-3/D+E)	—
Ki-67	ccg ggc tcc atc atc t ctc cag acg cca aaa taa gac	148	−4.3 (PC-3/D)	—
p21WAF1	ctg gag act ctc agg gtc gaa gga tta ggg ctt cct ctt gga	98	3.1 (PC-3/D)	2.1 (PC-3/D)
p27KIP1	cgc tcg cca gtc cat t aca aaa ccg aac aaa aca aag	187	5.4 (PC-3/D+E)	2.8 (PC-3/D+E)
PIR	cac tag ccc tcc atc ctc tac ggg tct gcc aat gct tct	151	−4.1 (PC-3/D)	—
MMP1	gct ttc ctc cac tgc tgc t aac ttg cct ccc atc att ctt	146	2.1 (PC-3/E)	—
STK6	tca gcg ggt ctt gtg t ctc ttt tgg gtg tta ttc agt	162	−2.1 (LN/D+E)	—
Survivin	cca ctg ccc cac tga gaa c acc gga cga atg ctt ttt atg	118	−3.7 (LN/T+E)	−3.1 (LN/T+E)
TRIP13	tct ggc agt gga caa gca gtt tgg gag acg gct gtg tgg	136	−4.6 (LN/D)	—
GAPDH	ctg cac cac caa ctg ctt ag ttc agc tca ggg atg acc tt	222	—	—
β-Actin	cca cac tgt gcc cat cta cg agg atc ttc atg agg tag tca gtc ag	99	—	—

NOTE: Fold change, positive values (increase); negative values (decrease). All samples were treated for 72 hours. PC-3/D+E, PC-3 cells treated with a combination of docetaxel and estramustine; LN/D+E, LNCaP cells treated with a combination of docetaxel and estramustine; PC-3/D, PC-3 cells treated with docetaxel alone; LN/D, LNCaP cells treated with docetaxel alone.

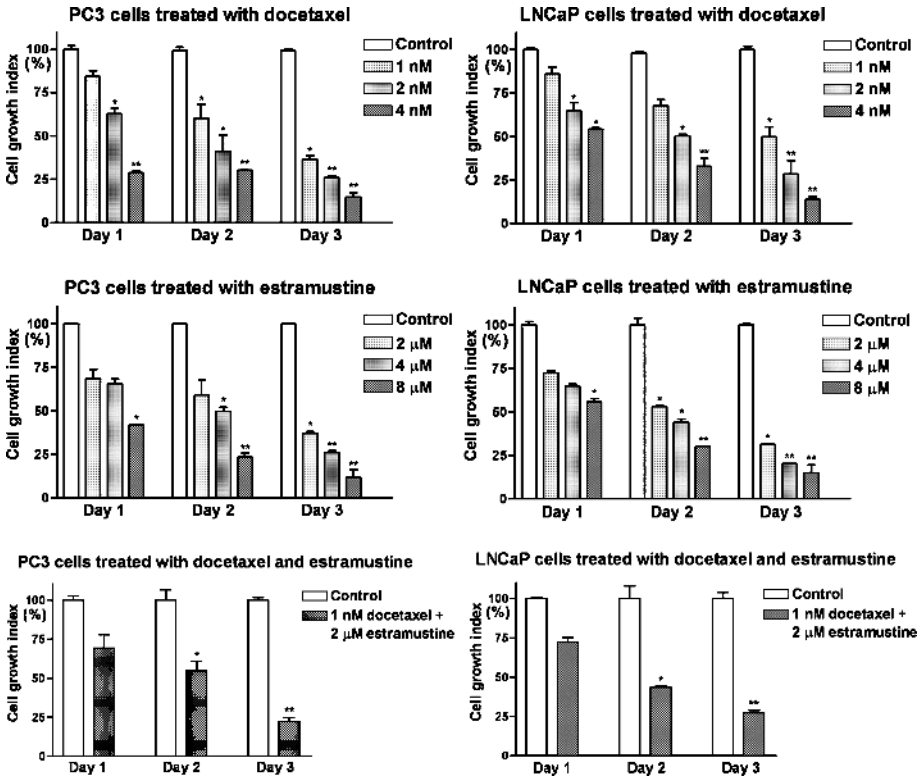

Figure 1. Effects of docetaxel and/or estramustine on the growth of PC-3 and LNCaP cells. PC-3 and LNCaP prostate cancer cells treated with docetaxel and/or estramustine resulted in a dose- and time-dependent inhibition of cell proliferation (*, $P < 0.05$; **, $P < 0.01$; $n = 3$).

Materials and Methods

Cell Culture and Growth Inhibition

PC-3 and LNCaP (both from American Type Culture Collection, Manassas, VA) human prostate cancer cells were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. Docetaxel (Aventis Pharmaceuticals, Bridgewater, NJ) was dissolved in DMSO to make a 4 μmol/L stock solution. Estramustine (Pharmacia Italia SpA, Milan, Italy) was dissolved in DMSO to make an 8 mmol/L stock solution. For growth inhibition, PC-3 and LNCaP cells were treated with docetaxel (1, 2, and 4 nmol/L), estramustine (2, 4, and 8 μmol/L), or 1 nmol/L docetaxel plus 2 μmol/L estramustine for 1 to 3 days. Control PC-3 and LNCaP cells received 0.1% DMSO at the same time points. After treatment, PC-3 and LNCaP cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/mL, Sigma, St. Louis, MO) at 37°C for 2 hours and then with isopropyl alcohol at room temperature for 1 hour. The spectrophotometric absorbance of the samples was determined using an ULTRA Multifunctional Microplate Reader (Tecan, Durham, NC) at 595 nmol/L. The concentrations of docetaxel and estramustine used for our *in vitro* studies are easily achievable in humans, suggesting that our experimental results are relevant for human applications. All experiments were repeated thrice and a *t* test was done to verify the significance of cell growth inhibition after treatment.

