The novel estrogen 17α-20*Z*-21-[(4-amino)phenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol induces apoptosis in prostate cancer cell lines at nanomolar concentrations *in vitro*

James A. Mobley,[1] James O. L'Esperance,[1] Mengchu Wu,[1] Carolyn J. Friel,[2] Robert H. Hanson,[2] and Shuk-Mei Ho[1]

[1]Department of Surgery, Division of Urology, University of Massachusetts Medical School, Worcester, Massachusetts and [2]Department of Chemistry, College of Arts and Sciences, Northeastern University, Boston, Massachusetts

Abstract

Prostate cancer remains the number one cause of non-cutaneous cancer, with 220,900 new cases predicted for the year 2003 alone. Of the more promising classes of compounds studied thus far for the treatment of prostate cancer, estrogens of various types have consistently exhibited antitumor activities both *in vitro* and *in vivo*. For this reason, we have synthesized and screened a library of unique 17α/11β modified 17β-estradiol (E_2) analogues designed for estrogen receptor β (ER-β) specificity and a potential for cytotoxic activity directed toward prostate cancer cells. From this library, the novel compound 17α-20*Z*-21-[(4-amino)phenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol ($APVE_2$) was identified as the primary lead, found to induce a high level (>90%) of cell death through an apoptotic mechanism, with an EC_{50} of 1.4, 2.7, and 16 nM in the LNCaP, PC3, and DU145 cell lines, respectively. $APVE_2$ was found to bind to ER-β, albeit weakly, with an EC_{50} of 250 nM and a binding activity of 6.2% relative to E_2, nearly two orders of magnitude less than the concentration required to induce apoptosis. $APVE_2$ bound preferentially to ER-β by 7-fold over ER-α, and did not induce growth in the MCF-7 cell line, thus indicating that it is not a classical ER agonist. Furthermore, the cytotoxic actions of $APVE_2$ were not reversed by co-treatment with a 50-fold excess E_2. In summary, a novel 17 modified estrogen $APVE_2$ was identified as a lead compound, capable of inducing apoptosis in three prostate cancer cell lines at low nanomolar concentrations, through a mechanism inconsistent with an ER-mediated mechanism. [Mol Cancer Ther 2004;3(5):587–95]

Introduction

Prostate cancer remains the number one cause of non-cutaneous cancer, and the second leading cause of cancer-related deaths in American men with 220,900 new cases predicted for the year 2003 alone (1). For patients diagnosed with localized disease, treatments such as extirpative surgery and radiation therapy are associated with promising long-term outcomes (2). However, 15–20% of these patients will go on to present with advanced metastatic disease (1, 2). The current treatment strategies for patients with metastatic prostate cancer revolve around inhibition of androgen biosynthesis, and/or direct blockade of the androgen receptor. This approach, referred to as hormone therapy, currently includes luteinizing hormone-releasing hormone (LHRH) agonists, androgen receptor antagonists, and direct inhibitors of androgen biosynthesis (2, 3). While the classic hormone therapy approaches are known to lengthen the time to symptomatic onset, there is no significant increase in life span (2–6). This unfortunate observation is a result of the inevitable selection of cancer cells capable of proliferating independent of androgen stimulation (6). Treatment strategies following this stage are generally considered palliative, as no survival benefits have been documented in a significant patient population to date (6).

Therefore, a number of novel therapeutic strategies are actively being investigated. Of the more promising classes of compounds emerging for the treatment of prostate cancer, estrogens of various types have exhibited antitumor activities both *in vitro* and *in vivo* (6, 7). For example, estramustine, diethylstilbestrol (DES), raloxifene, genistein, resveratrol, and licochalcone are estrogens that have all shown to be promising agents for the treatment or prevention of prostate cancer (7–13). Interestingly, the mechanism(s) reported to be responsible for the noted antitumor properties of this class are numerous, and yet have not been thoroughly defined *in vivo*. From this perspective, most estrogens are believed to primarily act on the hypothalamic-pituitary axis, thereby inhibiting testosterone synthesis (3, 14). However, some estrogens including DES, 2-methoxy-E_2, and estramustine have been found to exhibit a level of antitumor activity that is likely to be independent of this pathway (14–17). In addition, the pure antiestrogen ICI-182,780 and the selective estrogen receptor modulator raloxifene have been reported to decrease cell number and induce apoptosis in prostate cancer cell lines *in vitro* through what has been described as an estrogen receptor β (ER-β)-mediated mechanism

Received 9/8/03; revised 2/17/04; accepted 2/26/04.

Grant support: NIH (DK61084) and US Army Prostate Research Program (DAMD17-98-1-8606) (S-M. Ho), NIH (CA81049) (R.H. Hanson), and Grant Number 5 P30 DK32520 from the National Institute of Diabetes and Digestive and Kidney Diseases.

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Note: J.A. Mobley and J.O. L'Esperance contributed equally to this work.

Requests for Reprints: Shuk-Mei Ho, Department of Surgery, Division of Urology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324. Phone: (508) 856-1909; Fax: (508) 856-8699. E-mail: shuk-mei.ho@umassmed.edu

(18, 19). This is important, in that while the role of ER-β in prostatic tissues is not well defined, the heightened levels of expression of this receptor in the early and advanced stages of disease make it an attractive and novel drug target for the treatment of prostate cancer (20, 21). Therefore, ER-β-specific pro-apoptotic ligands have the potential to serve as a new class of antineoplastics for the treatment of prostate cancer.

