Evaluation of Cancer Chemopreventive Agents Using Clones Derived from a Human Prostate Cancer Cell Line

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Abstract. *Background: The successful use of clonal selection through fluctuation analysis of human cancer cells as a means for studying tumor progression has been previously reported. Materials and Methods: Three clones derived from a parental population of human prostate cancer (LNCaP) cells were selected based on proliferation, hormone sensitivity and anchorage-independent growth. The effects of five potential cancer preventive agents were evaluated using cell proliferation, anchorage-independent growth and apoptosis as end-points. Results: Clone 21 cells, which represent a presumptive normal phenotype, were generally more sensitive than Clone 17 and Clone 6 cells, which represent a more malignant phenotype, to fluasterone, 7β-HF, L-selenomethionine and troglitazone in assays for proliferation and/or apoptosis. Conclusion: The results confirm the efficacy of the above agents as cancer chemopreventive agents and support our contention that clonal selection of established human cancer cells provides a model to study the efficacy of chemopreventive agents.*

Prostate cancer is the most common male malignant disease and the second leading cause of male cancer death in the United States (1). The etiology of prostate cancer is not well understood; however, epidemiological data suggest that interaction of genetic and environmental factors may play a significant role in the development of prostate cancer. It has been reported that incidence and mortality rates of prostate cancer are quite low in most countries of the Pacific basin when compared to those in the Western world (2). When Asian men migrate to this country, however, their offspring develop prostate cancer at rates similar to those of American men (3). These observations suggest that at least some of the prostate cancer cases are likely to be preventable by changing the environmental and dietary factors.

Several agents, such as 9-cis retinoic acid, dehydroepiandrosterone (DHEA) analogue 8354 (fluasterone), L-selenomethionine and vitamin E, are thought to be useful as cancer prevention agents (4-10). However, the efficacy of these agents for prevention of prostate cancer remains to be determined. Ideally, an effective cancer preventive agent should be able to inhibit the growth and/or survival of transformed cells at concentrations that are not toxic to normal cells. This is particularly relevant for cancer prevention in targeted populations involving individuals who, although at increased risk of cancer, are presumed to be clinically cancer free while taking the cancer preventive agents. We have previously evaluated the effects of several potential cancer chemopreventive agents on cell growth or clonogenic survival using cultured normal human prostate epithelial cells, an immortalized but non-tumorigenic human prostate epithelial cell line (267B1), a human benign prostatic hyperplasia (BPH) cell line (BRF-55T), and a human prostate cancer cell line (267B1/Ki-ras) and demonstrated that 9-cis retinoic acid, liarozole fumarate, phenylenebis(methylene)-selenocyanate (p-XSC), and L-selenomethionine had much stronger growth inhibitory effects on prostate cancer cells than on the normal prostate epithelial cells (11).

While our previous studies demonstrated differential effects between the malignant and non-malignant human prostate cells, the observed effects were confounded by the different genetic backgrounds of the cells and cell lines, as well as the different media and culture conditions required to maintain these cells and cell lines. We have previously reported the use of clonal selection through fluctuation analysis of human cancer cells as a means for studying tumor progression. Specifically, we proposed that this approach

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permits the study of a genetic continuum, including normal through intermediate to the highly malignant cell phenotypes occurring within the parental cell population, and that the phenotypically normal clones represent an important tool to screen for the chemopreventive efficacy of potential agents during cancer progression. The present study was undertaken to further characterize the LNCaP cell clones established in our previous studies, using cell proliferation, anchorage independent growth and apoptosis as end-points, and evaluate the effects of 9-cis retinoic acid, fluasterone, 7β-HF, L-selenomethionine and troglitazone in LNCaP cell clones with different degrees of malignant phenotypes. The results further support our contention that clonal selection of established human cancer cells provide a model to study the efficacy of chemopreventive agents through their ability to inhibit progression of the phenotypically normal cell clones.