Microarray Analysis for Gene Expression Profiles

PC-3 and LNCaP cells were treated with 2 nmol/L docetaxel, 4 μmol/L estramustine, or 1 nmol/L docetaxel plus 2 μmol/L estramustine for 6, 36, and 72 hours. Total RNA from each sample was isolated by Trizol (Invitrogen) and purified by RNeasy mini kit and RNase-free DNase set (Qiagen, Valencia, CA) according to the manufacturer's protocols. RNA quality of all samples was tested by RNA electrophoresis and RNA LabChip analysis (Agilent, Palo Alto, CA) to ensure RNA integrity. cDNA for each sample was synthesized by Superscript cDNA synthesis kit (Invitrogen) using the T7-$(dT)_{24}$ primer instead of the oligo(dT) provided in the kit. Then, the biotin-labeled cRNA was transcripted *in vitro* from cDNA by using BioArray HighYield RNA transcript labeling kit (Enzo Biochem, New York, NY) and purified by RNeasy Mini Kit. The purified cRNA was fragmented by incubation in fragmentation buffer [40 mmol/L Tris-acetate (pH 8.1), 100 mmol/L KOAc, 30 mmol/L MgOAc] at 95°C for 35 minutes and chilled on ice. The fragmented labeled cRNA was tested on Test Chip (Affymetrix, Santa Clara, CA) to ensure that the control transcript 3′/5′ ratio was ~1. Then, the fragmented labeled cRNA was applied to Human Genome U133A Array (Affymetrix) and hybridized to the probes in the array. After washing and staining, the arrays were scanned. Two independent experiments were done to verify the reproducibility of results.

Table 2. Fold change of genes in PC-3 cells exposed to combination treatment or mono-treatment with docetaxel or estramustine