Defined differences exist within the ligand binding pockets of the two ER subtypes, ER-α and ER-β, which correspond primarily with two amino acids Met421 α/Ile336 β and Leu384 α/Met336 β, thereby allowing the design of receptor subtype-specific ligands as noted in numerous reports (22). These amino acids reside in the proximity of the 7α and 11β positions of the ERs bound to 17β-estradiol (E_2). In addition, ICI-182,780 and raloxifene both contain modifications that correspond to the 7 position of E_2. For this reason, we have synthesized and screened a series of unique 17α/11β modified estrogens with the ability to affect regions within the ER corresponding to the 11β and/or 7α domains and the potential to exhibit ER-β mediated pro-apoptotic activity toward prostate cancer cells (unpublished data) (23–28). Our initial screening results produced a lead compound, 17α-20*Z*-21-[(4-amino)phenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol (APVE$_2$), which was capable of inducing a high level of cell death. Therefore, we have further characterized the cytotoxic actions of APVE$_2$ through viability, apoptosis, and ER selectivity studies in prostate cancer-derived cell lines *in vitro*, the results of the analysis are reported within.

Materials and Methods

General Synthesis

All reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The synthesis of APVE$_2$ is described below (Fig. 1).

3-Acetoxy-(17α-20*Z*)-21-(tri-*n*-butylstannyl)19-norpregna-1,3,5(10)20-tetraene-17β-ol (**3**). Protection of the 3-OH phenol was carried out as previously described with changes (27, 29). Briefly, 17α-ethynylestradiol (EE) (**1**) (1.7 g, 5.7 mM) was stirred with excess acetic anhydride (1 ml) in pyridine (15 ml) at room temperature for 12 h. The reaction mixture was poured into ice water, stirred for 2 h, and filtered. The filtrate was collected and recrystallized from hexane:acetone (1:1) to give the carboxylate ester **2** as glassy crystals (1.8 g, 93%). The *cis*-vinyl-tributyltin-EE isomer was prepared for the following coupling step as previously reported with modifications. Briefly, to a solution containing **2** (6.76 g, 20 mM) in tetrahydrofuran (20 ml), was added tri-*n*-butyl tin hydride (8.5 ml, 31 mM) and 1 M triethylborane (1 ml, 8.8 mM). The reaction was stirred under nitrogen at room temperature for 10 h. The THF was removed under reduced pressure and the resulting oil was separated via silica gel column chromatography using hexane:ethyl acetate (5:1) as both the packing and eluting solvent. Product **3** was isolated as an oil, solidifying on standing [4.6 g, 36%; 63% based on recovered starting material; R_f = 0.68 (hexane:ethyl acetate, 5:1); mp 80–82°C].

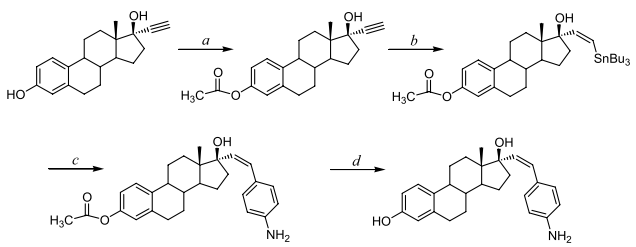

Figure 1. Synthesis of 17α-*Z*-*p*-aminophenylvinyl-17β-estradiol (APVE$_2$). *a*, 17α-Ethynyl-E_2, acetic anhydride, pyridine, room temperature, 12 h; *b*, **2**, tributyltin hydride, triethyl borane, N_2, room temperature, 10 h; *c*, *p*-iodoaniline, triphenylphosphine palladium (0) in refluxing toluene, 20 min added to **3**, 2,6-di-*t*-butyl-4-methylphenol, toluene, reflux, N_2, 17 h; 10% KF/H_2O, 1 h, room temperature; *d*, **4**, 10 N sodium hydroxide in methanol, 2 h, room temperature.

(17α, 20*Z*)-21-[(4-Amino)phenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol (**5**). The Stille coupling and deprotection were carried out as previously described with modifications (27). A mixture of *p*-iodoaniline (0.22 g, 5.7 mM) was stirred with Tetrakis (triphenylphosphine) palladium (0) (0.028 g, 0.02 mM), in refluxing anhydrous toluene (25 ml) for 20 min. A solution of **3** (1.7 g, 2.8 mM) and crystal of 2,6-di-*tert*-butyl-4-methylphenol in toluene (20 ml) was added. The reaction mixture was refluxed under nitrogen for 17 h and cooled to room temperature. A 10% KF/H_2O (20 ml) solution was added and the mixture was stirred for 1 h. The solution was filtered to remove Pd black, no $SnBu_3F$ precipitated, then partitioned between ethyl acetate:water (100:100 ml). The aqueous layer was extracted with ethyl acetate (2 × 50 ml). Organic layers were combined and washed with brine (100 ml) and water (2 × 100 ml), dried over magnesium sulfate, and concentrated. The residue was chromatographed on silica gel eluted with hexane:ethyl acetate (4:1) to afford **4** (0.06 g pure and 0.5 g mixture > 35%): R_f = 0.32 (hexane:ethyl acetate, 5:1). The crude acetylated product (~0.5 g) was stirred for 2 h in a solution containing 10 N sodium hydroxide in methanol and acidified with 4% acetic acid. The deprotected product was then partitioned between ethyl acetate and water, dried over magnesium sulfate, and purified by column chromatography to yield a dark orange solid **5** (0.34 g, 75.3%) followed by three rounds of recrystallization from acetone:chloroform (1:1) to yield a light orange powder; (0.31 g, 68%): R_f = 0.47 (1:1 hexane:ethyl acetate); mp 140–142°C; ^{1}H NMR ($CDCl_3$): 0.91 (s, 3H, 18-CH_3), 1.2–2.9 (m, 15H, steroid nucleus), 3.8 (s, b, 2H, -NH_2), 4.6 (s, b, 17-OH), 5.79 (d, 1H, $J_{20\text{-}21}$ = 12.90 Hz, 20-H), 6.40 (d, 1H, $J_{21\text{-}20}$ = 12.60 Hz, 20-H), 6.56 (d, 1H, $J_{4\text{-}2}$ = 2.7 Hz, 4-H), 6.64 (m, 4H, 2-H, 4-H, 24-H, and 26-H), 7.16 (d, 1H, $J_{1\text{-}2}$ = 8.4 Hz, 1-H), 7.26 (s, $CDCl_3$), 7.32 (d, 2H, J = 8.4 Hz, 23-H, and 27-H); ^{13}C NMR (acetone d_6) 15.04 (C-18), 24.17 (C-15), 27.79 (C-11), 28.66 (C-7), under acetone peak (C-6), 32.94 (C-12), 38.51 (C-16), 41.25 (C-8), 45.03 (C-9), 49.12 (C-13), 50.12 (C-14), 83.99 (C-17), 113.90 (C-2); 114.58 (C-24 and C-26), 116.25 (C-4), 127.41 (C-1), 131.14 (C-21), 132.33 (C-10), 132.66 (C-23 and C-27), 133.03 (C-20), 138.76 (C-5), 150.02 (C-25), 156.29 (C-3).

