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Silvestrol Regulates G_2/M Checkpoint Genes Independent of p53 Activity

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Abstract. *As previously reported, silvestrol, a rocaglate derivative isolated from* Aglaia foveolata, *has similar potency to paclitaxel and camptothecin against cultured human cancer cells. Furthermore, silvestrol can inhibit cancer cell growth in mice without noticeable toxicity when administered up to 5 mg/kg body weight (the highest dose tested). The purpose of the current study was to evaluate the mechanism of silvestrol's cytotoxicity in human prostate cancer cells (LNCaP). The molecular signature induced in LNCaP cells by silvestrol was evaluated using microarray analysis. The results revealed that 20 apoptosis and cell cycle related genes were significantly altered in LNCaP cells exposed to silvestrol. These included UBL-3, p21 and p300, which were up-regulated, and p53, which was down-regulated. Since p53 expression is governed primarily at the level of translation, p53 was also evaluated by Western blot. Silvestrol caused a dose-dependent decrease in p53 protein within 30 min of exposure with no p53 detectable after 6 h. Down-regulation of p53 by silvestrol was associated with down-regulation of MDM2 and not prevented by lactacystin suggesting that silvestrol-induced degradation of p53 is not mediated by the proteasome. A slight decrease in cyclin B was observed within 6 h of silvestrol exposure and phosphatase Cdc25C protein, which activates Cdc2, was also decreased. These data demonstrate that cytotoxicity induced by silvestrol in LNCaP cells is associated with a block in the cell cycle at the G_2/M checkpoint and alterations in the expression of genes regulating apoptosis and cell cycle in a manner independent of p53.*

Silvestrol is a rocaglate derivative that was isolated from the fruits and twigs of *Aglaia foveolata* by bioassay-guided fractionation (1, 2). Silvestrol exhibited potent *in vitro* cytotoxic activity against a panel of human cell lines derived from breast, prostate and lung cancers. The potency of silvestrol (ED_{50} 1.2 to 1.5 nM) was similar to that observed for paclitaxel (ED_{50} 0.7 to 4.7 nM) and camptothecin ($ED_{50} \approx 30$ nM). The *in vitro* studies were followed up with analysis of silvestrol *in vivo* using the hollow fiber test and the murine P388 leukemia model (1). In the hollow fiber assay, developed at the U.S. National Cancer Institute (3, 4), human cancer cells are propagated within fibers that are implanted either subcutaneously (*s.c.*) or intraperitoneally (*i.p.*) in immunodeficient mice (5). When tested at doses up to 5 mg/kg, silvestrol inhibited proliferation of all cell lines, but the most potent activity was against the human prostate cancer line designated LNCaP (up to 83% inhibition) with no detectable gross toxicity (1). Silvestrol was also tested in the P388 murine leukemia model using a previously described procedure (6). Silvestrol was active at 2.5 mg/kg when administered as five daily *i.p.* injections. A maximum increase in lifespan corresponding to a treatment/control of 150% was achieved. Silvestrol was active (treatment/control 129%) when injected intravenously (*i.v.*) on a twice daily schedule (2 qd x 5 for 2 mg/kg/day). Since silvestrol showed most potent activity against LNCaP cells with the hollow fiber model (1), this cell line was chosen for further mechanistic studies.

Gene expression microarray technology is a useful method to study the global effects of substances on gene expression in biological systems (7). Microarray technology

Abbreviations: Ct, threshold cycle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction.

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facilitates a more complete and inclusive experimental approach where alterations in the transcript level of entire genomes can be simultaneously assayed in response to a variety of stimuli. Once an overview of a substance's molecular signature is obtained for a particular biological system, such as a human cancer cell line, these data are confirmed by more conventional techniques, such as RNA or immunoblot or quantitative real-time PCR (8). The microarray data reported here indicates that silvestrol significantly altered the expression of cell cycle and apoptosis-associated genes. Furthermore, flow cytometry experiments illustrated that silvestrol induced arrest in the cell cycle at the G_2/M transition. These findings prompted us to further study silvestrol's effect on the transcription of genes regulating the G_2/M checkpoint in LNCaP cells.