Gene	Docetaxel			Estramustine			Docetaxel + Estramustine		
	6 h	36 h	72 h	6 h	36 h	72 h	6 h	36 h	72 h
Cell proliferation and apoptosis									
NM_006739 cell division cycle 46	NC	−1.5	−2.6	−1.2	−1.5	−2.1	−1.1	−1.3	−2.5
U77949 Cdc6-related protein (HsCDC6)	0.3	−1.9	−2.0	NC	−1.7	−2.0	1.4	−2.6	−1.9
NM_005983 S phase kinase-associated protein 2 (SKP2)	−0.2	−2.1	−3.7	−1.2	−3.0	−3.2	NC	−1.7	−2.1
NM_002634 prohibitin (PHB)	NC	−1.4	−3.0	NC	−1.1	−2.8	NC	−2.6	−3.0
NM_003132 spermidine synthase (SRM)	NC	−1.5	−2.5	NC	−1.4	−2.0	NC	−1.4	−2.3
NM_014303 pescadillo	NC	−1.5	−2.3	NC	NC	−2.8	1.4	−2.3	−2.3
NM_003242 transforming growth factor, β receptor II (TGFBR2)	−0.8	−1.6	−2.0	−2.8	−4.3	−1.7	−1.9	−1.3	−2.1
L49506 cyclin G_2 mRNA	−2.1	2.0	2.6	−1.9	1.4	3.2	−1.7	2.6	1.5
BC001971Similar to cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.1	1.6	2.3	1.3	1.6	2.1	NC	1.6	2.1
NM_001731 B cell translocation gene 1, antiproliferative (BTG1)	NC	1.9	4.0	1.1	2.3	3.7	−1.3	1.9	2.0
NM_006472 up-regulated by 1,25-dihydroxyvitamin D_3 (VDUP1)	1.3	2.1	−2.0	NC	2.0	1.3	1.1	1.7	2.1
NM_014330 growth arrest and DNA-damage-inducible 34 (GADD34)	1.6	1.5	9.8	2.0	2.5	4.9	2.1	2.1	1.7
NM_015675 growth arrest and DNA-damage-inducible, β (GADD45B)	1.2	2.5	6.5	1.3	2.5	4.9	1.4	2.6	4.0
AF087853 growth arrest and DNA damage inducible protein β (GADD45B)	NC	2.0	4.6	1.1	2.3	4.9	1.2	2.8	3.0
AF078077 growth arrest and DNA-damage-inducible protein GADD45β	1.5	2.0	5.3	1.1	1.1	6.1	1.3	2.8	3.7
NM_014456 programmed cell death 4 (PDCD4)	NC	1.7	3.7	1.1	1.9	3.0	NC	1.9	2.1
Oncogenesis and cancer aggressiveness									
NM_001511 GRO1 oncogene	2.0	−1.5	−3.0	1.9	−1.3	−2.6	1.9	−6.1	−11.3
NM_002090 GRO3 oncogene (GRO3)	2.8	−3.0	−4.0	2.5	−1.6	−2.5	2.5	−5.7	−8.0
NM_007045 FGFR1 oncogene partner (FOP)	−0.1	−1.6	−2.6	−1.1	−1.1	−2.5	NC	−2.0	−2.6
AF061832.1 M4 protein deletion mutant mRNA	NC	−1.7	−2.1	NC	−1.2	−3.7	NC	−3.2	−3.5
NM_004501 heterogeneous nuclear ribonucleoprotein U (HNRPU)	NC	−1.5	−2.5	NC	−1.5	−2.6	NC	−1.9	−2.5
NM_005789 proteasome activator subunit 3 (PA28 γ)	NC	−1.4	−2.6	NC	−1.2	−2.0	NC	−1.7	−2.8
NM_006392 nucleolar protein (NOP56)	NC	−2.0	−3.5	−1.1	−1.3	−4.3	NC	−2.6	−3.7
NM_006851 glioma pathogenesis-related protein (RTVP1)	2.0	−2.0	−1.7	2.0	−1.2	−2.0	2.3	−2.1	−1.7
M80261 apurinic endonuclease (APE)	1.1	−1.4	−3.0	−1.1	−1.3	−3.0	NC	−1.6	−2.1
NM_006850 suppressor of tumorigenicity 16 (ST16)	2.0	1.0	1.7	2.0	7.0	3.2	3.7	NC	NC
Transcription and translation									
NM_012068 activating transcription factor 5 (ATF5)	NC	−2.1	−3.5	NC	−2.1	−4.0	NC	−2.3	−2.5
NM_007111 transcription factor Dp-1 (TFDP1)	NC	−1.6	−3.2	NC	−1.7	−2.5	NC	−1.7	−2.3
NM_012251 transcription factor A, mitochondria (TFAM)	1.3	−1.5	−2.5	NC	−1.7	−2.1	NC	−2.3	−2.8
AF220509 transcription associated factor TAFII31L	NC	−2.3	−3.2	NC	−4.6	−1.7	NC	−1.7	−2.3
NM_001412 eukaryotic translation initiation factor 1A (EIF1A)	−0.1	−1.3	−2.3	NC	−1.1	−2.6	NC	−1.4	−2.3
NM_002546 osteoprotegerin	1.5	−1.3	−3.0	1.2	−1.3	−3.7	1.7	−2.1	−1.7
NM_001674 activating transcription factor 3 (ATF3)	1.4	1.9	14.9	1.7	3.7	7.5	1.4	1.9	2.5
Invasion, metastasis, differentiation, and others									
AF092095 metastasis-suppressor gene CC3 (TC3)	1.0	1.5	3.0	NC	1.3	2.6	1.1	3.2	3.7
AF021834 tissue factor pathway inhibitor β (TFPIβ)	1.1	5.7	9.8	NC	3.2	5.3	−2.1	2.6	4.3
AF021834 tissue factor pathway inhibitor β (TFPIβ)	NC	3.7	12.1	1.0	3.7	7.5	NC	3.0	4.0
NM_001908 cathepsin B (CTSB)	1.2	1.9	3.0	NC	1.5	2.1	1.0	1.5	2.0
NM_000930 plasminogen activator, tissue (PLAT)	1.1	2.5	4.6	1.2	2.3	2.1	1.4	2.0	2.0
NM_002658 plasminogen activator, urokinase (PLAU)	NC	2.3	3.5	1.1	2.3	4.0	1.1	2.6	2.5
M92934 connective tissue growth factor	2.6	2.5	2.5	3.0	2.6	2.8	2.1	NC	NC
AF003934 prostate differentiation factor mRNA	−1.5	4.3	26.0	−1.5	3.2	13.0	1.1	2.6	5.3
NM_005345 heat shock 70 kDa protein 1A (HSPA1A)	1.1	1.2	4.6	2.1	1.2	3.0	1.4	2.3	1.9
NM_005346 heat shock 70 kDa protein 1B (HSPA1B)	1.1	1.3	5.3	2.0	1.3	2.8	1.6	2.3	1.9
NM_000435 Notch (*Drosophila*) homologue 3 (NOTCH3)	NC	2.0	2.6	1.2	1.3	2.5	−2.1	2.1	2.6
U73191 inward rectifier potassium channel (Kir1.3)	NC	6.5	8.6	6.1	6.1	4.0	2.1	2.8	2.1
Resistance to chemotherapeutic agents									
NM_001894 casein kinase 1 (CSNK1E)	NC	1.7	3.2	NC	1.7	3.0	NC	1.6	2.1
AU154504 cytochrome *P*450, subfamily I (dioxin-inducible)	NC	1.1	2.1	NC	NC	2.5	NC	2.0	2.1
AU144855 cytochrome *P*450, subfamily I (dioxin-inducible)	NC	1.5	2.1	NC	1.3	2.5	NC	2.3	2.6
NM_005980 S100 calcium-binding protein P (S100P)	−1.2	4.9	24.3	1.1	7.0	13.9	−1.5	2.5	4.0

NOTE: The genes in this list have a >2-fold change in expression in at least one time point in both mono- and combination treatment ($P < 0.05$); NC, no change; negative values, decrease; positive values, increase.