Materials and Methods

Chemicals, reagents, medium and cancer preventive agents. The cancer preventive agents 9-cis retinoic acid and fluasterone were obtained from the Division of Cancer Prevention (DCP) Repository, the National Cancer Institute, at McKesson BioServices (Rockville, MD, USA). 7-β-hydroxyfluasterone (7β-HF) and L-selenomethionine (SeM) were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA), and troglitazone was obtained from Sankyo Co. (Tokyo, Japan). The stock solutions of 9-cis retinoic acid (50 mM) and troglitazone (1.0 or 0.5 M) were prepared in dimethylsulfoxide (DMSO) and stored at –20˚C. Fluasterone, 7β-HF and SeM were directly dissolved in the medium before use. RPMI-1640 medium and trypsin-EDTA were obtained from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) and hormone-depleted FBS were purchased from HyClone (Logan, UT, USA). ApopTag Peroxidase kit was purchased from Intergen Company (Purchase, NY, USA). Agar, Methocel (methyl mellulose 4000 CP) and other chemicals were purchased from Sigma Chemical Company, unless specified otherwise.

Cells and cell culture. LNCaP cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). LNCaP Clones 6, 17 and 21 were established from parental LNCaP cells, as described previously (12). These cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum for routine use. All cell culture incubation was carried out at 37˚C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cell proliferation assay and hormone sensitivity determination. Cell proliferation was determined using the sulforhodamine B (SRB) assay method, which was carried out essentially as previously described (13). To initiate the experiment, cells were dissociated by treatment with trypsin-EDTA and plated on 96-well plates at a density of 4,000 cells per well. After an overnight incubation for the cells to attach, the medium was changed and fresh medium containing one of the cancer preventive agents at specified concentrations was added into the wells. Media containing no cancer preventive agent or 0.2% DMSO alone were included in the experiments as negative and carrier controls, respectively. After every 48 h of incubation, one set of cell culture plates were fixed with 10% trichloroacetic acid at 4˚C for 1 h, washed four times with tap water and stained with 0.4% sulforhodamine B (dissolved in 1% acetic acid) for 15 min. The plates were then washed three times with 1% acetic acid and dried in air. After all plates were fixed, stained and dried, the cells in each well were solubilized with 200 μl of 10 mM Tris solution, and the plates were read with a PowerWave microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 540 nm. The absorbance (A540) readings, which were proportional to the number of cells in the wells (13), were compared among different treatment groups to assess the effects of the cancer preventive agents on the growth of normal prostate epithelial cells.

For a hormone sensitivity determination, some of the cell proliferation experiments were performed using medium supplemented with regular FBS and hormone-depleted FBS for a side-by-side comparison of cell proliferation in the presence or absence of testosterone hormone.

Anchorage-independent growth assay. For the anchorage-independent growth assay, cells were trypsinized and suspended in medium containing 0.8% methyl-cellulose and supplemented with or without the cancer preventive agents at specified test concentrations. The cells were plated in 24-well tissue culture plates at a density of $1x10^4$ cells/well. The bottoms of the wells were pre-coated with an agar layer prepared by adding 5% agarose to medium to give a final agarose concentration of 0.9% (14, 15). The plates were incubated at 37˚C and the medium was changed once a week. Twenty-four hours after plating, the cultures were examined under a microscope and wells containing cell aggregates were marked off so that only wells without visible cell aggregates were evaluated for colony formation. Four wells per treatment group were stained with neutral red 21 days after plating and counted under a dissection microscope to calculate the colony formation efficiency.

Apoptosis induction assay. The ability of the cancer preventive agents to induce apoptosis was determined in parental LNCaP cells and LNCaP Clones 6, 17 and 21 cells. To start the experiments, the cells were plated into 96-well tissue culture plates and cultured in medium without the cancer preventive agent. Each cancer preventive agent was diluted in the medium to five non-toxic concentrations determined from the cell proliferation assays. When the cells were confluent, the medium was changed and fresh medium containing one of the test cancer preventive agents or carrier was added to the wells. The cells were incubated for an additional 24 h, then gently washed with phosphate buffered saline (PBS) and fixed with standard 10% (v/v) neutral buffered formalin. The cells were then washed with PBS and incubated with proteinase K (20 μg/ml, 100 μl/well) for 15 min to digest proteins, which may hinder the labeling of the DNA 3'-OH ends.