Materials and Methods

Test material. Silvestrol was obtained by bioassay-guided fractionation from extracts derived from the fruits and twigs of *Aglaia foveolata* (1, 2). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

Cell culture. Human prostate carcinoma LNCaP cells were purchased from the American Type Culture Collection (Manassas, VA, USA). LNCaP cells were maintained in RPMI 1640 medium with charcoal-stripped 10% heat-inactivated calf serum and PSF (100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, 250 ng/ml amphotericin B) supplemented with 0.1 nM testosterone. Cell culture media and supplements were obtained from Life Technologies, Inc. (Grand Island, NY, USA).

Microarray data collection and analysis. LNCaP cells were seeded ($1.8x10^4$ cells/cm^2), incubated for 24 h and exposed to silvestrol (120 nM, 24 h). Total RNA of treated and untreated cells was isolated using an RNeasy Mini kit (Qiagen). RNA purity was verified by spectrophotometry ($A_{260}/A_{280} \geq 1.9$) and RNA integrity was checked by gel electrophoresis. From total RNA, double stranded cDNA and biotin labeled cRNA was synthesized. After cRNA was sheared, GeneChip hybridization with sample was preformed. GeneChip® Human Genome Focus Array (Affymetrix, Santa Clara, CA, USA), which represents over 8,500 verified human sequences from the NCBI RefSeq database was employed. Detection of bound probe was achieved using a scanning confocal laser microscope (Probe Array Scan, Agilent Technologies). Data normalization was performed by multiplying raw signal by a scaling factor, such that the trimmed mean of signals is always the same for the arrays being compared. Web based NIA array Analysis Tool (http://lgsun.grc.nia.nih.gov/ANOVA) was further used to identify a statistically significant subset of genes differentially expressed between the two sample types. Threshold for false discovery rate (FDR) equals to 0.01. ANOVA analyses produced a subset of 2,828 significantly changed genes. Functional annotation for these genes was performed with the use of the web based annotation tool DAVID (http://apps1.niaid.nih.gov/david). Genes were annotated according to Gene Ontology Consortium (http://www.geneontology.org/) classification.

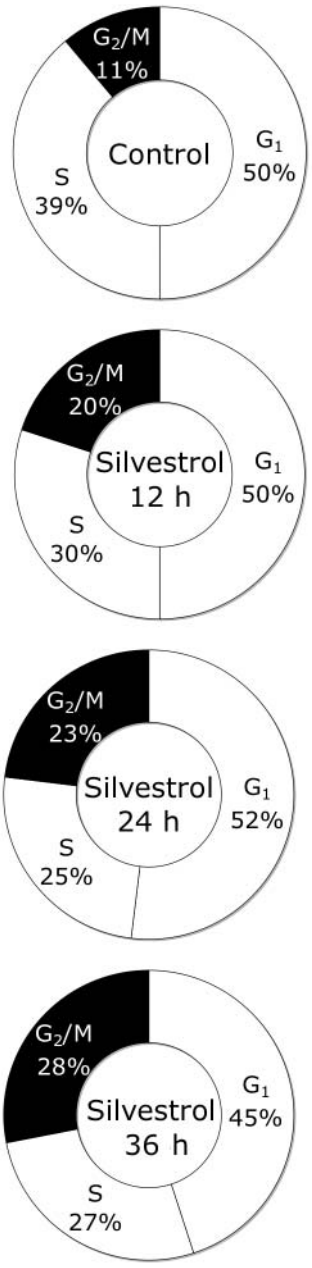

Figure 1. *Silvestrol induced cell cycle arrest in LNCaP cells. LNCaP cells were exposed to vehicle or 120 nM silvestrol for the times indicated, harvested and analyzed by flow cytometry. Note the increase in the fraction of cells in G_2/M over time as a function of silvestrol treatment.*

Cell-cycle analysis. LNCaP cells were seeded and incubated for 24 h prior to being treated with 120 nM silvestrol for the durations indicated in Figure 1. Cells were harvested by trypsinization, fixed with 70% ethanol and incubated with staining solution containing trypsinase inhibitor (30 μg/mL), RNase A (30 μg/mL) and propidium iodide (50 μg/mL) in Triscitrate buffer (pH 7.2). The cellular DNA content was then analyzed by flow cytometry.