Microarray Data Normalization and Analysis

The gene expression levels of samples were normalized and analyzed by using Microarray Suite, MicroDB, and Data Mining Tool software (Affymetrix). The absolute call (present, marginal, or absent) and average difference of 22,215 gene expressions in a sample, and the absolute call difference, fold change, average difference of gene expressions between two or several samples were normalized and identified using this software package. Statistical analysis of the mean expression average difference of genes, which show >2-fold change based on a log normalization, was done using a *t* test between treated and untreated samples. Clustering and annotation of the gene expression were analyzed by using Cluster and TreeView (21), Onto-Express (22), and GenMAPP (23). Genes that were not annotated or not easily classified were excluded from the functional clustering analysis.

Real-time Reverse Transcription-PCR Analysis for Gene Expression

To verify the alterations of gene expression at the mRNA level, which appeared on the microarray, we chose representative genes (Table 1) with varying expression profiles for real-time reverse transcription-PCR analysis. Two micrograms of total RNA from each sample were subjected to reverse transcription using the Superscript first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. Real-time PCR reactions were then carried out in a total of 25 μL reaction mixture (2 μL of cDNA, 12.5 μL of 2× SYBR Green PCR Master Mix, 1.5 μL of each 5 μmol/L forward and reverse primers, and 7.5 μL of H_2O) in SmartCycler II (Cepheid, Sunnyvale, CA). The PCR program was initiated by 10 minutes at 95°C before 40 thermal cycles, each for 15 seconds at 95°C and 1 minute at 60°C. PCR amplification efficiency and linearity for each gene including targeted and control genes were tested. Data was analyzed according to the comparative Ct method and was normalized by β-actin or glyceraldehyde-3-phosphate dehydrogenase expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

Western Blot Analysis

We also conducted Western blot analysis to verify the alterations of genes at the level of translation for selected genes with varying expression profiles. The PC-3 and LNCaP cells were treated with 1 and 2 nmol/L docetaxel or 2 and 4 μmol/L estramustine for 24, 48, and 72 hours. After treatment, the cells were lysed in 62.5 mmol/L Tris-HCl and 2% SDS, and protein concentration was measured using bicinchoninic acid protein assay (Pierce, Rockford, IL). The proteins were subjected to 10% or 14% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. The membranes were incubated with anti-$p21^{WAF1}$ (1:500, Upstate, Lake Placid, NY), anti-$p27^{KIP1}$ (1:250, Novocastra, Newcastle upon Tyne, United Kingdom), anti-survivin (1:200, Alpha Diagnostic, San Antonio, TX), anti-annexin A (1:200, Santa Cruz, Santa Cruz, CA), anti-cyclin A (1:250, NeoMarkers, Union City, CA), anti-cathepsin C (1:200, Santa Cruz), anti-cathepsin K (1:100, Santa Cruz), and anti-β-actin (1:10,000, Sigma) primary antibodies and subsequently incubated with secondary antibodies conjugated with fluorescence dye (Molecular Probes, Eugene, OR). The signal was then detected and quantified using Odyssey IR imaging system

Table 3. Fold change of genes in LNCaP cells exposed to combination treatment or mono-treatment with docetaxel or estramustine

Gene	Docetaxel			Estramustine			Docetaxel + Estramustine		
	6 h	36 h	72 h	6 h	36 h	72 h	6 h	36 h	72 h
Cell proliferation and apoptosis									
NM_001255 CDC20 (cell division cycle 20)	−1.9	−90.5	−147	−1.4	−1.7	−3	NC	−1.2	−2.3
NM_001237 cyclin A2 (CCNA2)	−1.2	−5.7	−12.1	NC	−1.4	−2.5	NC	−1.5	−2.1
D55716 cdc47	−1.2	−5.3	−5.3	−1.3	−1.4	−2	−1.1	−1.5	−2
NM_016426 G_2 and S phase expressed 1 (GTSE1)	−1.1	−4.9	−8.6	NC	−1.3	−2	−1.1	−1.2	−2.1
NM_005030 polo (*Drosophila*)-like kinase (PLK)	−1.4	−5.3	−5.3	NC	−1.3	−2.1	NC	−1.1	−2
NM_001168 survivin	−1.1	−27.9	−294.1	NC	−1.2	−2.3	NC	−1.1	−2.3
NM_001924 growth arrest and DNA-damage-inducible, (GADD45A)	1.4	7.5	7.5	1.0	1.3	2.5	1.0	1.6	2.6
NM_014454 p53 regulated PA26 nuclear protein (PA26)	1.1	4.0	3.0	1.1	1.9	2.5	1.3	1.5	2.0
Transcription factor and other									
NM_021953 forkhead box M1 (FOXM1)	−1.2	−13.9	−42.2	−1.3	−1.5	−2.3	−1.1	−1.3	−2.3
NM_014264 serinethreonine kinase 18 (STK18)	−1.4	−8.6	−16	−1.1	−1.3	−2.3	−1.1	−1.3	−2
NM_003158 serinethreonine kinase 6 (STK6)	−1.7	−11.3	−9.8	−1.1	−1.6	−2.3	NC	−1.1	−2
NM_001647 apolipoprotein D (APOD)	1.3	8.0	12.1	1.4	1.6	2.0	−1.2	1.5	2.3
NM_001147 angiopoietin 2 (ANGPT2)	3.5	3.5	8.0	2.6	4.3	9.2	1.0	8.6	34.3
AF187858 angiopoietin-2 isoform-1	NC	2.6	4.3	NC	NC	3.0	−1.6	3.5	18.4
AF270487 androgen-regulated serine protease TMPRSS2 precursor	NC	1.7	2.0	NC	1.7	2.1	−1.1	2.0	2.6
NM_001070 tubulin, 1 (TUBG1)	−1.1	−4.6	−3.2	−1.1	−1.2	−2.5	NC	−1.3	−2