DNA fragmentation was measured using the ApopTag Peroxidase kit. To perform the experiment, the fixed cells treated with proteinase K were incubated with 3% hydrogen peroxide in PBS for 5 min to quench endogenous peroxidase activity. The cells were then incubated in equilibration buffer for 5 min, followed by a one-hour incubation with a working strength solution of TdT containing digoxigenin-11-dUTP and dATP at a ratio optimized for detection by anti-digoxigenin antibody. After the incubation, the plates were washed and incubated in a wash buffer for 10 min, then incubated with peroxidase conjugated anti-digoxigenin antibody for

Table I. *Anchorage-independent growth capabilities of cloned LNCaP cells.*

LNCaP cell clone	Colony formation efficiency (%)	Number of experiments	Number of replicates
6	0.47±0.09	2	10
11	0.57±0.07	2	10
17	0.40±0.07	2	10
18	0.20±0.05	2	10
20	0.20±0.04	2	10
21	0.02±0.02	3	29
34	0.02±0.03	3	18

Table II. *Hormone sensitivity of LNCaP cell clones.*

LNCaP cell clone	Cell doubling time (hours)			Student's *t*-test (*p*-value)
	Regular FBS	Hormone depleted FBS	% Increase	
6	31.92±0.39	89.30±30.69	180%	<0.05
11	35.65±0.55	61.66±7.98	73%	<0.01
17	32.89±0.33	85.51±10.67	160%	<0.01
18	105.92±4.38	>196*	>160%*	N/A
20	87.18±5.89	184.05±41.39	111%	<0.01
21	41.21±0.76	109.62±15.69	166%	<0.01
34	113.93±8.70	191.36±46.35	68%	<0.05

*Cell population failed to double at the end of an 196-h incubation

30 min. The plates were washed again with PBS, incubated with TMB substrate solution for 15 min and read with a microplate reader at a wavelength of 650 nm.

Data and statistical analysis. For cell proliferation and hormone sensitivity determinations, A540 values (representing cell density) measured at each time point were plotted against incubation time to generate a growth curve for each LNCaP cell clone cultured in medium supplemented with regular FBS or hormone-depleted FBS. The cell population doubling time was calculated using the data from the logarithmic phase of the growth curve. The population doubling times for each LNCaP cell clone cultured in medium supplemented with regular FBS or hormone-depleted FBS were compared by the Student's *t*-test to determine the hormone sensitivity of the cells.

To determine the effects of cancer preventive agents on cell proliferation, the A540 values measured for cells cultured in medium containing the cancer preventive agent at different concentrations were measured at the time when the cells in the medium control group were confluent. The dose response curve for each test agent in each LNCaP cell clone was fitted with a sigmoidal dose-response curve fit model. For each cancer preventive agent that inhibited cell proliferation by more than 50% at the highest concentration tested, the 50% inhibition concentration (IC_{50}) and the associated 95% confidence interval calculated from the dose-response curve were reported.

For the soft agar colony formation assay, the number of colonies in each well was divided by the number of cells plated to calculate the anchorage-independent colony formation efficiency. For each LNCaP cell clone, the colony formation efficiency data from two or three experiments were pooled to calculate the mean colony formation efficiency. The results are expressed as mean ± standard deviation (SD). To determine the effects of cancer preventive agents on anchorage-independent growth, the colony formation efficiency results of LNCaP cell clones 6 and 17 cells treated with each cancer preventive agent were analyzed by one-way ANOVA followed by the Dunnett multiple comparison test. The lowest effective concentration of the cancer preventive agent that significantly reduced the anchorage-independent colony formation efficiency is reported.

For the apoptosis induction assay, the A650 values were adjusted by subtracting the mean A650 value of unlabeled (TdT omitted) control, and apoptosis in each treatment group is expressed as a percentage of apoptosis in the medium (no cancer preventive agent treatment) control group. The lowest effective concentration of the cancer preventive agent that resulted in a significant induction of apoptosis was reported.

The sigmoidal dose-response curve fitting and one-way ANOVA, Dunnett multiple comparison test and *t*-test were performed using GraphPad Prizm (version 2.01) and GraphPad InStat (version 3.00) statistical softwares (GraphPad Software, San Diego, CA, USA), respectively.