PCR. LNCaP cells were seeded ($1.8x10^4$ cells/cm^2), incubated for 24 h as described above, and exposed to silvestrol for 24 h at 120, 240 and 480 nM. Total RNA was isolated from cultured LNCaP cells with TRIzol reagent and quantified by UV absorbance. The reverse transcription of RNA was performed by using SUPERScript™ Preamplification System (Life Technologies, Inc., Rockville, MD, USA) as described previously (9). As an internal control, 0.125 μmol of primers for GAPDH (5'-CGG GAA GCT TGT GAT CAA TGG-3'; 5'-GGC AGT GAT GGC ATG GAC TG-3' for 336 bp; or 5'-ACC CAG AAG ACT GTG GAT GG-3', 5'-AGG GGT CTA CAT GGC AAC TG-3' for 598 bp) were added. The primers and cell cycle condition for each gene were as follows. For UBL-3 the primers were 5'-GTT GGC CCA GTA ATT AAC TTC-3' and 5'-CTT TCA TGC TGC TGG GAC A-3' with an initial denaturation at 94˚C for 4 min followed by 30 cycles of 94˚C 30 sec, 52˚C 30 sec and 72˚C 30 sec. For p21 the primers were 5'- CCT GGC ACC TCA CCT GCT CTG CTG-3' and 5'-GCA GAA GAT GTA GAG CGG GCC TTT-3' with an initial denaturation at 96˚C for 3 min followed by 30 cycles of 96˚C 30 sec, 57˚C 30 sec and 70˚C 50 sec. For p300 the primers were 5'-GAA TTA ATC AAC TCT ACA GA-3' and 5'- CAA TTT ATC AAA CCT AAT CC-3' with an initial denaturation of 94˚C for 5 min followed by 35 cycles of 94˚C 60 sec, 60˚C 60 sec, 72˚C 90 sec. For p53 the primers were 5'-CAG CCA AGT CTG TGA CTT GCA CGT AC-3' and 5'-CTA TGT CGA AAA GTG TTT CTG TCA TC-3' with an initial denaturation of 94˚C for 4 min followed by 35 cycles of 94˚C 60 sec, 55˚C 120 sec, 72˚C 45 sec. After the final cycle, a 10 min elongation step at 72˚C was performed. Aliquots of PCR products were electrophoresed on 2% agarose gels, and PCR fragments were visualized with ethidium bromide staining.

For real-time PCR, the cDNA synthesis was performed using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The reaction was performed for 10 min at 25˚C, followed by 48˚C for 30 min and a 5-min incubation step at 95˚C. The PCR and subsequent analyses were performed in the GeneAmp 5700 Sequence Detection System (Applied Biosystems). Real-time quantitation was performed using the TaqMan technology of Applied Biosystems. p21 primers and probe sequences were 5'-CCC GTG AGC GAT GGA ACT-3', 5'-CGC TCC CAG GCG AAG TC-3' and 5'-CGA CTT TGT CAC CGA GAC ACC ACT GG-3', respectively.

PCR reaction was performed in triplicate in MicroAmp® Optical 96-well Reaction Plate with ABI PRISM™ Optical Caps. The PCR reaction mixture contained different concentrations of primers and probe depending on the gene of interest, and 1 x TaqMan Universal Master Mix. Concentrations used for primers are 900 nM for both forward and reverse primers and 150 nM for probe. The reactions were first incubated at 50˚C for 2 min, followed by 10 min at 95˚C. The PCR itself consisted of 40 cycles with 15 sec at 95˚C and 1 min at 60˚C each. The fluorescence signal was measured during the last 30 sec of the annealing/extension phase. After the PCR, a fluorescence threshold value was set and threshold cycle (Ct) values were determined as the fractional cycle at which the fluorescence signal reached a plateau. These values were used for further calculations.