NOTE: The genes in this list have a >2-fold change in expression in at least one time point in both mono- and combination treatment ($P < 0.05$); NC, no change; negative values, decrease; positive values, increase.

Table 4. Comparison of difference in gene expression between combination treatment and mono-treatment in PC-3 and LNCaP cells

Gene	Docetaxel + Estramustine		
	6 h	36 h	72 h
Decrease in combination treatment in PC-3 cells, No change or increase in mono-treatment			
U63131 CDC37 homologue, proliferation-related	NC	−2.1	−1.6
NM_012121 Cdc42 effector protein 4 (CEP4), proliferation-related	−2.1	NC	NC
NM_002006 fibroblast growth factor 2 (basic; FGF2), proliferation-related	1.9	−2.1	−2
NM_005627 serum glucocorticoid-regulated kinase (SGK), proliferation-related	1.6	−1.6	−2.3
U78525 eukaryotic translation initiation factor (eIF3), proliferation-related	NC	−1.5	−2
BC001173 eukaryotic translation initiation factor 3, proliferation-related	NC	−1.6	−2
NM_001814 cathepsin C (CTSC), invasion-related	NC	−1.5	−2
NM_002532 nucleoporin 88 kDa (NUP88), oncogenesis-related	NC	−2	−1.9
NM_003155 stanniocalcin 1 (STC1), oncogenesis-related	−1.5	−1.6	−2.1
NM_002774 kallikrein 6 (neurosin, zyme; KLK6), oncogenesis-related	−1.1	−1.9	−2
NM_001216 carbonic anhydrase IX (CA9), oncogenesis-related	−2.3	1.7	NC
L78132 prostate carcinoma tumor antigen (pcta-1), oncogenesis-related	−2	NC	NC
NM_001145 angiogenin, ribonuclease, RNase A family, 5 (ANG), angiogenesis-related	−2.6	NC	NC
NM_001038 sodium channel, nonvoltage-gated 1α (SCNN1A), cell size and shape-related	−2.1	NC	1.7
Increase in combination treatment in PC-3 cells, no change or decrease in mono-treatment			
AI056359 microtubule-associated protein, differentiation-related	1.7	2.6	4.9
X79683 laminin, β2, differentiation-related	1.1	2	1.9
AI812030 thrombospondin 1, angiogenesis-related	2.1	−1.9	−2.5
NM_000396 cathepsin K (pycnodysostosis; CTSK), invasion-related	NC	2	3.5
AA020826 cathepsin B, invasion-related	1.1	2	2
M99422 glutathione transferase (GST), drug resistance-related	1.1	1.6	2.1
X08020 glutathione S-transferase subunit 4, drug resistance-related	NC	1.6	2
M21940.1 cytochrome *P*450 S-mephenytoin 4-hydroxylase (P-450mp), drug resistance-related	1.1	1.9	2
M15331 cytochrome *P*450 S-mephenytoin 4-hydroxylase (P-450mp), drug resistance-related	1.7	2.3	2.3
Decrease in combination treatment in LNCaP cells, no change or increase in mono-treatment			
NM_003461 zyxin (ZYX), proliferation-related	−2.0	−1.1	NC
AF274954 PNAS-29, proliferation-related	−1.1	−1.6	−2.0
NM_007034 heat shock protein 40 (HLJ1), stress-related	−1.7	−2.1	−2.1
BG403660 heat shock 105 kDa, stress-related	NC	−1.9	−2.0
Increase in combination treatment in LNCaP cells, no change or decrease in mono-treatment			
U02520.1 collagen type IV 3, differentiation-related	1.2	2.1	2.3
NM_020348 cyclin M1 (CNNM1), proliferation-related	2.3	2.0	1.9
BF197655 caveolin 2, oncogenesis-related	2.3	−2.0	2.8
NM_001657 amphiregulin (AREG), apoptosis-related	4.3	5.3	4.0
M33987 carbonic anhydrase I (CAI), metastasis-related	2.6	3.2	3.2
NM_000928 phospholipase A2, group IB (pancreas; PLA2G1B), oncogenesis-related	11.3	3.0	7.5
NM_004039 annexin A2 (ANXA2, drug resistance-related	2.0	2.6	2.3
X08020.1 glutathione S-transferase subunit 4, drug resistance-related	1.1	2.1	1.6
NM_014139 sodium channel, voltage-gated, type XII, (SCN12A), cell size and shape-related	2.5	6.1	3.2

NOTE: NC, no change; negative values, decrease; positive values, increase ($P < 0.05$).

