RESEARCH ARTICLE

Differential effects of resveratrol and its naturally occurring methylether analogs on cell cycle and apoptosis in human androgen-responsive LNCaP cancer cells

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Stilbenes are phytoalexins that become activated when plants are stressed. These compounds exist in foods and are widely consumed. Resveratrol is a grape-derived stilbene, which possesses a wide range of health-promoting activities, including anticancer properties. Several other stilbenes structurally similar to resveratrol are also available in food, but their biological activities remain largely unknown. In this study, we compared the effects of resveratrol and its natural derivatives pterostilbene, *trans*-resveratrol trimethylether, *trans*-pinostilbene and *trans*-desoxyrhapontigenin on androgen-responsive human prostate cancer LNCaP cells. We found that these compounds exert differential effects on LNCaP cell growth, cell cycle and apoptosis. *Trans*-resveratrol trimethylether appeared to be the most potent compound among the stilbenes tested. Treatment of LNCaP cells with *trans*-resveratrol trimethylether resulted in G2/M blockage while other compounds, including resveratrol, induced G1/S arrest. Moreover, different from other compounds, *trans*-resveratrol trimethylether induced apoptosis. At the molecular level, the effects of these compounds on cell cycle correlated with induction of the cyclin-dependent kinase inhibitor 1A and B mRNA levels. Additionally, these compounds also inhibited both androgen- as well as estrogen-mediated pathways. These results provide mechanistic information on how resveratrol and its methylether analogs may act to contribute to potential antiprostate cancer activity.

Keywords:
Androgen / Cancer prevention / Estrogen / Phytochemicals / Stilbenes

1 Introduction

Prostate cancer is the second leading cause of cancer death among American men [1]. Although the etiology of prostate cancer remains unknown, elevated levels of steroid hormones such as androgens and estrogens, as well as growth factors such as insulin-like growth factor 1, are considered to be important risk factors [2–4]. These hormones and growth factors have been shown to promote proliferation of prostate cancer cells through the activation

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Abbreviations: ARG, androgen-responsive gene; **CDKN,** cyclin-dependent kinase inhibitor; **CDS,** charcoal dextran-treated FBS; **DESO,** desoxyrhapontigenin; **DHT,** dihydrotestosterone; **FBS,** fetal bovine serum; **PI,** propidium iodide; **PINO,** pinostilbene; **PSA,** prostate-specific antigen; **PTER,** pterostilbene; **RES,** resveratrol; **RTE,** resveratrol trimethylether

of receptor-mediated signaling pathways [2–4]. Therapeutic as well as preventive strategies have explored modulation of these pathways as potential approaches to prevent or control prostate cancer [5–7].

Bioactive food components, in particular, are increasingly being evaluated as potential prostate cancer chemopreventive agents because of their presumed safety [8]. One such compound is resveratrol (3, 4′, 5-trihydroxystilbene, RES) [9], a polyphenol categorized as a phytoalexin [10], found principally in the skin of grapes as well as in peanuts and other plant species [11]. Red wine, often mentioned as a good source of RES, contains 1–10 mg of RES/L (4–40 μM) [12]. Recent studies attributed a variety of health benefits to consumption of foods containing RES, including protection against cancers, cardiovascular disease, and aging [13].

Results from rodent carcinogenesis models suggested that RES could inhibit initiation, promotion, and progression of cancer [14]. Moreover, molecular studies showed that RES possesses anticancer activities, including acting as an antioxidant [15], possessing anti-inflammatory properties [11, 13], and functioning as a weak estrogen [16]. *In vitro* experiments using prostate cancer cell lines provided support for RES to serve as a candidate prostate cancer preventive agent. RES has been shown to inhibit prostate cancer cell growth in culture [17, 18], to inhibit DNA synthesis [19], and to increase apoptosis in LNCaP cells, a human androgen-responsive prostate cancer cell line [20]. It has been reported that RES increased expression and serine phosphorylation levels of the tumor suppressor protein p53 [21], thereby affecting activation of p53-dependent signaling pathways, such as inhibition of cell cycle progression and induction of apoptosis. RES has also been found to decrease expression of prostate-specific antigen (PSA), an androgen-responsive gene (ARG) often used as a marker for prostate cancer cell growth [17]. Moreover, a recent microarray study revealed that RES exerted global effects on ARG expression in LNCaP cells [22]. ARGs such as PSA play important roles in cellular functions, including cell cycle regulation, transcription, cell proliferation, differentiation as well as metabolism [23, 24]. We previously showed that estrogen as well as androgen can regulate ARG expression [25]. Others have reported modulation of estradiol-mediated effects on Akt pathway by RES [26, 27]. Overall, the effect of RES on ARGs appeared to be through modulation of steroid hormone-mediated pathways. Given the roles of androgen and estrogen in prostate cancer development [2, 3], modulation of these pathways may contribute to RES's protective effects against prostate cancer. Recently, using the TRAMP mouse prostate cancer model, Harper *et al.* [28] reported a protective effect of RES on prostate cancer development that correlated with inhibition of insulin-like growth factor 1 pathway and upregulation of estrogen receptor-β. However, despite these findings, precise mechanisms underlying the effects of RES on prostate cancer remain largely unknown.