β-Actin was used as an internal control to correct for variability in total RNA used for each reaction and to compensate for different levels of inhibition during reverse transcription of RNA into cDNA. p21 and β-actin expression were related to a standard curve derived from a serial dilution of control cDNA with dH_2O. Also, p21 and β-actin quantities were expressed in terms of ng of RNA yielding the same level of expression. Subsequently, normalization was achieved by dividing the expression level of p21 by the β-actin expression level. Finally, results were expressed as a percentage, where the level of p21 observed in the control sample was set as 100%.

Analysis of protein expression. Western blots were performed on extracts of LNCaP cells exposed to silvestrol at the concentrations and for the durations indicated in the figures. Cells were seeded as described above ($1.8x10^4$ cells/cm^2) and harvested using cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin). Equivalent amounts of protein (as determined by BCA assay, Pierce Biotechnology, Rockford, IL, USA) were electrophoresed by SDS-PAGE in 7.5-12.5% Tris-glycine acrylamide gels (9). To assure accurate loading, β-actin was probed using an anti-β-actin anibody from Sigma Chemical, St. Louis, MO, USA. Following transfer to polyvinylidene difluoride membranes and blocking with 5% non-fat dry milk, the membranes were incubated with antibodies against p53, MDM2, HSP90α/β, Cdc2, Cdc25C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and cyclin B (Oncogene Research Products, Inc., San Diego, CA, USA). The antibody for androgen receptor was kindly provided by Gail Prins (Department of Urology, University of Illinois at Chicago, IL, USA), analyzed by HRP-linked secondary antibodies (Cell Signaling) and visualized using an ECL detection system (Amersham Life Science, Piscataway, NJ, USA).

Results

Microarray data collection and analysis. LNCaP cells were seeded ($1.8x10^4$ cells/cm^2), incubated for 24 h and exposed to 120 nM silvestrol for an additional 24 h. The cells were then harvested and mRNA expression was analyzed as described above. The results from these studies afforded an overview of silvestrol's effect on LNCaP cells (Table I). In particular, it was evident that a number of proteins involved in the regulation of apoptosis and the transition from G_2 to M in the cell cycle were affected in LNCaP cells by silvestrol treatment. We have grouped the genes presented in Table I into two broad categories: genes that code for apoptosis related proteins and genes that code for proteins involved in cell cycle regulation.

Apoptosis related genes included caspases -9, -4 and -10, which were up-regulated 3.2-, 2.4- and 2.0-fold, respectively. Furthermore, activating transcription factor 3 (ATF3), which enhances caspase activation and apoptosis (10), was up-regulated 25-fold. ATFs 5, 1 and 2 were also up-regulated 2.8-, 1.7- and 1.5-fold by silvestrol exposure. Also, the cell death activator CIDE-3, a member of the cell-death-inducing DNA-fragmentation-factor family (11), was up-regulated by silvestrol treatment 1.9-fold. Conversely, inhibitors of apoptosis were down-regulated. Examples include Aven, an inhibitor of caspase activation (12), and apoptosis inhibitor 5 (API5 or AAC- 11) (13).

Table I. *Summary of microarray results*[a].

Affymetrix Probe Set ID	Gene Description	Gene Symbol	Fold Change
NM 001674.1	activating transcription factor 3	ATF3	25.340
NM 015675.1	growth arrest and DNA-damage-inducible, beta	GADD45B	17.734
NM 004091.1	E2F transcription factor 2	E2F2	8.293
NM 002892.2	retinoblastoma binding protein 1	RBBP1	6.028
AV 727101	E1A binding protein p300	EP300	3.451
U 60521.1	caspase-9, apoptosis-related cysteine protease	CASP9	3.179
M 96577	E2F transcription factor 1	E2F1	2.979
NM 012068.2	activating transcription factor 5	ATF5	2.833
U 25804.1	caspase 4, apoptosis-related cysteine protease	CASP4	2.411
U 25804.1	ubiquitin-like 3	UBL3	2.109
NM 001230.1	caspase-10, apoptosis-related cysteine protease	CASP10	2.032
NM 022094.1	cell death activator CIDE-3	CIDE-3	1.956
AI 434345	activating transcription factor 1	ATF1	1.746
W 58696	growth arrest-specific 1	GAS1	1.560
AA 029873	cyclin-dependent kinase inhibitor 1A (p21, Cip 1)	P21	1.410
NM 001237.1	cyclin A2	CCNA2	-1.733
NM 006595.1	apoptosis inhibitor 5	API5	-1.749
NM 020371.1	apoptosis, caspase activation inhibitor	AVEN	-1.768
NM 000546.2	tumor protein p53 (p53)	TP53	-1.895

[a]Microarray data collection and analysis were performed as described in "Materials and Methods". The false discovery rate for each gene was less than 0.001. Statistically significantly regulated genes were indicated as the Fold Change (Treatment/Control).