Results

This study was undertaken to evaluate the effects of five potential cancer preventive agents on cell proliferation, anchorage-independent growth and apoptosis in LNCaP cell clones with varying degrees of malignant potential, as represented by anchorage-independent growth capability. Of the 7 clones evaluated, Clones 6, 11 and 17 were the most malignant with a soft agar colony formation efficiency of 0.4% or higher, whereas Clones 21 and 34 were least malignant, with a soft agar colony formation efficiency of only 0.02% (Table I). All 7 clones were hormone sensitive and their population doubling time increased by 68% or more when the regular FBS used in the medium was replaced with hormone-depleted FBS (Table II). Based on the anchorage-independent growth capability of the cells, Clones 6, 17 and 21 were selected for cancer preventive agent evaluation.

The effects of 9-cis retinoic acid, fluasterone, 7β-HF, L-selenomethionine and troglitazone on cell proliferation were determined based on the concentration required to inhibit cell proliferation by 50% (IC_{50}). The results (Table III) indicate that Clone 6 was most sensitive to treatment with 9-cis retinoic acid (IC_{50}=85.4 μM) and L-selenomethionine (IC_{50}=33.9 μM), Clone 21 was most sensitive to treatment with fluasterone (IC_{50}=77.3 μM), 7β-HF (IC_{50}=0.7 μM) and troglitazone (IC_{50}=26.0 μM), while Clone 17 was less sensitive

Table III. *Effects of cancer preventive agents on cell proliferation.*

Agent	Mean IC_{50} (95% confidence interval)		
	LNCaP Clone 6	LNCaP Clone 17	LNCaP Clone 21
9-cis retinoic acid	85.4 (79.2-92.0)	>100	>100
Fluasterone	>100	>100	77.3 (46.0-130.1)
7-β-hydroxyfluasterone	>3	>3	0.7 (0.2-.3)
L-selenomethionine	33.9 (22.8-50.4)	>100	66.0 (46.1-95.7)
Troglitazone	>100	>100	26.0 (22.2-30.3)

than Clones 6 and 21 to treatment with 9-cis retinoic acid, fluasterone, 7HF, L-selenomethionine and troglitazone, as its proliferation was not inhibited by more than 50% at the highest concentration of the any of the five agents evaluated.

The effects of 9-cis retinoic acid, fluasterone, 7β-HF, L-selenomethionine and troglitazone on anchorage-independent growth were determined in LNCaP Clones 6 and 17, which are capable of forming anchorage-independent colonies in soft agar. The soft agar colony formation efficiency of LNCaP Clone 6 cells was inhibited by 27.9%, 38.0%, 20.9%, 60.5% and 43.2%, respectively (Table IV), by treatment with 9-cis retinoic acid (3 μM), fluasterone (100 μM), 7β-HF (1 μM), L-selenomethionine (30 μM) and troglitazone (10 μM). The soft agar colony formation efficiency of LNCaP Clone 17 cells was inhibited by 25.6%, 49.8% and 42.3%, respectively, by treatment with 9-cis retinoic acid (1 μM), fluasterone (100 μM) and L-selenomethionine (30 μM) but was not significantly affected by treatment with 7β-HF (up to 3 μM) or troglitazone (up to 50 μM).

The effects of 9-cis retinoic acid, fluasterone, 7β-HF, L-selenomethionine and troglitazone on apoptosis were evaluated in parental LNCaP cells and Clones 6, 17 and 21. The results (Table V) show that the level of apoptosis increased by 127% in parental LNCaP cells treated with fluasterone (40 μM) but was not significantly affected by treatment with 9-cis retinoic acid (up to 10 μM), 7β-HF (up to 3 μM), L-selenomethionine (up to 15 μM) and troglitazone (up to 15 μM). The level of apoptosis did not significantly increase in Clones 6 and 17 cells treated with any of the five agents at the concentrations tested. For Clone 21 cells, the level of apoptosis increased by 92%, 73%, 124%, 75% and 73%, respectively, after treatment with 9-cis retinoic acid (0.3 μM), fluasterone (10 μM), 7β-HF (1 μM), L-selenomethionine (1.5 μM) and troglitazone (0.5 μM), respectively.