(LI-COR, Lincoln, NE). The ratios of $p21^{WAF1}$, $p27^{KIP1}$, survivin, annexin A, cyclin A, cathepsin C, or cathepsin K against β-actin were calculated by standardizing the ratios of each control to the unit value.

Results

Cell Growth Inhibition

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that the treatment of PC-3 and LNCaP prostate cancer cells with docetaxel, estramustine, or lower concentrations of docetaxel plus estramustine resulted in the inhibition of cell proliferation in a dose- and time-dependent manner (Fig. 1), demonstrating the inhibitory effect of docetaxel and estramustine on the growth of both PC-3 and LNCaP prostate cancer cells. Compared to mono-treatment, similar but more growth inhibition was achieved by the combination treatment with lower concentrations of docetaxel and estramustine (Fig. 1).

Regulation of mRNA Expression by Docetaxel and Estramustine Treatment

Microarray analysis showed that the alteration of gene expression occurred as early as 6 hours after mono-treatment or combination treatment and was more evident with a longer treatment period (Tables 2 and 3).

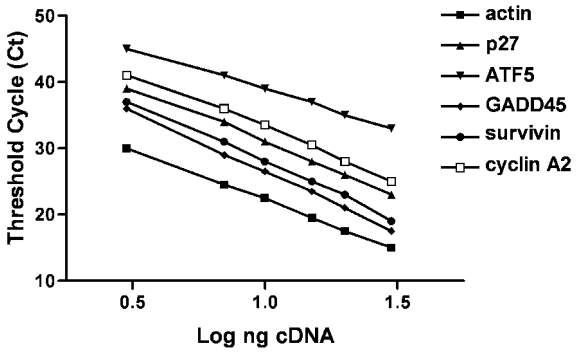

Figure 2. Real-time reverse transcription-PCR amplification from different amounts of cDNA input from selected genes showed high linearity.

Clustering analysis based on gene function showed down-regulation of some genes for cell proliferation and apoptosis inhibition (cyclin A2, CDC20, CDC46, CDC47, survivin, etc.), transcription factors (transcription factor A, ATF5, TAF1131L, FOXM1, etc.), and oncogenesis (GRO1 oncogene, GRO3 oncogene, etc.) in prostate cancer cells with mono-treatment and combination treatment (Tables 2 and 3). In contrast, mono-treatment and combination treatment up-regulated some genes that are related to the induction of apoptosis (GADD34, GADD45A, GADD45B, PA26, etc.), inhibition of cell proliferation (BTG1, p27KIP1, VDUP1, etc.), and tumor suppression (suppressor of tumorigenicity 16, TC3, TFPIβ, etc.; Tables 2 and 3).

Combination treatment with docetaxel and estramustine also altered expression of some genes, which showed no change in mono-treatment and are related to cell proliferation, oncogenesis, angiogenesis, invasion, and differentiation (Table 4). Down-regulation of CDC37, CDC42 effector protein 4, PNAS-29, FGF2, eIF3, cathepsin C, prostate carcinoma tumor antigen, and angiogenin, and up-regulation of collagen IV, laminin, and microtubule-associated protein were observed only in combination treatment, suggesting the possible synergic effects of combination treatment on the promotion of differentiation and the inhibition of cell proliferation, oncogenesis, angiogenesis, and invasion. These results provide, for the first time, molecular evidence in support of combination treatment for better tumor cell killing.

Docetaxel and estramustine in mono-treatment and combination treatment also up-regulated some genes (S-100P, casein kinase, p450, etc.) responsible for chemotherapeutic resistance, suggesting the induction of cancer cell resistance to these agents (Tables 2 and 3). These results also suggest that inactivation of these genes by novel approaches may provide optimal therapeutic strategies when docetaxel and estramustine are used in combination for the treatment of prostate cancer.

Target Verification by Real-time Reverse Transcription-PCR and Western Blot

To verify the alterations of gene expression at the mRNA level, which appeared on the microarray, we chose representative genes with varying expression profiles for real-time reverse transcription-PCR and Western blot analysis. Real-time PCR amplification from 3 to 30 ng cDNA input of each gene showed high linearity (Pearson correlation coefficient, $r > 0.99$; Fig. 2). The results of real-time reverse transcription-PCR for these selected genes were in direct agreement with the microarray data (Tables 1–3). The same alternations of gene expression were observed by real-time reverse transcription-PCR analysis, although the fold change in the expression level was not exactly the same between these two different analytic methods. The results of Western blot analysis were also in direct agreement with the microarray and real-time reverse transcription-PCR data [Fig. 3; Tables 1–4; and our earlier report (11)]. These results support the findings obtained from microarray experiments, supporting and providing molecular evidence for combination of docetaxel and estramustine for the treatment of prostate cancer.