Silvestrol also had a significant affect on genes that regulate the cell cycle in LNCaP cells. For example, the E2F family of transcription factors, which regulate the expression of genes involved in progression through the restriction point (14), were up-regulated: E2F2 8.3 fold and E2F1 3.0 fold. The retinoblastoma tumor suppressor pRB (RBBP1) protein is well known to govern progression of the cell through the restriction point (14). The expression of this tumor suppressor gene was up-regulated 6-fold by silvestrol. In addition, the product of the tumor suppressor gene growth arrest-specific gene 1 (GAS1) inhibits the G_0/G_1 transition (15). Silvestrol treatment induced GAS1 expression 1.9-fold. Cyclin A2 promotes both G_l/S and G_2/M transitions in somatic cells (16) and decreases in cyclins A2 and B1 levels in SCC97 cells lead to G_2 arrest (17). Cyclin A2 was down-regulated 1.7 fold by silvestrol treatment.

Some of the most striking results observed from the array data were in the expression of genes involved in the G_2/M transition (Table I). For example, Gadd45b and Gadd45g are Cdc2/cyclinB1 kinase inhibitors. Gadd45b and Gadd45g, as well as Gadd45a, interact with both Cdc2 and cyclinB1 inhibiting the kinase activity of the Ccd2/cyclinB1 complex (18). Gadd45B was up-regulated 17-fold in response to silvestrol. The tumor suppressor gene p53 is a key protein regulating progression through G_2/M and apoptosis (19). Surprisingly, p53 expression was down-regulated about 1.9-fold in response to silvestrol. Furthermore, p21, which is regulated in part by p53, was up-regulated about 1.4-fold.

Cell-cycle analysis. In a parallel set of experiments, it was observed by flow cytometry that silvestrol (Figure 1) and a structural analog of silvestrol, rocaglaol (20), both block the cell cycle of LNCaP cells in the G_2/M transition. Figure 1 contains pie charts that represent the percentage of cells in each phase of the cell cycle as a function of vehicle or silvestrol treatment. By 12 h, the percentage of cells in the G_2- or M-phase increased from 11% in the control cells to 20% in the silvestrol treated cells. This trend continued through 36 h of silvestrol exposure at which time 28% of the cells were in G_2/M. Based on these flow cytometry results and the array data described above, we decided to focus on genes involved in the G_2/M transition for the present study.

PCR. In order to confirm our array results, PCR experiments were performed for UBL-3 (Figure 2A), p21 (Figure 2B), and p300 (Figure 2C) and p53 (Figure 2D). Silvestrol was administered to LNCaP exactly as in the array experiments with additional higher concentrations evaluated for potential dose response effects. Expression of the housekeeping gene GAPDH served as control. As shown in Figure 3, the PCR results confirmed the array data (Table