Molecular Modeling

Local energy minima conformers of $APVE_2$ were initially determined using Chemdraw 3D v.7.0 (CambridgeSoft, Cambridge, MA), and ''fitted'' into the ligand-binding domains of ER-α (1ERE) and ER-α (1QKM) following manual superimposition using Deepview Swiss-PDB Viewer v.3.7 (GlaxoSmithKline and the Swiss Institute of Bioinformatics, http://us.expasy.org/spdbv). Crystal structures were downloaded from the protein database.

Cell Culture

DU145, LNCaP, PC3, and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). DU145, PC3, and MCF-7 cells were maintained in DMEM/F-12 supplemented with 5% heat inactivated fetal calf serum and penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA). LNCaP cells were maintained in RPMI 1640 supplemented in the same fashion. Cells were cultured in 75-cm^2 flasks, 24- and 96-well plates (Falcon, BD Biosciences, Bedford, MA) at 37°C and 5% CO_2. For the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) and bromodeoxyuridine (BrdUrd) assays, 5000 cells and 800 cells were plated in each well of a 24- and 96-well plate, respectively, and allowed to attach for a 48-h period in the media described, and changed to a defined medium for 24 h before beginning the treatment (DMEM/F-12 or RPMI 1640) supplemented with insulin (BD Biosciences). A 1000× solution of the compound of interest solubilized in DMSO (Sigma, St. Louis, MO) was diluted in the defined media just before starting each experiment, and media were refreshed every 48–72 h throughout the experiments.

MTS

Cell viability assays were carried out with five logs of concentration between 0.1 nM and 1 μM for $APVE_2$, including controls in DU145, LNCaP, PC3, and MCF-7 cell lines. On completing 5 days of treatment, MTS activity was measured as per the manufacturer's instructions (Promega, Madison, WI). All experiments were carried out in quadruplicate; control wells were treated with the carrier DMSO. E_2 (Steraloids, Newport, RI) at 1 μM and resveratrol (Sigma) at 10 μM were used as negative and positive controls, respectively.

ER competition studies were carried out using the MTS assay in DU145 cells with E_2 as the competitor. The concentration of $APVE_2$ used was derived from the EC_{50} concentration. Treatment with E_2 at concentrations of 1 μM was performed at 0 and 1 h before the addition of 16 nM (EC_{50}) $APVE_2$. Each experiment was carried out with carrier, E_2, E_2 with $APVE_2$, and $APVE_2$ alone.

BrdUrd Uptake Assay

Proliferation was measured through the uptake of BrdUrd as measured by the cell proliferation Biotrak ELISA system kit (Amersham Biosciences, Arlington Heights, IL). Cells were plated and treated in triplicate with 16 nM and 1 μM $APVE_2$, 1 μM E_2, or carrier alone (no treatment, NT) over a 5-day period. The assay was carried out as per the manufacturer's instructions in a 96-well plate format.

ER Binding

Binding affinities were determined by fluorescence polarization with the Victor 2 1420 FP analyzer set to FP @ 485 nm (Ex), 530 nm (Em) (Perkin-Elmer, Wellesley, MA), in conjunction with ER-α and ER-β kits (Pan Vera, Madison, WI) containing a fluorescent estrogen (E2S). All assays were carried out as per the manufacturer's instructions with the following modifications. Briefly, 30 μl of competitor diluted in 10% DMF and the binding buffers supplied with the kit were combined with 30 μl of a solution containing 30 nM ER and 2 nM E2S in each well of the microplate (Corning 384 well black NBS # 3654). Each concentration was carried out in quadruplicate at 25°C after equilibration for 2 h, with E_2, 4-hydroxytamoxifen (4-OH-TAM), and DES used as controls. The effective concentration at 50% maximal activity (EC_{50}) was calculated for each compound and used to calculate the relative binding activities.