Discussion

In the present study, seven LNCaP cell clones established in our previous study (12) were further characterized and were found to differ greatly in their ability to grow and form colonies in soft agar, although they were all sensitive to hormone deprivation in the medium. These clones are believed to represent different stages of prostate carcinogenesis in a multi-step process, as proposed previously (16, 17). The presence of multiple intermediate stages in cancer development is supported by experimental and epidemiological evidence (18-22). Cells at different stages of cancer development may possess different biological properties and respond differently to treatments with cancer preventive agents. Thus, it is important to evaluate cancer preventive agents in cells at the different stage of carcinogenesis.

Clone 6 was established from cells packed in multi-layered patches on the original plate, from which the parental LNCaP cells were cloned by the limiting dilution method (12). Clone 17 was also derived from cells that formed mainly multi-layered patches on the cell culture plate (12). These two clones are presumed to represent a more malignant phenotype based on the tendency to form multi-layered cell patches in culture and the high colony formation efficiency in soft agar observed both in this study and the previous study (12). Clone 21 was established from cells growing as a monolayer on the original plate, from which the parental LNCaP cells were cloned (12). Clone 21 is presumed to represent a less malignant phenotype, based on its monolayer morphology and the low colony formation efficiency in soft agar observed in the previous study (12) and confirmed in the present study. Clones 6, 17 and 21 were selected in this study for evaluation of the effects of five cancer preventive agents on proliferation, anchorage-independent growth and/or apoptosis.

Based on the 95% confidence intervals of the mean IC_{50} for cell proliferation, treatment with fluasterone, 7β-HF and troglitazone had a significantly stronger inhibitory effect on Clone 21 than on Clones 6 and 17. Treatment with L-selenomethionine had a significantly stronger inhibitory effect on Clone 21 cells than on Clone 17 cells, although the strongest inhibitory effect was observed on Clone 6 cells. The inhibitory effect of 9-cis retinoic acid was significantly stronger on Clone 6 cells than on cells from Clones 17 and 21. Overall, the proliferation of Clone 21 cells was more sensitive to treatment with fluasterone, 7β-HF and troglitazone than the proliferation of cells from Clones 17 and 6. In addition, all five agents tested exhibited stronger effects in inducing apoptosis in Clone 21 cells than in cells from Clones 6 and 17. Assuming that Clone 21 represents prostate epithelial cells at an early stage of carcinogenesis whereas Clones 6 and 17 represent prostate epithelial cells at a more advanced stage of carcinogenesis, the stronger effects of these agents on Clone 21 cells suggest that they are probably more effective as cancer preventive agents than as cancer therapeutic agents.

When soft agar colony formation was used as the endpoint for evaluation, cells from Clone 6 were more

Table IV. *Effects of cancer protective agents on anchorage-independent growth.*

Agent	Concentration evaluated (μM)	Lowest effective concentration (μM)	Decrease in colony formation efficiency (%)	Dunnett multiple comparison *p*-value
LNCaP Clone 6 cells				
9-cis retinoic acid	0.01-3	3	27.9±11.6	<0.05
Fluasterone	0.01-100	100	38.0±15.4	<0.05
7-β-hydroxyfluasterone	0.01-3	1	20.9±8.8	<0.05
L-selenomethionine	0.03-30	30	60.5±14.1	<0.01
Troglitazone	0.01-50	10	43.2±12.4	<0.01
LNCaP Clone 17 cells				
9-cis retinoic acid	0.01-3	1	25.6±12.5	<0.05
Fluasterone	0.01-100	100	49.8±7.8	<0.01
7-β-hydroxyfluasterone	0.01-3	>3	n.s.*	n.s.*
L-selenomethionine	0.03-30	30	42.3±18.7	0.050
Troglitazone	0.01-50	>50	n.s.*	n.s.*

*Not statistically significant.