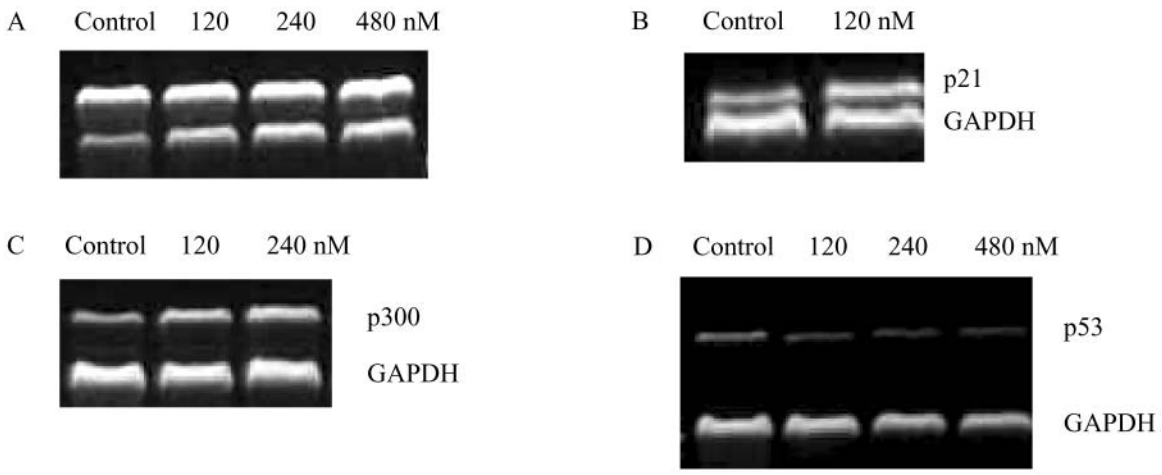

Figure 2. *RT-PCR analysis of UBL3, p21, EP300 and p53 mRNA expression. LNCaP cells were seeded at 1.8x10^4 cells/cm^2 and incubated for 24 h. The cells were then treated with the indicated concentrations of silvestrol for 24 h prior to isolation of RNA performance of RT-PCR.*

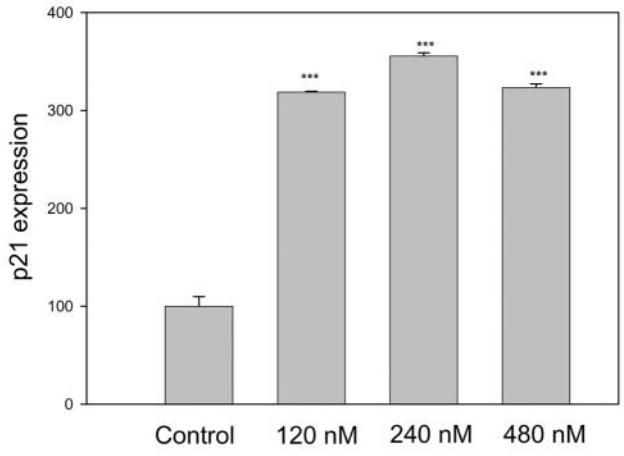

Figure 3. *Real-time PCR analysis of p21 expression in LNCaP cells exposed to silvestrol for 24 h. LNCaP cells were seeded at 1.8x10^4 cells/cm^2 and incubated for 24 h. The indicated concentration of silvestrol was then added for an additional 24 hours prior to harvest and analysis for p21 expression.*

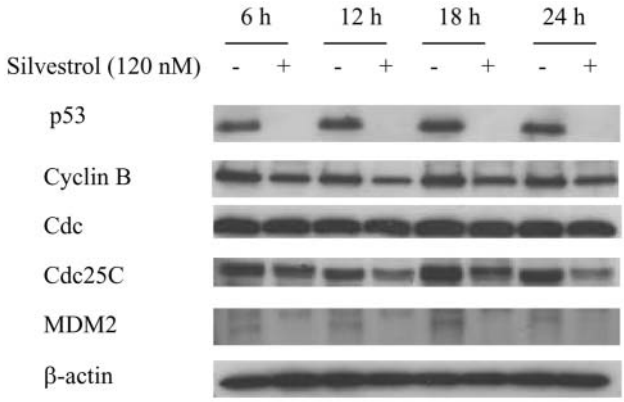

Figure 4. *Time-course analysis of the expression of cell cycle related proteins in LNCaP cells exposed to vehicle or 120 nM silvestrol. LNCaP cells were seeded at 1.8x10^4 cells/cm^2 and incubated for 24 h. Silvestrol was then added for the indicated times. The cells were then harvested for western analysis as described in Materials and Methods.*

I). A surprising result, consistent with the array data, was the decrease in p53 mRNA, which was inhibited in a dose related fashion (Figure 2D) by silvestrol treatment. As noted above, expression of p21 mRNA, which is known to be up-regulated by p53, was actually increased by about 1.4-fold. Quantitative real-time PCR was used to evaluate p21 expression in response to silvestrol (120, 240, 480 nM for 24 h). As illustrated in Figure 4, silvestrol treatment significantly increased the expression of p21 (over 3-fold; $p<0.0001$).