Discussion

From gene expression profiles, we found that cellular and molecular responses to docetaxel and estramustine combination treatment are complex and are likely to be mediated by a variety of regulatory pathways. Docetaxel and estramustine combination regulated the expression of important genes that control cell growth, apoptosis, transcription, translation, cell signaling, differentiation, oncogenesis, invasion, and metastasis (Fig. 4). These regulations may be responsible for inhibiting the progression of prostate cancers. Compared to the mono-treatment gene expression results, combination treatment with lower doses of each drug altered the expression of more genes involved in the control of cell proliferation, oncogenesis,

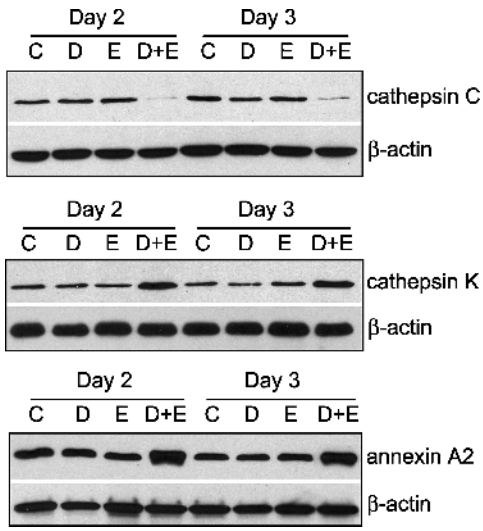

Figure 3. Western blot analysis showed the altered expression of specific genes in docetaxel- and estramustine-treated PC-3 and LNCaP cells (*C*, control; *D*, docetaxel treatment; *E*, estramustine treatment; *D + E*, docetaxel and estramustine combination treatment).

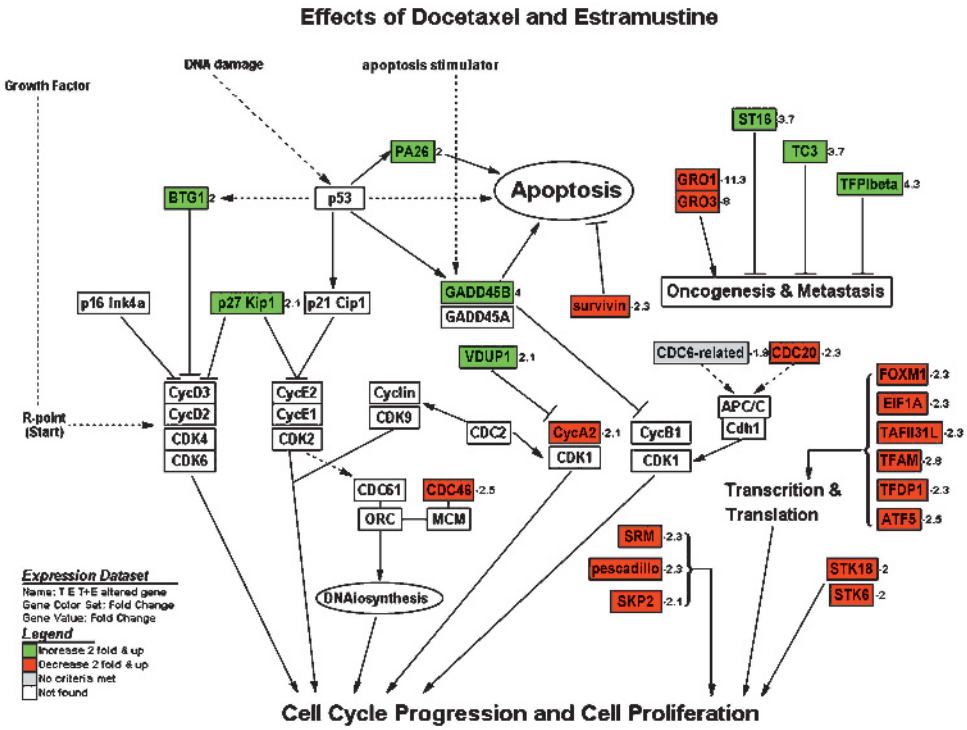

Figure 4. Effects of docetaxel and estramustine on pathway-related gene expression analyzed and visualized by GenMAPP software integrated with cDNA microarray data (*positive values,* increase in fold change; *negative values,* decrease in fold change).

angiogenesis, invasion, and differentiation. This data suggests that combination treatment may exert more inhibitory effects on prostate cancer cells, and these effects may mechanistically correspond with the improved antitumor activity of combination treatment observed in clinical studies (19).

By gene expression profiling, we found that docetaxel and estramustine combination treatment inhibited the expression of cyclin A2, CDC20, CDC46, CDC47, pescadillo, spermidine synthase, and polo-like kinase, and up-regulated the expression of $p27^{KIP1}$, BTG1, and VDUP1, all of which are involved in the regulation of cell cycle and cell proliferation. It has been well known that CDCs regulate the molecules related to the cell cycle initiation and progression, and that cyclins associate with cyclin-dependent protein kinases and CDCs to control the cell cycle process (24, 25). The cyclin-dependent protein kinase inhibitors including $p27^{KIP1}$ have been shown to arrest the cell cycle and inhibit the growth of cancer cells (24, 25). Pescadillo, spermidine synthase, and polo-like kinase have been shown to promote cell proliferation, whereas BTG1 and VDUP1 have been shown to inhibit cell growth (26–30). Our results suggest that docetaxel and estramustine combination treatment may inhibit prostate cancer cell growth through regulation of expression of these important genes related to cell cycle and cell proliferation.