In addition to RES, other stilbenes structurally similar to RES also exist in various plant species, including *Vaccinium* berries [29–35]. Pterostilbene (PTER), a dimethylether analog of RES, was found to possess anticancer effects similar to RES [36]. Although a recent report described growth inhibitory effects of selected stilbenes on prostate cancer cells *in vitro* [37–39], the molecular mechanisms and structure–function relationship of these stilbenes in prostate cancer carcinogenesis remain largely unclear.

In this study, we compared the cellular and molecular effects of a group of naturally occurring stilbenes that are methylether analogs of RES (Fig. 1) in the human androgen-responsive prostate cancer cell LNCaP. We investigated structure–function relationships, efficacy on cell growth, cell cycle, apoptosis, and the molecular targets that are important in prostate carcinogenesis for these compounds. We found that these compounds exert differential effects on multiple pathways important in prostate cancer development.

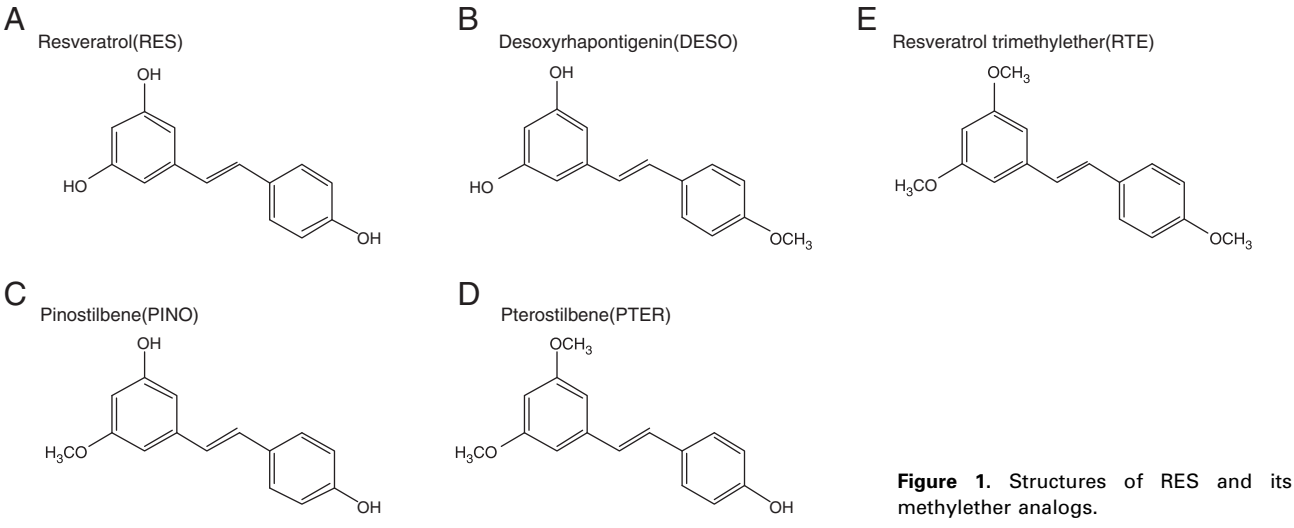

Figure 1. Structures of RES and its methylether analogs.

2 Materials and methods

2.1 Chemicals

RES, 17β-estradiol, dihydrotestosterone (DHT), 4-methoxybenzaldehyde, *n*-butyllithium, and DMSO were from Sigma Chemical (St. Louis, MO, USA).

2.2 Synthesis of stilbenes

Although natural analogs of RES can be isolated from various plant materials, they were chemically synthesized to provide a defined source for structure and function studies:

2.3 Synthesis of PTER

PTER was synthesized by following the previously published procedures [40].

2.4 Synthesis of resveratrol trimethyl ether

To a cold solution (−78°C) of (3,5-dimethoxybenzyl)-triphenylphosphonium bromide (300 mg, 0.608 mmol) in THF, *n*-butyllithium (1.6 mol in hexanes, 0.608 mmol) was added and the reaction stirred under inert atmosphere for 2 h [40]. 4-Methoxybenzaldehyde (75 μL, 0.608 mmol) dissolved in THF was then added and the reaction stirred at room temperature for 12 h. The resulting suspension was poured into water and extracted with dichloromethane twice. The organic phase was combined, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified through automated flash purification and eluted with hexanes/ethyl acetate (86:14) to give 15 mg (9%) of resveratrol trimethylether (RTE) as a white solid plus the *cis*-isomer.

2.5 Synthesis of pinostilbene

A solution of RTE (30 mg, 0.11 mmol) and lithium thioethoxide (135 mg, 1.33 mmol) in dimethylformide was heated at 160°C for 2 h [41]. After cooling, 15 mL of 0.1 M HCl was slowly added and the solution washed with water (3 × 10 mL) and extracted with dichloromethane twice. The organic phase was dried over $MgSO_4$ and the solvent removed under reduced pressure. The crude product was purified using flash chromatography eluting with hexanes/ethyl acetate (3:2) to give 18 mg (67%) of pinostilbene (PINO).