Protein expression. In a time-course study, treatment of LNCaP cells with 120 nM silvestrol for p53 protein was undetectable by 6, 12, 18 and 24 h after 120 nM silvestrol treatment (Figure 4). A 30-min dose-response study further indicated that silvestrol caused a dose dependent decrease of p53 expression from 7.5 to 480 nM (Figure 5A). To characterize whether silvestrol targets p53 at the mRNA or protein level, a 30 min dose response RT-PCR study was performed. No significant change in p53 mRNA expression was detected (Figure 5B).

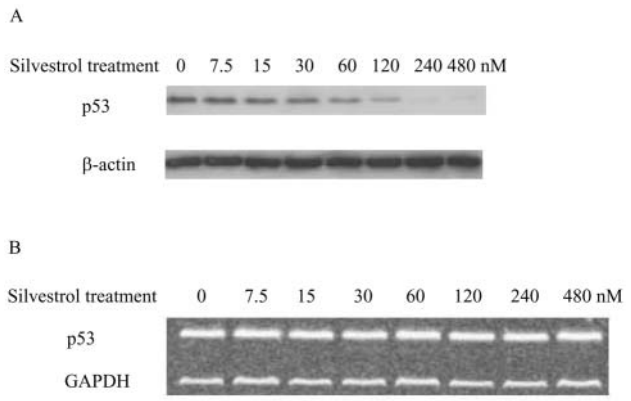

Figure 5. *p53 protein expression is down-regulated by silvestrol in a dose-dependent manner. LNCaP cells were seeded at 1.8x10^4 cells/cm^2 and treated with indicated concentrations of silvestrol for 30 min. Cells were then harvested and evaluated by Western blot (A) and RT-PCR (B) as detailed in the text.*

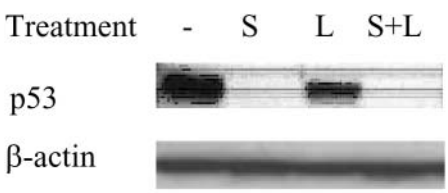

Figure 6. *p53 degradation in LNCaP cells is independent of the proteasome. LNCaP cells were seeded at 1.8x10^4 cells/cm^2 and incubated for 24 h. The cells were then exposed for 24 h to silvestrol (S, 120 nM) and/or the proteasome inhibitor lactacystin (L, 1000 nM).*

To test the hypothesis that the loss of p53 protein was mediated by the proteasome pathway, MDM2, which is a ubiquitin ligase E3 that targets p53 for catalysis by the proteasome (21), was measured in a time course study. Surprisingly, treatment of 120 nM silvestrol also caused MDM2 protein to be degraded within 6 h of silvestrol exposure (Figure 4). Furthermore, degradation of p53 was not blocked by the potent proteasome inhibitor lactacystin (1.0 mM, Figure 6), which was incubated for 24 h together with silvestrol (120 nM).

The Cdc2/cyclin B complex, originally defined as the maturation-promoting factor (MPF), is required for progression from G_2 to M (22). To assess the effect of silvestrol on the Cdc2/cyclin B complex, a time course experiment was conducted. As illustrated in Figure 4, cyclin B decreased somewhat in response to silvestrol at all time points evaluated, but Cdc2 was not affected. However, protein phosphatase Cdc25C, which dephosphorylates Cdc2, decreased after 6 h of silvestrol treatment (Figure 4).

Since LNCaP cells are dependent on androgens for proliferation and survival, the effect of silvestrol on the molecular chaperone HSP90, which binds androgen receptor, as well as androgen receptor itself, was examined. However, silvestrol had no effect on the expression of either HSP90 or androgen receptor (120 nM, up to 24 h; data not shown).