Western Blot Analysis

DU145 cells were cultured as described. Protein extraction was carried out using the MPER extraction kit (Pierce, Rockford, IL) containing the protease inhibitor III cocktail kit (Calbiochem, San Diego, CA) as per the manufacturer's instructions. Protein was quantified in quadruplicate using a BCA kit (Pierce) along with BSA (Pierce) standard curve. Twenty-five micrograms of protein were added to each well of a 10-well 7.5% pre-poured acrylamide gel (Bio-Rad, Hercules, CA) and analyzed in triplicate. To determine specificity and relative sensitivities, increasing concentrations up to 10 ng of each recombinant protein ER-α_r and ER-β_r (Pan Vera) were blotted in the presence of each ER-specific antibody used. A kaleidoscope molecular weight standard (Bio-Rad) was used to assess the relative size of the proteins. The gel was electroblotted to a PVDF membrane (Whatman, Newton, MA), washed in PBST (PBS + 0.1% Tween 20), preblocked with 10% milk for 30 min, and placed on one of three primary antibodies. ER-β (Biogenex, San Ramon, CA), ER-α (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma) were incubated for a period of 24 h at 250:1, 2 h at 500:1, and 1 h at 1000:1, respectively at 4°C. Antibodies were diluted in 0.1% BSA, and 1.0% milk in PBST, and the blots were placed in sealed Kapak pouches. β-Actin was incubated with membranes pre-blotted and stripped as per standard protocol. The membranes were further washed in PBST for 30 min and incubated with a donkey anti-rabbit secondary antibody (Amersham, Piscataway, NJ) in PBST for a period of 1 h at 5000:1. Membranes were then rinsed and washed for 4 h, and incubated in ECLplus (Amersham) for 10 min before imaging with a Storm 840 Scanner set to blue laser mode (Amersham). Kodak 1D software was then used to quantify the relative protein concentrations and molecular weight.

Flow Cytometry/Apoptosis

DU145 cells were treated with $APVE_2$ at the EC_{50} concentration of 16 nM for 0, 1, and 2 days before the 5-day treatment. Apoptosis was assessed with the Annexin V-FITC apoptosis detection kit (Oncogene Research Products, Boston, MA) as per the manufacturer's instructions

with E_2 and camptothecin (Calbiochem) as negative and positive controls, respectively. Cell cycle distribution, DNA content, and apoptosis analysis were assayed according to standard methods using a FACS sorter, FACScan flow cytometer (Becton Dickinson, Mountain View, CA), and analyzed by the Cell Cycle Multi-Cycle system (Phoenix Flow System, San Diego, CA). Approximately 15,000 singlet events were collected, and cell-cycle distribution was determined using Modfit LT version 2 software (Verity House, Inc., Topsham, ME). All analyses were carried out in quadruplicate.

Caspase Assay

Cells were treated with 1 μM of each of the following compounds: E_2, etoposide, $APVE_2$, or carrier alone (no treatment, NT). Treatment times were for 4 days, with the exception of the positive control etoposide dosed for 24 h on day 2. The presence of apoptotic cells was further determined in duplicate through the measurement of caspase 3 using the BD ApoAlert Caspase 3 assay (BD Biosciences Clonetech, Palo Alto, CA) as per the manufacturer's instructions.

Statistical Analyses

All statistical calculations, EC_{50} values, mean ± SD, and *P* values were carried out on GraphPad Prism software version 2.0, and calculated using a nonlinear regression fit and the unpaired *t* test at 95% confidence interval, respectively.

Results

ER Relative Binding Affinities

The relative binding of $APVE_2$ was assessed by fluorescence polarimetry as described. The controls, DES, and 4-OH-TAM were found to exhibit relative binding activities that agree with previously published results for this assay, and $APVE_2$ was found to exhibit low relative binding activities to both of the receptors, ER-α and ER-β at 0.87% and 6.2%, respectively, with a preference for ER-β of ~7-fold (Table 1).

Molecular Modeling

Locally minimized structures of $APVE_2$ were fitted into the ligand binding domains of the crystal-derived structures of ER-α and ER-β (Fig. 2). The final derivation depicting the lowest energy model predicts poor binding, yet preferential affinity for ER-β, as a result of steric restrictions corresponding primarily to amino acid Met421, which resides within the ligand binding domain of ER-α. In addition, the minimized structure for $APVE_2$ corresponds well with previously published NMR-derived structures previously published for this series of compounds (30).

MTS Assay, Dose Curves

Following 5 days of growth, all cell lines were found to proliferate appreciably to 70–80% confluence under the defined, non-treated conditions mentioned in ''Materials and Methods'' section. Measurement of cell number by hemocytometry revealed an increase from approximately 10k with a range of ±2k to an average of 52k with a range of ±5k for all cell lines tested in 24-well plates. Treatment with $APVE_2$ led to a 92 ± 0.9%, 81 ± 0.8%, and 69 ± 2.3% decrease in cell number, as determined by the MTS assay, in the DU145, PC3, and LNCaP cell lines, respectively, following 5 days of treatment at the highest concentration of 1 μM (Fig. 3). The negative control, consisting of 1 μM E_2 induced no effect on MTS activity with an average of no more than 0 ± 3.4% difference over non-treated cells, while the positive control resveratrol induced a maximal average of 65 ± 5.3% decrease in cell number following 5 days of treatment (data not shown). Resveratrol was chosen as it was found to cause appreciable, but not complete cell death in all cell lines chosen in a very reproducible manner. E_2 and resveratrol were added to each plate to control for any unforeseen variability. Incremental adjustments in $APVE_2$ concentrations from 10^{-10} to 10^{-6} M resulted in a dose response with high statistical significance (r^2 = 0.98–0.99) for each prostate cancer cell line tested with EC_{50} values of 16, 1.4, and 2.7 nM in the DU145, LNCaP, and PC3 cell lines, respectively (Fig. 3). In addition, a 20% increase in cell proliferation occurred over non-treated controls at 1 nM in the PC3 cell line, but not in DU145 or LnCaP cell lines. Treatment of MCF-7 cells did not cause an increase in proliferation, but did induce cell death in a similar fashion to that of the other cell lines (data not shown).

