Combination of Resveratrol and Antiandrogen Flutamide Has Synergistic Effect on Androgen Receptor Inhibition in Prostate Cancer Cells

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Abstract. *Agents targeting the androgen receptor (AR) axis are critical for chemoprevention and treatment of prostate cancer (PCa) at all stages of the disease. Combination molecular targeted therapy may improve overall efficacy. The combination of dietary compound resveratrol with known therapeutic agents, such as the antiandrogen flutamide, may be particularly attractive due to the pharmacological safety of resveratrol. Materials and Methods: Resveratrol, 5α-dihydrotestosterone and flutamide were used in various experiments using mostly LNCaP cell line. Quantitative reverse transcription polymerase chain reaction (qRT-PCR), Western blots, and luciferase assay were utilized to examine the levels of AR mRNA, and protein and transcriptional activity in response to treatments. Growth proliferation assays were performed in three cell lines (LNCaP, PC3 and Du145). Results: Treatment of LNCaP cells with resveratrol (1-100 μM) resulted in the inhibition of androgen-promoted growth, inhibition of AR transcriptional activity and decrease in the AR and prostate-specific antigen protein levels through degradation pathways. The combination of resveratrol with flutamide had a synergistic effect on down-regulation of AR. Conclusion: Resveratrol works in concert with antiandrogen flutamide to reduce the amount and activity of AR, suggesting new therapeutic strategies for the treatment of PCa.*

The central role of androgen receptor (AR) signaling in prostate cancer (PCa) is defined by the fact that PCa develops and progresses under the influence of androgens. Remarkably, AR continues to be expressed and active in hormone-refractory androgen-independent tumors ultimately using alternative signaling pathways (1-3). Therefore, agents other than traditional antiandrogens with the ability to inhibit AR production and block AR signaling are of great interest for both chemopreventive and therapeutic strategies for all stages of PCa.

Diet-derived polyphenols are attractive clinical candidates for cancer prevention and treatment because they are pharmacologically safe. Resveratrol (3,5,4'-trihydroxystilbene) is a natural phytoalexin that is synthesized in several plants as a defensive response against fungal infection and environmental stress. It is produced in the skin and seeds of grapes, with its further accumulation in red wine during the fermentation process. Recently, epidemiological studies have demonstrated a reduced relative risk for PCa associated with an increased level of red wine consumption (4), which was partly attributed to resveratrol content.

Resveratrol, also well known as an antioxidant, has received increasing attention during the last fifteen years for its potential chemopreventive and antitumor effects. From accumulated data, it is clear that resveratrol exhibits numerous biological activities, such as inhibition of cancer cell proliferation, migration, invasion and angiogenesis and induction of apoptosis (5-11) by acting through multiple signaling pathways.

The goal of the current study was to examine the effects of resveratrol on AR and its signaling (androgen-inducible target gene prostate-specific antigen, PSA) in PCa cells and to assess if combined treatment with the conventional endocrine agent flutamide would result in superior effects. Here, we report that resveratrol inhibits AR production and blocks AR signaling and acts as a 5α-dihydrotestosterone (DHT) antagonist in concert with pure antiandrogen flutamide. This makes resveratrol a very attractive and promising pharmacologically safe compound to be used in combination settings for improved therapeutic outcomes.

Materials and Methods

Materials. Resveratrol, DHT, flutamide, proteasome inhibitor N-[(phenylmethoxy)carbonyl]-L-leucyl-N-[(1R)-1-formyl-3-methylbutyl]-L-leucinamideN-benzoyloxy-carbonyl (MG132) and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Caspase-3 inhibitor Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (Z-DEVD-FMK) was purchased from Imgenex (San Diego, CA, USA). Compounds used in the experiments were dissolved in 100% ethanol or high purity dimethyl sulfoxide. Resveratrol was stored in the dark at –20˚C to ensure its stability.

Cell culture. LNCaP, PC3 and DU145 cells (ATCC, Manassas, VA, USA) were grown in RPMI- 1640 containing 10% fetal bovine serum and 1× penicillin-streptomycin-amphotericin B at 37˚C/5% CO_2 as previously described (8, 12). For experiments involving treatment with resveratrol, DHT and flutamide, media were replaced with phenol red-free RPMI-1640 containing 5% charcoal-stripped serum 16 hours prior to treatment to eliminate steroidal background that can interfere with their action. RPMI-1640 was obtained from Gibco BRL (Gaithersburg, MD, USA).