We have previously found that docetaxel or estramustine mono-treatment regulated the expression of apoptosis-related genes (11, 17). In this study, we found that combination treatment with lower doses of docetaxel and estramustine also regulated the expression of genes that are critically involved in the apoptotic processes. Among these genes, survivin acts as an important inhibitor of apoptosis (31). GADD45A and GADD45B have been known to promote apoptosis and regulate G_2-M arrest (32). PA26 is a target of the p53 tumor suppressor and is a member of the GADD family with an apoptosis-inducing property (33). We found a decrease in the expression of survivin and an increase in the expression of GADD45A, GADD45B, and PA26 in docetaxel- and estramustine-treated prostate cancer cells, suggesting their effects in the induction of apoptosis. The induction of apoptosis mediated by these genes could be another molecular mechanism by which docetaxel and estramustine combination treatment inhibits the growth of prostate cancer cells.

Docetaxel and estramustine combination treatment also showed down-regulation of the expression of genes that control transcription (ATF5, transcription factor A, transcription factor Dp-1, forkhead box M1, transcription-associated factor TAFII31L), translation (EIF1A), and cell signaling (STK6, STK18). These results suggest that both docetaxel and estramustine may inhibit prostate cancer cell growth by regulation of the molecules that are important in the processes of cell signal transduction, transcription, and translation.

Metastasis-suppressor gene CC3, tissue factor pathway inhibitor β, suppressor of tumorigenicity 16, and connective tissue growth factor have been known to inhibit cancer development, invasion, and metastasis (34–36). Both docetaxel and estramustine increased the expression of these genes in mono-treatment and combination treatment, suggesting their inhibitory effects on oncogenesis,

invasion, and metastasis. Moreover, we also observed increased level of cathepsin B, tissue-type, and urokinase-type plasminogen activator in docetaxel- and/or estramustine-treated PC-3 cells. Therefore, more experimental studies are needed to ascertain the overall effect of docetaxel and estramustine on invasive and metastatic processes. These results, however, were not observed in androgen-sensitive LNCaP cells, suggesting the different effects mediated through different cell signaling transduction pathways.

Although both docetaxel and estramustine target microtubules, it seems that docetaxel mono-treatment exerted stronger inhibitory effects on the expression of tubulin (11). Estramustine mono-treatment showed effects on the expression of genes related to the control of iron homeostasis and cell shape (17). In the gene expression profiles, we found that docetaxel and estramustine combination treatment also inhibited the expression of tubulin and induced the expression of inward rectifier potassium and voltage-gated sodium channel, which are related to iron homeostasis (37, 38). These results suggest that the combined effects of docetaxel and estramustine are better on the microtubule formation and the changes of cell size and shape.

It is important to note that docetaxel and estramustine in mono-treatment and combination treatment also up-regulated the expression of some genes which are known to induce cell resistance to chemotherapeutic agents and to favor cell survival. Among these genes, calcium-binding protein, S100P, has been found to be highly expressed in cells which develop acquired resistance to antitumor agents (39), and casein kinase 1 modulates drug resistance in tumor cells (40). The up-regulation of these molecules by docetaxel and estramustine could induce cell resistance to chemotherapeutic agents. Also, docetaxel and estramustine in mono-treatment and combination treatment were found to up-regulate the expression of Notch 3, angiopoietin, activating transcription factor 3, and apurinic endonuclease, all of which could favor cell survival (41–43). These results may represent intrinsic cellular response to these agents as defensive survival mechanisms and further suggest that strategies must be devised to disable these molecules to achieve the optimal therapeutic benefit of docetaxel and estramustine combination for successful treatment of prostate cancer. However, further in-depth mechanistic studies are needed to address these issues. The investigation on overcoming these unbeneficial effects with other agents is ongoing in our laboratory.

Both docetaxel and estramustine in mono-treatment and combination treatment showed no effect on androgen receptor expression in LNCaP cells. However, the genes altered by mono-treatment or combination treatment with respect to the control of cell growth, apoptosis, transcription, oncogenesis, and metastasis in androgen-insensitive PC-3 cells are different from those in androgen-sensitive LNCaP cells, suggesting that the effects of docetaxel and estramustine may be mediated by both androgen receptor-dependent and -independent signaling pathways.

In conclusion, docetaxel and estramustine combination treatment directly and indirectly caused changes in the expression of many genes that are critically involved in the control of cell proliferation, apoptosis, transcription, translation, oncogenesis, angiogenesis, metastasis, and drug resistance. These findings provided molecular information for further investigation on the mechanisms by which docetaxel and estramustine exert their pleiotropic effects on prostate cancer cells. These results could also be important in devising mechanism-based and targeted therapeutic strategies for prostate cancer, especially in devising combination therapy for drug resistant prostate cancers. Nevertheless, it is clear that our findings provide, for the first time, novel molecular targets of docetaxel and estramustine combination treatment in prostate cancer cells. However, further in-depth investigations are needed in order to establish cause and effect relationships between these altered genes and treatment outcome.

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Molecular Cancer Therapeutics

Gene expression profiling revealed novel molecular targets of docetaxel and estramustine combination treatment in prostate cancer cells

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