2.6 Synthesis of desoxyrhapontigenin

To a solution of (4-methoxybenzyl)triphenylphosphonium bromide (500 mg, 1.11 mmol) in THF, *n*-butyllithium (1.6 mol in hexanes, 694 μL, 1.11 mmol) was added [42]. The reaction was stirred for 1 h and 3,5-*bis*(tert-butyldimethylsilyloxy)benzaldehyde (408 mg, 1.11 mmol) dissolved in THF was then added. The reaction mixture was stirred for an additional 12 h and then poured into ice water and extracted with ethyl acetate. The organic phase was combined and dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified using flash chromatography eluting with hexanes/ethyl acetate (94:6) to obtain 452 mg (86% yield) of a mixture *cis*- and *trans*-(5-(4-methoxystyryl)-1,3-phenylene)*bis*(oxy)*bis*(tert-butyldimethylsilane). To the mixture of isomers (452 mg, 0.96 mmol) in anhydrous THF (10 mL), tetrabutylammonium fluoride (2.5 mL, 2.5 mmol) was added. The solution was stirred for 45 min, poured into water and extracted with ether. After removal of the solvent, the resulting crude mixture was purified using flash chromatography (7:3 hexanes/ethyl acetate) and resulted in 23 mg (8%) of *trans*-desoxyrhapontigenin (DESO) plus the *cis*-isomer.

The structures of the stilbenes synthesized were confirmed from their spectroscopic data and validated with values reported in the literature.

2.7 Cells and cell culture

LNCaP human prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Media A (RPMI 1640 medium with phenol red (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin (BioSource International, Camarillo, CA, USA) with 10% fetal bovine serum (FBS) (Invitrogen)). In some cultures, Media A was replaced with Media B, which had the same composition as Media A except that FBS was replaced with 10% charcoal dextran-treated FBS (CDS, Hyclone, Logan, UT, USA). Cells were incubated in the presence of 5% CO_2 in air at 37°C.

2.8 Cell growth assay

LNCaP cells (5×10^4 cells/well) were plated in 24-well plates (Costar); 24 h later treatments began. Cells were treated with 0, 1, 2.5, 5, or 25 μM of the test compounds (DMSO as the vehicle) for 0–72 h and the medium containing RES or its analogs was replaced every 24 h. DMSO added as the vehicle was at a concentration of 0.05%. Cell growth was analyzed using the sulforhodamine B assay as described previously [25].

2.9 Cell cycle analysis using flow cytometry

LNCaP cells (3×10^6 cells) were seeded into T-175 flask (NUNC, Rosklide, Denmark) in Media A. Twenty-four

hours later, the media were changed with that containing vehicle (DMSO) or varied concentrations of the test compounds. DMSO added as the vehicle was at a concentration of 0.05%. RES, PINO, and PTER were used at concentrations of 0, 1, 5, or 25 μM, and RTE at 0, 1, 2.5, and 5 μM. Cells were treated with RES, PINO, and PTER for 72 h, and RTE for 24 h. Cells were treated and harvested by scraping, transferred into centrifuge tubes (50 mL polypropylene) pelleted at $1000 \times g$, washed $1\times$ in PBS (without calcium or magnesium), and pelleted again. Cell pellets were then resuspended in 1.5 mL PBS. To these resuspended cells, 15 mL of 70% ethanol was added and vortexed gently with the cap on the tube. The ethanol-fixed cells were pelleted and washed once in PBS before staining the DNA with propidium iodide (PI) in the presence of RNase. The cellular DNA was then analyzed by flow cytometry. DNA content of the cells was determined by flow cytometry using a FACScalibur cytometer (Becton Dickinson, San Jose, CA, USA). Washed cells were fixed in ethanol and stained for DNA content using PI [43]. Flow cytometric data were collected and analyzed using the CELLQuest program (Becton Dickinson). A total of 10 000 cell events were collected for DNA analyses. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software (Version 3.0, Verity Software House, Topsham, ME, USA). Calibration standards (LinearFlow Green and DNA QC Particle Kit) for verification of instrument performance were purchased from Molecular Probes (Eugene, OR, USA) and Becton Dickinson, respectively.

2.10 Determination of the effects of RES and analogs on gene expression in LNCaP cells using RT-PCR

To examine the effects of test compounds on cyclin inhibitors CDNK1A and CDNK1B, or PSA mRNA levels, LNCaP cells were plated in six-well plates (0.25×10^6 cells/well) in Media A and after 24 h the media was removed and replaced with fresh media containing vehicle or varied concentrations of test compounds RES, PINO, and PTER were used at concentrations of 0, 1, 5, or 25 μM, and RTE at 0, 1, 2.5, and 5 μM. For experiments using the androgen DHT or 17β-estradiol, LNCaP cells were plated in six-well plates (0.25×10^6 cells/well) in Media A, and switched to Media B 24 h after plating to minimize the effect of serum hormones. The cells were then incubated in Media B for an additional 24 h before the treatments began. Twenty-four hours later, the medium was replaced with fresh medium containing 1 nM DHT or 17β-estradiol with or without test compounds. Concentration of RES, PINO, and PTER was 5 μM and RTE at 2.5 μM. For all experiments, fresh medium containing the test compounds was changed daily and cells were harvested for total RNA isolation using the Trizol method (Invitrogen) after 48 h (25). Taqman real-time PCR was used to quantify expression of the mRNA [25]. Taqman real-time PCR Primer and probes for glyceraldehydes-3-phosphate dehydrogenase, PSA, cyclin-dependent kinase inhibitor (CDKN) 1A and 1B were purchased from Applied Biosystems (Foster City, CA, USA).