Western Blot Analysis

A control blot was carried out with recombinant proteins ER-α_r and ER-β_r to determine the specificity of each antibody. No cross-reactivity was observed for either of the two blots shown, and the calculated molecular weights agreed with the expected values at M_r 69,000 for ER-α_r and M_r 54,000 for ER-β_r (data not shown). To determine the level of each ER at the protein level under the noted

Table 1. Relative binding activities to the two estrogen receptor subtypes

Compound	ER-α EC_{50}[a]	% Relative Binding Activities[b]	ER-β EC_{50}[a]	% Relative Binding Activities[b]	ER-α/ER-β
E_2[c]	3.19	100	15.5	100	1.0
$APVE_2$[c]	365	0.87	250	6.2	0.14
4-OH-TAM[c]	13.2	24.2	59	26.3	0.92
DES[c]	4.2	75.9	18.4	84.2	0.90

[a]Effective concentrations at 50% half maximum are depicted in nanomolars.
[b]Relative binding is based on the ratio of EC_{50} values as compared to E_2.
[c]Average of four measurements at half-log concentrations from log −5.3 to −10.

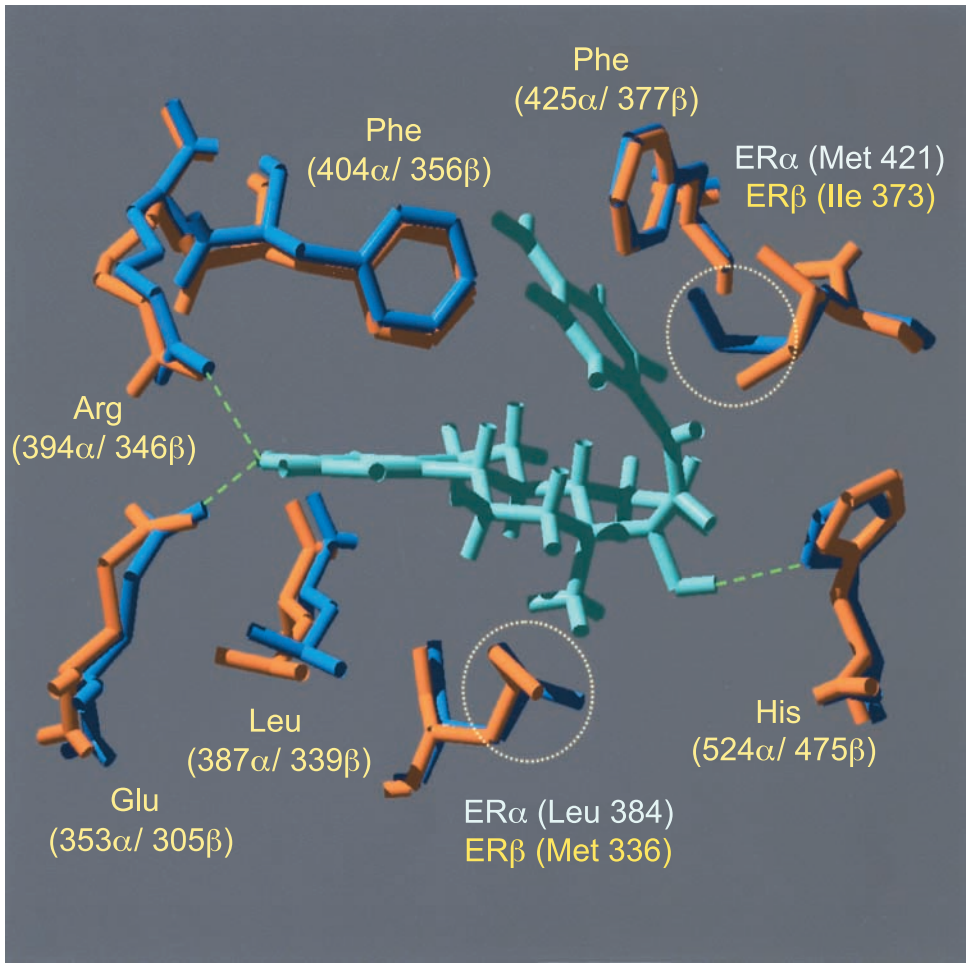

Figure 2. An energy minimized depiction of $APVE_2$ is shown bound within the ligand binding pocket of ER-β (*orange*) and ER-α (*blue*). Hydrogen bonds are illustrated as *dotted lines*. The phenyl moiety was predicted to cause a higher level of steric hindrance within the ligand binding pocket of ER-α as a result of Met421 as opposed to the corresponding Ile373 for ER-β.

cell culture conditions, Western analysis was carried out following a no-treatment control and after a treatment with 1 μM E_2 for 12 h. The level of ER-α was found to be nearly undetectable, while ER-β was expressed strongly, with no effect on receptor levels induced by treatments with E_2 (Fig. 4). In addition, β-actin was used as a control for loading, and was found to be equal in all samples tested.