Discussion

Evaluation of a drug candidate's molecular signature by microarray technology is proving to be a useful tool for drug discovery and development, since it affords investigators a broad overview of the types of pathways affected by a novel agent (7). In the present study, 8,500 verified human sequences were queried to obtain a summary of silvestrol's molecular mechanism of cytotoxicity in LNCaP cells. Twenty apoptosis and cell cycle regulated genes were found to be significantly modulated by silvestrol (Table I). Based on these array data and flow cytometry data indicating that silvestrol (Figure 1) and the structurally similar rocaglaol block the G_2/M transition of the LNCaP cell cycle, we evaluated the effect of silvestrol on the expression of genes that regulate progression of the cell cycle from G_2 to M.

The cell division cycle is tightly regulated at specific checkpoints by the products of a family of cell division cycle (Cdc) genes. The Cdc gene products are regulated by a family of serine and threonine kinases known as cyclins by virtue of the periodicity of their synthesis and degradation during the cell cycle. Entry into mitosis is governed by activation of Cdc2 through its association with cyclin B, which together form the M-phase promoting factor. Cdc2 activity, and entry into mitosis, can be inhibited several ways. One is by down-regulation of Cdc2 from cyclin B. However, silvestrol treatment did not result in significant decrease either Cdc2 itself or cyclin B protein (Figure 4). Alternatively, Cdc2 activity may be suppressed, if not dephosphorylated by Cdc25C. Silvestrol treatment did produce a substantial decrease in Cdc25C protein (Figure 4). Another means by which Cdc2 could be inhibited is by increased activity of cyclin dependent kinase inhibitors such as p21 or Gadd45 (23). While p21 inhibits Cdc2 directly, Gadd45 dissociates Cdc2 from cyclin B1. As illustrated in Figures 2 and 3, p21 expression was significantly increased in LNCaP cells by silvestrol exposure. Also, Gadd45B expression was dramatically increased (approximately 18 fold, Table I). These data suggest that a decreased abundance of Cdc25C dephosphorylase and increase in p21 and Gadd45B expression may contribute significantly to the silvestrol-induced disruption in the LNCaP cell cycle at the G_2M transition.

The expression of both p21 and Gadd45B genes are known to be regulated by p53 (23). Furthermore, p53 plays

an important role in regulating the G_2/M checkpoint (23) and inducing apoptosis (24) in LNCaP cells, which are homozygous for wild-type p53 (25). Therefore, we hypothesized that the G_2/M block and induction of p21 and Gadd45 were driven by p53 induction. However, we were surprised to discover that silvestrol treatment caused p53 protein to be down-regulated in a dose- (Figure 5A) and time- (Figure 4) dependent manner. While p53 protein levels declined, mRNA levels remained constant as judged by RT-PCR even at high levels of silvestrol exposure (Figure 5B), which is consistent with p53 expression being regulated at the level of translation. Under normal physiologic conditions, cellular p53 protein has a short half-life of about 20 min (26). P53 is ubiquinated for degradation by the ubiquitin/proteasome system (27). Ubiquitination and degradation of p53 is dependent on MDM2 (28, 29), which is an E3 ubiquitin ligase that can recruit an E2 ubiquitin conjugating enzyme to ubiquitinate p53 (30). MDM2 is known to interact with p300 to effect the normal turnover of p53 (30). While p300 mRNA expression was increased, MDM2 protein did not increase (Figure 4) nor was MDM2 mRNA expression altered in the microarray studies (data not shown). Guillot *et al.* (31) have previously reported that tamoxifen treatment of MCF-7 cells, which are wild-type for p53, causes a gradual decrease of p53 protein, but not mRNA. Results from studies on the synthesis rate and half-life of p53 were measured by [^{35}S]methionine incorporation led these investigators to conclude that the decline in p53 protein in MCF-7 cells exposed to tamoxifen was due to a decreased rate of protein synthesis.

In summary, as previously observed, silvestrol and the structurally similar rocaglaol are cytotoxic to human prostate cancer (LNCaP) cells with similar potency to paclitaxel or camptothecin. In the current study, it was observed that silvestrol arrests the LNCaP cell cycle at the G_2/M transition. Also, we report that silvestrol induces p21 and Gadd45B expression while decreasing Cdc25C expression, which could contribute to G_2/M arrest. Furthermore, these effects are independent of p53 activity.

Acknowledgements

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