2.11 Apoptosis assay

Activation of caspase was used as an additional method to flow cytometry to detect apoptosis. LNCaP cells (1×10^6 cells/well) were plated in six-well plates and 24 h later the test compounds (5 μM final concentration) were added. The concentration was chosen based on our previous observation of 5 μM as higher limit of RES concentration in plasma of a rodent model [44]. After an additional 24 h of treatment with the test compounds, cells were washed with PBS once and lysed in cell lysis buffer (BioSource International). Protein was determined using the BCA method (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Fifty micrograms of lysate was used for determination of caspase activity using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) following the manufacturer's protocol.

2.12 Statistical methods

StatView (SAS Institute, Cary, NC, USA) software was used for statistical analysis. Multiple group data were analyzed using ANOVA followed by *post hoc* analysis with the

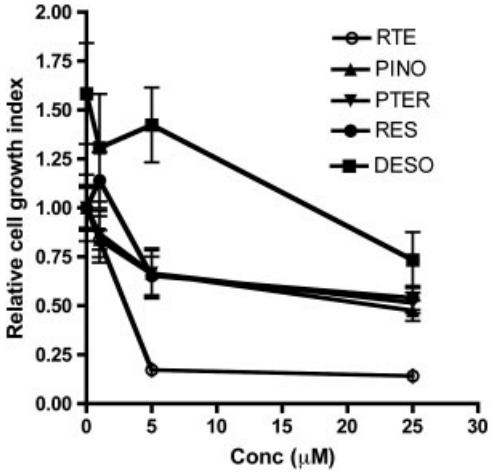

Figure 2. Concentration-dependent effects of RES and its methylether analogs on LNCaP cell growth. LNCaP cells (0.25×10^6 cells/well) were plated in six-well plates and 24 h later, the cells were treated with varied concentrations (0–25 μM) of test compounds. Cells were treated for an additional 72 h and cell number determined as described in Materials and methods. The test compounds are: RES, RTE, PINO, DESO, and PTER. Results expressed as relative proliferation index ±SD ($n = 6$) where vehicle control is 1.

Bonferroni test. The unpaired Student's *t*-test was used to compare experiments between the two groups. Values were considered significant at $p < 0.05$.

3 Results

3.1 Differential effects of RES and analogs on LNCaP growth

We first compared the effects of RES and its methylether analogs on cell growth. Human androgen-responsive prostate cancer cell LNCaP was treated with 0–25 μM of RES or its analogs (Fig. 1) for 72 h. As shown in Fig. 2, treatment of LNCaP cells with RES, RTE, PINO, and PTER led to growth inhibition. Significant growth inhibitory effect ($p < 0.05$) was observed for these compounds at 5 μM. There appeared to be no significant difference between RES, PTER, and PINO. However, RTE appeared to be unique; after 72 h treatment there were much less cells at 5 or 25 μM compared with those treated with the other stilbenes. We also observed significant growth inhibition *versus* control at 1 μM RTE treatment ($p < 0.05$). Different from the other four stilbenes, the 4′-methoxy analog DESO appeared to be less effective in inhibiting LNCaP cell growth, with an $EC_{50} > 25$ μM.