E_2 Competition Assay

Because E_2 was previously found not to induce any apparent growth or cytotoxic-related effects in DU145 cells, this compound was used to determine if the cytotoxic action of $APVE_2$ could be competed by a classical estrogen. Our results indicate that the level of cytotoxicity induced by 16 nM $APVE_2$ is not reversed by co- or pre-treating DU145 cells with 1 μM E_2 at 0 or 1 h, respectively (Fig. 5).

BrdUrd Assay

Carrier- and E_2-treated controls exhibited a similarly progressive increase in BrdUrd uptake of approximately 2-fold over a 5-day period (Fig. 6). Conversely, $APVE_2$-treated cells showed a marked decrease in uptake as compared to non-treated controls by the 3rd day with 16 nM and 1 μM to 25% and 78%, respectively, and by the 5th day with 16 nM and 1 μM to 0.1% and 0.0%, respectively.

Flow Cytometry/Apoptosis

Cell cycle analysis was carried out on DU145 cells with propidium iodide (PI) staining following treatments with 16 nM $APVE_2$. There was a significant increase in G_1-G_0 arrest by day 3 of 135% with a corresponding decrease in S phase to 32%, and no change in G_2-M as compared to non-treated controls (Fig. 7A). By the 4th day, cell morphology began to change, with a rounding of cells and decreased adherence, corresponding with concomitant changes in cell cycle (non-treated: sub-G_1 = 0%, G_1-G_0 = 73.9%, S = 18.6%, G_2 = 7.5%; $APVE_2$-treated: sub-G_1 = 19.2%, G_1-G_0 = 35.5%, S = 13.6%, G_2 = 31.5%). This change equated to a sharp increase in G_2-M arrest of 420%, a decrease in G_1-G_0 to 48%, and S phase to 73% over non-treated controls. An increase in apoptotic fraction was also noted and therefore measured by co-staining live cells with PI and an Annexin V conjugated to a fluorescent tag. These results illustrate that the $APVE_2$-treated cells underwent G_2-M arrest, which corresponded to a change in morphological appearance on the 4th and 5th days of treatment correspond with approximately 76% of the cells staining heavily with both dyes as compared to 1–2% in the non-treated control, thus equating to late-stage apoptosis (Fig. 7B). E_2 exhibited no

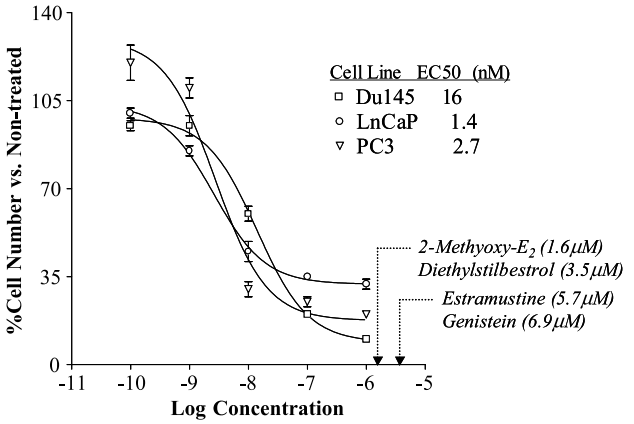

Figure 3. The change in cell number is shown after a 5-day treatment with varying concentrations of APVE$_2$ is illustrated as compared to the carrier-treated control for three commonly used prostate cancer cell lines, LNCaP, DU145, and PC$_3$ (mean, SD, $n = 4$). The effective concentrations at 50% of the maximum cell death ($EC_{50}s$) are illustrated for APVE$_2$ as well as for other pharmacologically active estrogens based on values derived from literature (15, 32, 47, 52). The negative control consisting of 1 μM E$_2$ caused virtually no effect on MTS activity with an average of no more than 0 ± 3.4% difference over non-treated cells throughout the 5-day treatment in any one cell line (data not shown).

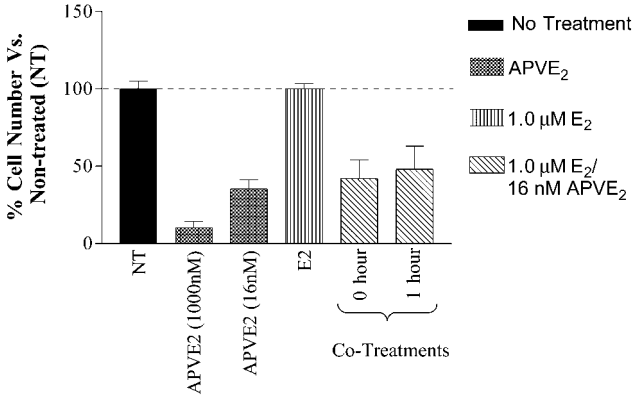

Figure 5. The change in cell number is shown after a 5-day treatment with 1.0 μM and the EC$_{50}$ concentration of 16 nM APVE$_2$ alone, 1.0 μM E$_2$ alone, and both as compared to the carrier-treated control in DU145 cells ($n = 4$; *, $P < 0.05$). E$_2$ exhibited no effect on the cell number, and the cytotoxic action of 16 nM APVE$_2$ was not reversed by co-treating with 1 μM E$_2$ at that same time or pretreating the cells 1 h before the addition of APVE$_2$ as shown.

cell cycle changes or Annexin V staining over non-treated cells following 5 days of treatment; conversely, camptothecan induced a significant 52% increase in Annexin V and PI-positive cells over non-treated controls at the same time point (data not shown).

Caspase Assay

The initiation of apoptosis, as measured by caspase 3 activity following a 4-day treatment, was not increased by E$_2$ as compared to non-treated controls. Conversely, treatments with the positive control etoposide and APVE$_2$ induced an increase in caspase activity by 142% and 210% over non-treated cells, respectively (Fig. 8).