Table V. *Effects of cancer preventive agents on apoptosis.*

Agent	Concentration evaluated (μM)	Lowest effective concentration (μM)	Apoptosis (% Control)	Dunnett multiple comparison *p*-value
Parental LNCaP cells				
9-cis retinoic acid	0.1-10	>10	n.s.*	n.s.*
Fluasterone	10-50	40	127±32	<0.05
7-β-hydroxyfluasterone	0.3-3	>3	n.s.*	n.s.*
L-selenomethionine	0.15-15	>15	n.s.*	n.s.*
Troglitazone	0.15-15	0.15	n.s.*	n.s.*
LNCaP Clone 6 cells				
9-cis retinoic acid	0.1-10	>10	n.s.*	n.s.*
Fluasterone	10-50	>50	n.s.*	n.s.*
7-β-hydroxyfluasterone	0.3-3	>3	n.s.*	n.s.*
L-selenomethionine	0.15-15	>15	n.s.*	n.s.*
Troglitazone	0.15-15	>15	n.s.*	n.s.*
LNCaP Clone 17 cells				
9-cis retinoic acid	0.1-10	>10	n.s.*	n.s.*
Fluasterone	10-50	>50	n.s.*	n.s.*
7-β-hydroxyfluasterone	0.3-3	>3	n.s.*	n.s.*
L-selenomethionine	0.15-15	>15	n.s.*	n.s.*
Troglitazone	0.15-15	>15	n.s.*	n.s.*
LNCaP Clone 21 cells				
9-cis retinoic acid	0.1-10	0.3	92±9	<0.05
Fluasterone	10-50	10	73±21	<0.05
7-β-hydroxyfluasterone	0.3-3	1	124±45	<0.05
L-selenomethionine	0.15-15	1.5	75±18	<0.05
Troglitazone	0.15-15	0.5	73±10	<0.05

*Not statistically significant.

sensitive to treatment with 7β-HF and troglitazone whereas cells from Clone 17 were more sensitive to treatment with 9-cis retinoic acid. The order of the sensitivity of Clones 6 and 17 cells to the five cancer preventive agents measured using soft agar colony formation efficiency as the endpoint does not correlate with the order of the sensitivity

measured using cell proliferation as the endpoint, indicating that the effect of a cancer preventive agent on cell proliferation does not necessarily predict its effect on anchorage-independent growth. The inference from this observation is that treatment with a combination of different cancer preventive agents is likely to be more effective since individual cells in a heterogeneous pre-malignant cell population may not all respond to a particular cancer preventive agent.

The potentials of 9-cis retinoic acid, fluasterone, 7β-HF, L-selenomethionine and troglitazone as cancer preventive agents have been demonstrated previously. It has been reported that 9-cis retinoic acid inhibits mammary carcinogenesis induced by N-nitroso-N-methylurea in Sprague-Dawley rats (23), suppresses aberrant crypt foci formation induced by azoxymethane (24), stimulates LNCaP prostate cancer cell differentiation (25) and induces apoptosis in MCF-7 human breast cancer cells (26). DHEA is an adrenocortical steroid that inhibits tumor initiation and promotion, probably by inhibiting glucose-6-phosphate dehydrogenase and the pentose phosphate pathway (5). Compared with DHEA, fluasterone (the DHEA analogue 8354) has enhanced cancer preventive activity with little or no steroid precursor like activity and without the peroxisome proliferating effect of DHEA (9, 27). L-selenomethionine is an organoselenium compound with cancer preventive activity (28), which is presumably related to increased glutathione peroxidase activity in cells and tissues after treatment with the organoselenium compound (29). It has been reported that L-selenomethionine inhibits the growth of MCF-7 breast cancer cells, DU-145 prostate cancer cells and UACC-375 melanoma cells at micromolar concentrations (30). Troglitazone belongs to a novel class of antidiabetic agents that decrease blood glucose levels in diabetic animal models and in patients with non-insulin-dependent diabetes mellitus through alleviating insulin resistance (31). It has also been shown that troglitazone, alone or in combination with LG10068, a ligand for retinoid X receptors, is effective in preventing the induction of preneoplastic lesions by 7,12-dimethyl-benz[a]anthracene (DMBA) in a mouse mammary gland organ culture model system (32).

The present study compared the efficacy of these agents in inhibiting proliferation and anchorage-independent growth of the prostate cells with varying degrees of malignant potential. In addition, the LNCaP cell clones characterized in this study can be valuable *in vitro* systems for evaluation of prostate cancer preventive and therapeutic agents.

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