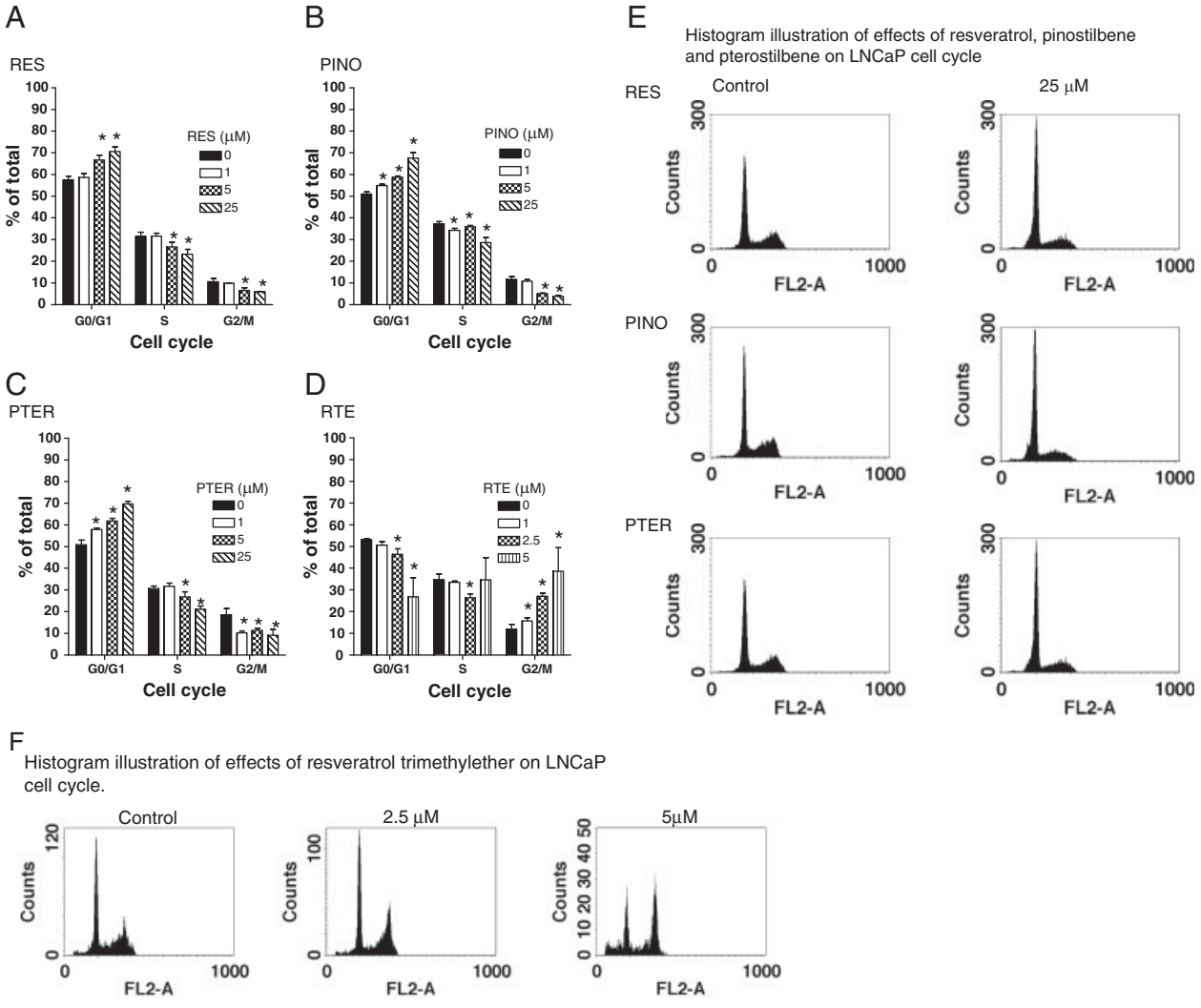

Figure 3. Differential effects of RES and its methylether analogs on cell cycle. LNCaP cells (3×10^6 cells) were plated in T-175 flask and treated for cell cycle analysis as described in Materials and methods. RES, PINO, and PTER were used at concentrations of 0, 1, 5, or 25 μM, and RTE at 0, 1, 2.5, and 5 μM. Cells were treated with RES, PINO, and PTER for 72 h, and RTE for 24 h. (A) RES. Results are expressed as % of total cells (mean ± SD, $n = 3$). (B) PINO. Results are expressed as % of total cells (mean ± SD, $n = 3$). (C) PTER. Results are expressed as % of total cells (mean ± SD, $n = 3$). (D) RTE. Results are expressed as % of total cells (mean ± SD, $n = 3$). (E) Representative cell cycle histogram of results for LNCaP cells treated with vehicle control or with 25 μM of RES, PINO, or PTER. (F) Representative cell cycle histogram of LNCaP cells treated with 0, 2.5, or 5 μM of RTE.

3.2 Differential effects of RES and analogs on LNCaP cell cycles and apoptosis

To gain mechanistic information on how RES and its analogs can lead to inhibition of LNCaP cell growth, we analyzed the effects of stilbenes on cell cycle progression using flow cytometry. Treatment of LNCaP cells with RES (Fig. 3A), PINO (Fig. 3B), and PTER (Fig. 3C) appeared to affect the cell cycle. These compounds arrested LNCaP cell cycle progression at G1/S after a 72 h treatment. These three compounds did not significantly differ in activity in terms of their effective concentration. By contrast, RTE treatment led to a significant effect on the G2/M phase after 24 h treatment of the LNCaP cells (Fig. 3D), and the effects of RTE on cell cycle were observed at 1 μM.

Flow cytometry analysis also revealed that RES, PINO, and PTER exhibited very little apoptosis, as indicated by a lack of PI staining of DNA < 2N (Fig. 3E). However, after 24 h treatment of LNCaP cells with RTE, significant accumulation of sub-2N DNA (16%) was observed at 5 μM (Fig. 3F), indicating that apoptosis occurred and in a concentration-dependent manner. The apoptosis-inducing effect of RTE was further confirmed by measuring activation of apoptosis-related caspase 3/7 activity [45]. As shown in Fig. 4, consistent with flow cytometry results after 24 h incubation with the test compounds (5 μM), only treatment of LNCaP cells with RTE led to induction of caspase activity.

Consistent with the cell growth result, DESO did not significantly affect cell cycle or apoptosis, and was not included in subsequent analyses.

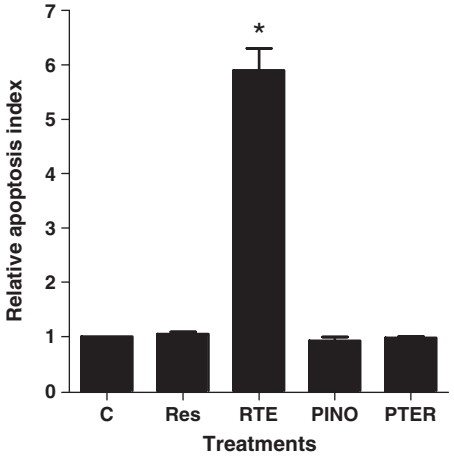

Figure 4. Differential effects of RES and its methylether analogs on apoptosis. LNCaP cells (1×10^6 cells/well) was plated in six-well plates and 24 h later, cells were treated with 5 μM of test compounds for an additional 24 h. Cells were harvested and caspase assay performed as described in Materials and methods. Fluorescence intensity that corresponded to caspase activity were normalized to control and expressed as relative apoptosis index (mean ± SD, $n = 3$). * Represents significant difference from control at $p < 0.05$.