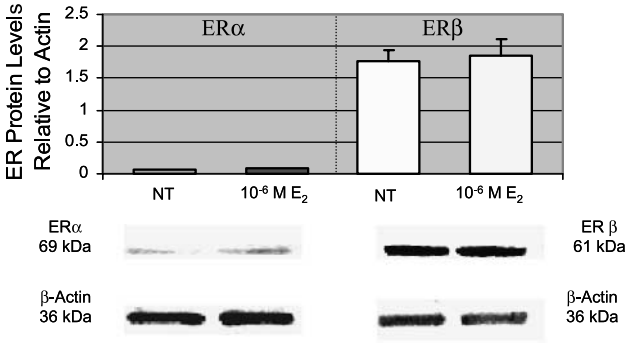

Figure 4. Estrogen receptor status in DU145 cells. Each *lane* was loaded with 25 μg of protein extract from DU145 cells with and without a 12-h treatment with 1 μM E$_2$. No change in the expression of either receptor was noted following treatments with E$_2$ as shown ($n = 3$). In addition, high levels of ER-β were observed, with nearly undetectable levels of ER-α while cultured under defined media conditions. In respect to the measurement of each receptor level, the *graph* is meant to be qualitative only, and not a quantitative comparison. β-Actin was used to control for equal loading. In addition, standard recombinant proteins for each receptor were used as positive controls (data not shown). In this respect, no cross-reactivates were observed between the estrogen receptor antibodies and sensitivities were found to be similar by ECL.

Discussion

Our initial objective included the design and synthesis of a series 17α modified estrogen analogues with the potential to exhibit ER-β-driven apoptosis in prostate cancer epithelial cells. Cell viability assays were carried out on this library of compounds in metastatic prostate cancer-derived DU145, PC3, and LNCaP cell lines.[3] These cell lines were chosen, in part because they have previously been shown to express ER-β with little to no ER-α expression (18, 19). The outcome of these initial studies indicated that APVE$_2$ was an exceptionally potent antitumor agent, found to cause a loss in cell viability as measured by the MTS assay at EC$_{50}$ concentrations of 1.4, 2.7, and 16 nM in the LNCaP, PC3, and DU145 cell lines, respectively. Therefore, APVE$_2$ was selected for further testing. Cell cycle and apoptosis studies were conducted more completely in DU145 cells, as this cell line was previously shown to express appreciable levels of ER-β and yet no ER-α at the transcript level (18, 19). We wished to further establish this point at the protein level by carrying out Western blot analysis using antibodies specific to each receptor. In addition, because estrogens are known to affect the level of stability and expression of both ERs in other cell lines, we also measured ER status following treatments with E$_2$ for up to 12 h. Our results, while not intended to be quantitative, indicate that ER-α is expressed at nearly undetectable levels, while ER-β is highly expressed at the protein

[3] Unpublished data.

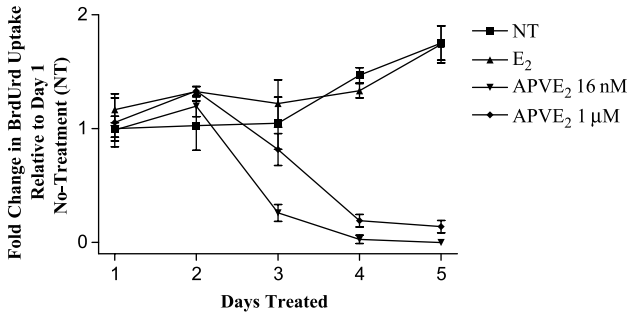

Figure 6. Carrier- and E_2-treated controls exhibited a similarly progressive increase in BrdUrd uptake of approximately 2-fold over a 5-day period. Conversely, $APVE_2$-treated cells showed a marked decrease in uptake as compared to non-treated controls to 0.1% and 0.0% uptake as compared to no-treatment controls by 16 nM and 1 μM on the 5th day of treatment.

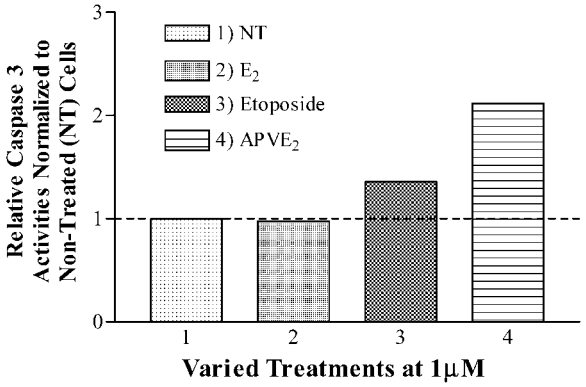

Figure 8. DU145 cells were treated for 4 days with the compounds shown. There was no increase in caspase activity observed for E_2, as compared to non-treated controls. However, treatments with the positive control etoposide and $APVE_2$ induced an increase in caspase activity by 142% and 210% over non-treated cells, respectively.

level in early passage DU145 cells while cultured under the defined conditions noted. In addition, there was no change in the level of either receptor following treatments with E_2.

Further testing was then completed with $APVE_2$ at a concentration equivalent to the EC_{50} value of 16 nM, which was established in the viability assay using the DU145 cell line. A significant and progressive decrease in BrdUrd uptake to 0% was observed as compared to non-treated controls following a 5-day treatment period. Cell cycle analysis revealed a slight but significant increase in G_1 arrest by 136%, and pronounced decrease in S phase by 68% at days 2 and 3, which was then followed by a 420% increase in G_2-M accumulation and a 79% increase in

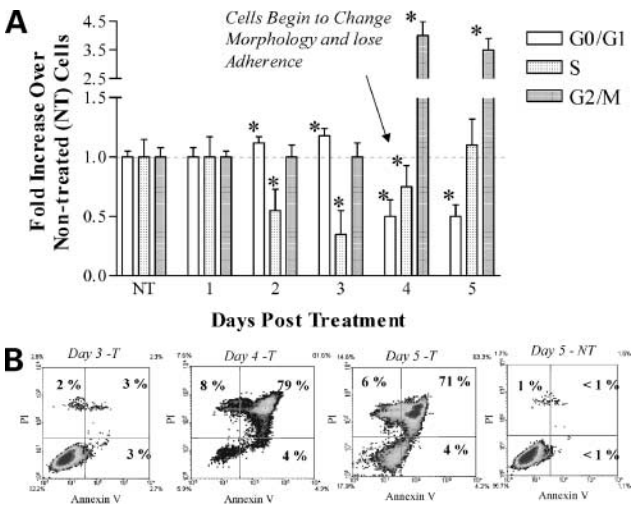

Figure 7. Cell cycle analysis including the Annexin V assay were carried out on DU145 cells after treatment with 16 nM $APVE_2$ over various time points as shown (n = 3). **A,** accumulation in the G_1 phase of 135% was noted by the 2nd and 3rd day of treatment with a concomitant loss in S phase by up to 68%. Treatment for 4 – 5 days led to an increase in G_2-M phase by 420% with a loss of G_1 by over 48%. **B,** treatments with 16 nM $APVE_2$ caused a significant increase in late-stage apoptosis of up to 79% by the 4th day, which correlated with the switch to G_2-M and a change in cellular morphology. The intensity of each stain is shown with Annexin V values corresponding to the *abscissa* and PI values to the *ordinate*.

apoptosis by Annexin V by days 4 and 5 that coincided with decreased cell adhesion. This is an important finding, because most pro-apoptotic estrogens including DES are active in the micromolar range, three orders of magnitude greater than that exhibited by $APVE_2$ (12, 14, 15, 31, 32). To further assure that the apoptosis pathway was indeed activated, caspase 3 activity was tested and found to be appreciably increased by 210% over non-treated controls following a 4-day treatment with $APVE_2$. In addition, this compound was capable of inducing apoptosis independent of p53, which has been shown to be mutated in the DU145 and PC3 cell lines (33).

Interestingly, $APVE_2$ was found to bind weakly to ER-β with an EC_{50} of 250 nM and a relative binding activity of 6.2%, a concentration far less than that required for cytotoxic activity. Furthermore, the cytotoxic actions of $APVE_2$ were not reversed by co-treating cells with a 50-fold excess concentration of E_2. The combination of weak affinity for ER-β, cytotoxic activities in the low nanomolar range, and the inability to compete-out activity with E_2 is not consistent with an ER-β-dependent mechanism in mediating apoptotic induction by this compound. In addition, as predicted *in silico*, there was a preferential affinity for ER-β by 7-fold over that for ER-α, likely due to steric restrictions that correspond to amino acid Met421 residing within the ligand binding domain of ER-α. These data also indicate that this class of 17α-phenylvinyl modified E_2 analogues may prove to be useful probes for the study of ER selective ligands. Furthermore, there was no growth response exhibited by $APVE_2$ in the MCF-7 cell line (results not shown), indicating that this compound is not a classic ER-α agonist.

It is our conclusion that $APVE_2$ did not induce apoptosis through an ER-β-mediated mechanism. Nevertheless, the results are striking, and the lack of ER-α activity may avoid common undesirable side effects associated with estrogen agonists such as increased thrombosis, cardiotoxicity, and decreased libido (2, 3). Results from flow analysis were consistent with mitotic spindle disruption; a pathway

affected by various estrogen-based cytotoxic agents, including a number of E_2 analogues modified in the 2 position, as well as estramustine (14, 15, 31, 34, 35). However, for the many estrogens found to exhibit cytotoxic activities, nearly as many mechanisms have been implicated, including inhibition of topoisomerase II, SOD, 5α-reductase, aromatase, 17β-hydroxy-steroid dehydrogenase, protein tyrosine kinases, and tubulin polymerization (15, 36–40). In addition, antiestrogen binding sites, type II binding sites, and the orphan estrogen receptor-related proteins, have all been implicated as well (41–46). The downstream pathways reportedly associated with the cytotoxic actions of estrogens are equally varied and include the regulation or activation of MMPs, BCL-2/BCLxl, BAX, MAPK, p21, p27, p34, p53, DR-5, FasL, and JNK (10, 47–51). Confusion may arise from the fact that a number of these pathways were studied at relatively high concentrations, which may prove difficult to achieve in a clinical setting (10, 52). To date, only one estrogen-related compound reported thus far, raloxifene, has exhibited activity nearing low nanomolar concentrations (53). Therefore, the ability to achieve cell kill at 1.4–16 nM in prostate cancer cells that exhibit varied levels of resistance to apoptosis, including the P53-mutated DU145 and PC3 cell lines, is of great interest (6). Further *in vitro* studies will help to shed light on key pathways involved in the underlying activity of this compound, while *in vivo* studies will help to determine if APVE$_2$ is a feasible candidate for the clinical application.

Acknowledgments

We thank Heisyuku L'Esperance for carrying out cell culture experiments, which led to the identification of the lead compound.

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Molecular Cancer Therapeutics

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Mol Cancer Ther 2004;3:587-596.