3.3 Differential effects of RES and its analogs on cyclin inhibitors expression

Given the observed changes in cell cycle progression, we queried what molecular effects contributed to these changes. The effect of the test compounds on CDKN1 A and B mRNA expression was examined because transcriptional regulation of these cyclin inhibitors is critical to the regulation of G1/S or G2/M transition [46, 47]. The effects of RES (Fig. 5A), PINO (Fig. 5B), and PTER (Fig. 5C) appeared to be similar and upregulated both CDKN1A and B mRNA levels in LNCaP cells at high (25 μM) concentration. Treatment of LNCaP cells with RTE also led to an increase in CDKN1A and B mRNA levels (Fig. 5D). However, the threshold for CDKN1A mRNA induction by RTE (1 μM) appeared to be lower than that for CDKN1B mRNA (5 μM).

3.4 RES and its analogs inhibit PSA mRNA expression in LNCaP cells cultured in 10% FBS

PSA, a classic ARG, can be activated at the transcriptional level by multiple serum factors such as androgen and estrogen in LNCaP cells (25), and was used as a surrogate end point to assess the effect of stilbenes on possible hormone-responsive pathways in LNCaP cells. As shown in Fig. 5, treatment of LNCaP cells with RES, RTE, PINO, and PTER all led to inhibition of PSA mRNA expression in cells cultured in 10% FBS. RES appeared to have a slightly lower threshold than PINO or PTER. RTE appeared to be similar to RES; the effect of these compounds on PSA mRNA can be seen at 1 μM.

3.5 Effect of RES and its analogs on androgen and estrogen induction of PSA mRNA levels

An effect of test compounds on PSA mRNA in LNCaP cells cultured in FBS suggested modulation of androgen-mediated or estrogen-mediated events. We have previously shown that mRNA for several ARGs, including that of PSA, can be induced by both estradiol- and androgen-mediated events when cultured in 10% CDS [25]. To further elucidate the effect of stilbenes on these steroid sex hormones, the effect of stilbenes on DHT- and 17β-estradiol-induction of PSAmRNA expression was examined. LNCaP cells were treated with 5 μM of RES, PINO, PTER, or 2.5 μM RTE in 10% CDS in the presence and absence of DHT (1 nM) or 17β-estradiol (1 nM). As shown in Fig. 7, treatment of LNCaP cells with all four compounds led to attenuation of 17β-estradiol as well as DHT induction of PSA mRNA levels. The compounds appeared to exert a stronger inhibitory effect on 17β-estradiol induction of PSA mRNA than on DHT induction.

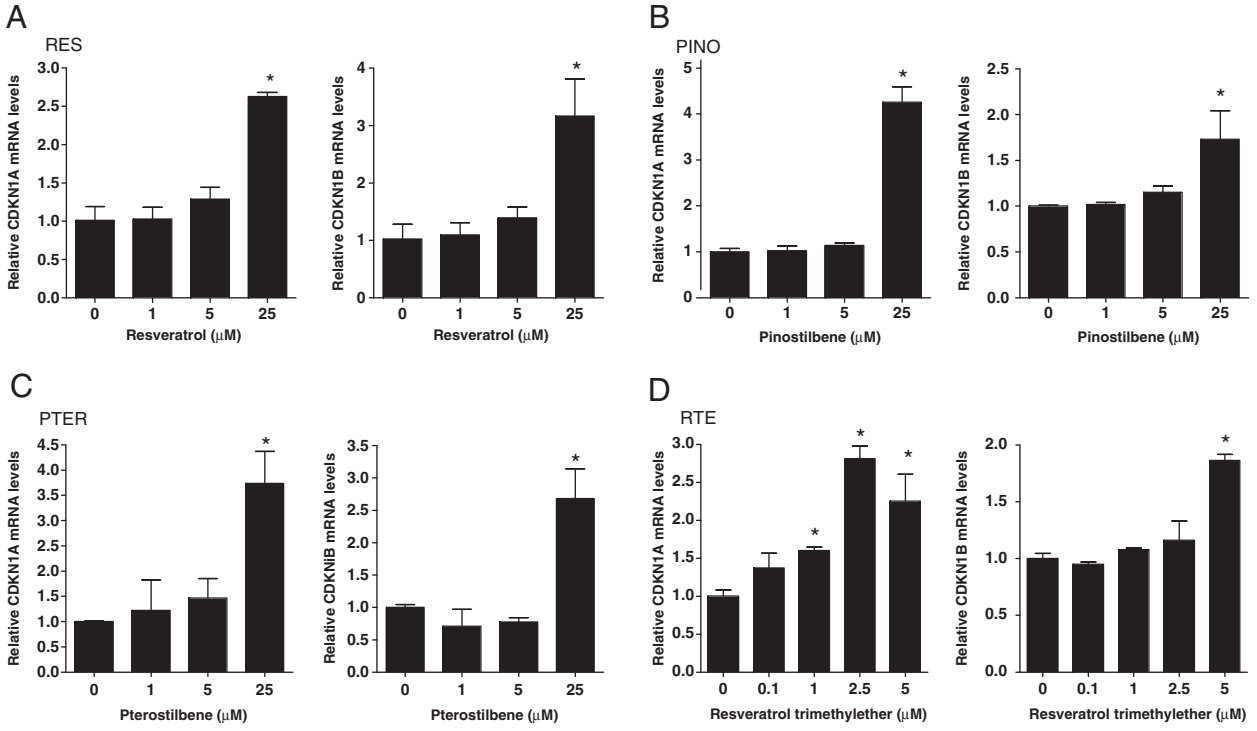

Figure 5. Effects of RES and its methylether analogs on cyclin inhibitors mRNA levels. LNCaP cells (0.25×10^6 cells/well) were plated on six-well plate and 24 h later the cells were treated with test compounds. RES, PINO, and PTER were used at concentrations of 0, 1, 5, or 25 μM, and RTE at 0, 1, 2.5, and 5 μM. After an additional 48 h treatment, cells were harvested for RNA and determination of CDKN1A and B mRNA levels using real-time PCR performed as described in Materials and methods. Results are expressed as relative mRNA levels (mean ± SD, $n = 3$) where vehicle control is 1. * Represents significant difference from control at $p < 0.05$.

4 Discussion

This study demonstrated that methyl ether analogs of RES that are found in edible plants exhibit differential effects on various cellular and molecular endpoints in the human androgen-responsive prostate cancer cell line LNCaP. Consistent with recent reports [37–39], we observed growth inhibitory effects of the methylether analogs of RES (Fig. 1). Of note, RTE appeared to be the most potent among the compounds studied similar to that found by Cardile *et al.* [38]. The effect of these compounds on cell growth appeared to be in part due to an effect on cell cycle. RES, PINO, and PTER treatment of LNCaP cells led to cell cycle arrest at G1/S (Figs 3A–C). Different from these three compounds, RTE appeared to exert its effects at G2/M (Fig. 3D). DESO, in contrast, appeared to exert little effect on cell growth as well as cell cycle. Temporally, the compounds also differed in their effects. The effects of RTE were observed as early as 24 h (Figs 3D and F). These results support the notion that, despite structural similarity (Fig. 1), RES and its analogs exert differential effects on LNCaP cells. Consistent with an effect on cell cycle, we also observed upregulation of CDKN1A and B mRNA (Fig. 5). Both CDKN1A and B mRNA upregulation are known to lead to cell cycle arrest at G1/S as well as G2/M [46, 47]. Hence, upregulation of these cyclin inhibitors is consistent with the cell cycle data shown in Fig. 3. Modulation of cyclin inhibitors likely contributed to the effects of the compounds at high (25 μM) concentrations of RES, PINO, and PTER (Figs 5 A–C). In contrast, treatment of LNCaP cells for 24 h with 1 μM RTE induced CDKN1A mRNA and with 5 μM RTE induced CDKN1B mRNA (Fig. 5D).

RTE exerted potent effects on LNCaP cell growth as well as cell cycle (Figs 2 and 3D). The effects of RTE occurred much quicker compared to the other stilbenes. RTE was the only compound that induced apoptosis as determined by both PI DNA staining and caspase 3/7 assays (Figs 3F and 4). Contrary to another report [27], we did not observe RES-induced apoptosis of LNCaP cells under the conditions used in this study.

The molecular effects of the RES and its analogs that may contribute to growth and cell cycle inhibition appeared to be not limited to regulation of the cyclin inhibitors. As shown in Fig. 6, LNCaP cells treated with RES and its analogs also downregulated PSA mRNA expression. The effect of the test compounds on PSA mRNA appeared to be due to inhibition of both estrogen- and androgen-mediated events, as exposure of LNCaP cells to the stilbenes led to inhibition of both DHT- (Fig. 7A) and 17β-estradiol- (Fig. 7B) induced increases in PSA mRNA levels. In addition to its responsiveness to the androgen DHT, LNCaP also responded to estradiol with

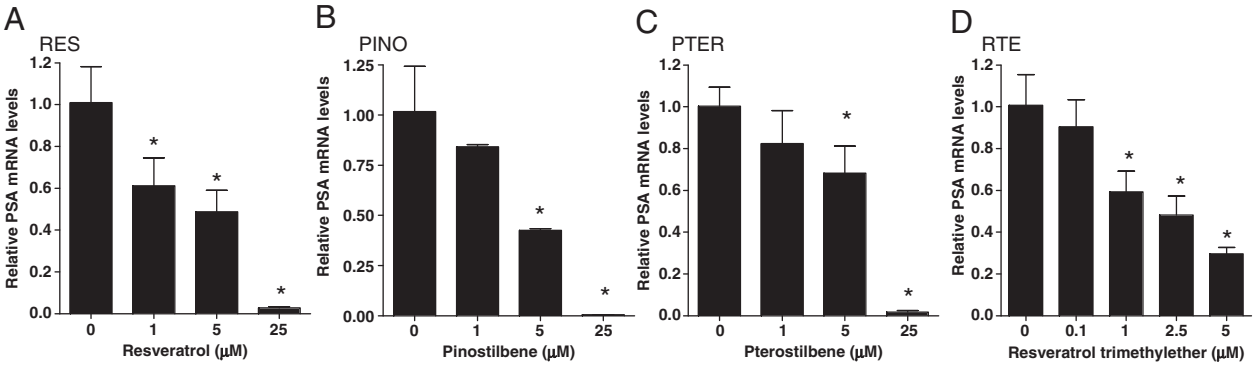

Figure 6. Effects of RES and its methylether analogs on classic ARG PSA mRNA levels. LNCaP cells (0.25×10^6 cells/well) were plated on six-well plate and 24 h later, the cells were treated with test compounds. RES, PINO, and PTER were used at concentrations of 0, 1, 5, or 25 μM, and RTE at 0, 1, 2.5, and 5 μM. After an additional 48 h treatment, cells were harvested for RNA and determination of PSA mRNA levels using real-time PCR performed as described in Materials and methods. Results are expressed as relative mRNA levels (mean ± SD, $n = 3$) where vehicle control is 1. * Represents significant differences from control at $p < 0.05$.

enhanced cell growth and gene expression [25]. Both androgen and estrogen are thought to be involved in prostate carcinogenesis [2, 3]. Estradiol acts through signal transduction events involving both estrogen receptor-β and androgen receptor to upregulate expression of ARGs [25]. Exposure to RES, RTE, PINO, and PTER inhibited the effects of DHT and 17β-estradiol, supporting the notion that an effect on these pathways may also contribute to the overall growth/cell cycle inhibitory effects. The effect of the stilbenes on DHT and estradiol seemed to be different, as the stilbenes exhibited stronger inhibitory effect on the estradiol-mediated induction of PSA mRNA (Fig. 7). Our results on steroid sex hormones are consistent with RES-induced inhibition of prostate cancer cells [17–22, 26–28, 37, 38], and provide new information that RTE, PINO, and PTER inhibit androgen and estrogen activity on LNCaP cells. Considering that the stilbenes showed effect on hormone-responsive pathways at relatively low concentrations (1–5 μM) compared to concentration led to cell cycle arrest or induction of apoptosis (> 5 μM), it is possible that these compounds may exert their prostate cancer-protective effects through interactions with the steroid sex hormone axis at physiological levels.

Methylether analogs of RES may be physiologically and/or pharmacologically important. Resveratrol is known to go through metabolism and circulate as glucuronides or sulfates [48]. Glucuronidation and sulfation occurred on the hydroxyl groups. The methylether analogs of RES may thus be important in providing protection against these processes and may be more bio-available. However, the physiological concentrations of methylether analogs of RES remain unknown. Hence, additional studies are needed to elucidate the physiological and/or pharmacological effects of these compounds.

In summary, this study provides molecular information on the effects of RES and its analogs on LNCaP cells. The molecular targets identified include modulations of cyclin inhibitors and modulation of androgen- and estrogen-mediated pathways.

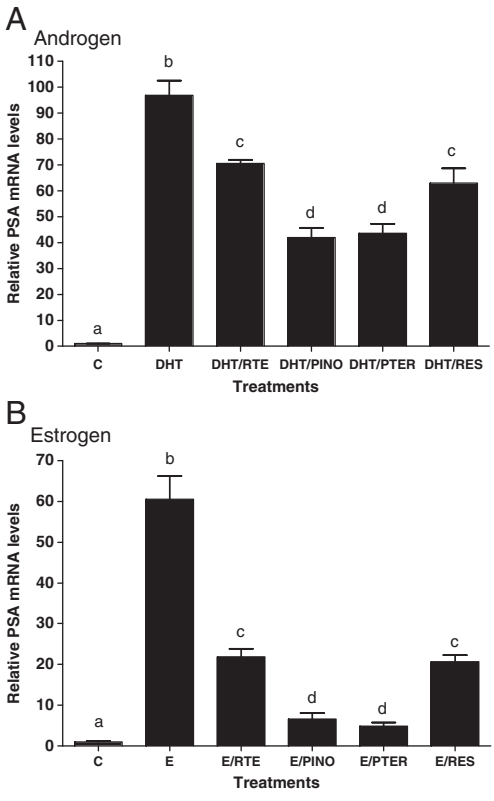

Figure 7. Effect of RES and its methylether analogs on DHT and 17β-estradiol induction of PSA mRNA levels. LNCaP cells were plated in six-well plates and 24 h later, the media was switched to Media B, which contained 10% CDS for an additional 24 h. Cells were then treated with or without DHT (1 or 17β-estradiol (1 nM) in the presence or absence of test compounds for 48 h. Total RNA was then isolated, and PSA mRNA levels determined as described in Materials and methods. RES, PINO, and PTER) were used at a concentration of 5 μM, and RTE at 2.5 μM. Results are expressed as relative mRNA levels (mean ± SD, $n = 3$) where vehicle control is 1. Bars with different letters are significantly different from each other at $p < 0.05$.

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