Western blot analysis. Western blot was carried out as described previously. Briefly, LNCaP cells were treated with different concentrations of resveratrol or other compounds for 24h. the cells were lysed by radioimmunoprecipitation assay (RIPA) buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCI, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate and supplemented with protease and phosphatase inhibitors (Phosphatase Arrest II and Mammalian Protease Arrest, G-Biosciences, St Louis, MO, USA).The protein concentration was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (40-70 μg) were resolved in 7-10% Tris-HCl Ready gels (Bio-Rad Laboratories) and transferred to a nitrocellulose membrane by Mini Trans-Blot Electrophoresis Transfer System (Bio-Rad Laboratories). The membranes were then blocked and subsequently probed with AR (Thermo Fisher Scientific, Fremont, CA, USA) and PSA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. The blots were also probed with β-Actin antibodies (Sigma-Aldrich) as a loading control. Signals were visualized using enhanced chemiluminescence. Images were quantified using Image J software, a public domain program at http://rsb.info.nih.gov/nih-image/ . Nuclear extracts from LNCaP cells were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce, Rockford, IL, USA).

Cell proliferation assay. Du145 and PC3 cells were seeded at 5×10^5 cells/well density in 24-well plates using RPMI-1640 media. LNCaP cells were seeded at 1×10^6 cells/well density in 24-well plates using the same media as above. The media were changed to phenol red-free with 5% charcoal-stripped serum 16 h prior to treatments. Media containing different concentrations of resveratrol or its combination with DHT were added and refreshed every other day for 6 days. The cells were then lysed by sonication using microsonicator (Triad Scientific, Manasquan, NJ, USA). DNA content was measured as described previously (13) using a VersaFluor™ Fluorometer (Bio-Rad Laboratories).

Luciferase reporter assay. LNCaP cells cultured in phenol red-free media with charcoal-stripped serum were transiently co-transfected with pGL3-GRE-Luc and pRL-tk-Luc reporter constructs using FuGene (Roche Applied Sciences, Indianapolis, IN, USA). The pGL3-GRE-Luc expression plasmid encodes for the luciferase reporter gene under the control of the AR responsive element, whereas pRL-tk-Luc serves as an internal control for luciferase activity. Cells were then treated with different compounds for 24 h and harvested for the Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA). Luminometer Bio-TEK Synergy HT (Bio TEK, Winooski, VT, USA) was used for measuring light emission. Renilla luciferase activity was used for background normalization.

RNA isolation and quantitative RT-PCR (qRT-PCR). RNA was isolated using RNeasy Kit (Qiagen, Valencia, CA, USA) from the resveratrol-treated cells. We performed qRT-PCR on a MiniOpticon Real-Time PCR System (Bio-Rad Laboratories) using the protocol of the SuperScript III platinum two-step qRT-PCR kit with SYBR green (Invitrogen, Carlsbad, CA, USA). Briefly, first-strand cDNA was synthesized using 1 μg total RNA from treated cells. The PCR reaction was conducted in a reaction mix of 25 μl Platinum SYBR Green qPCR SuperMix-UDG with 1 μl cDNA template and 0.5 μl primers (10 μM each). The PCR reaction was run for 40 cycles at 95˚C for 30s, 60˚C for 30s and 72˚C for 30s. Primers were purchased from IDT (Coralville, IA, USA). The gene-specific primers for AR and PSA were: forward: 5'-GGA CTT CAC CGC ACC TGA TG -3; reverse: 5'-CTG GCA GTC TCC AAA CGC AT -3' and forward: 5'-GCC TCT CGT GGC AGG GCA GT -3'; reverse: 5'- CTG AGG GTG AAC TTG GGC AC -3', respectively. β-Actin was amplified in parallel as the internal control. The analysis was performed using Opticon Monitor software version 3.1. The quantitation was carried out using the ΔΔCt method (14). Briefly, ΔCt for AR or PSA was calculated by subtracting the Ct of β-Actin mRNA from the Ct of *AR* or *PSA* mRNA. ΔΔCt was then calculated by subtracting the ΔCt of the ethanol-treated control from the ΔCt of the resveratrol-treated samples. Fold change of the expressions of AR and PSA mRNA were calculated by the equation $2^{-\Delta\Delta Ct}$.

Statistical analysis. Each experiment was reproduced at least three times. Values are expressed as the mean±SEM of triplicate measurements unless otherwise stated. Student's two tailed paired t-test was used to analyze differences between treated and control cells. Combination index (CI) values (quantitative definition for additive effect (CI=1), synergism (CI<1), and antagonism (CI>1) in drug combinations) for combination experiments were calculated by Chou-Talalay method using CompuSyn software (ComboSyn, Inc. Paramus, NY, USA).

Results

Resveratrol inhibits cell proliferation of both AR-negative and AR-positive PCa cells and acts as an anti-androgen in LNCaP cells. We examined the effect of Res on the growth of DU145, PC3 (AR-negative) and LNCaP (AR-positive) cells. As shown in Figure 1A, resveratrol inhibited the growth of all three cell lines in a dose-dependent manner, albeit with different potency. The IC_{50} for DU145 and PC3 were 7 and 15 μM respectively, whereas for LNCaP it was 25 μM. These results demonstrate that resveratrol inhibited proliferation of PCa cells *via* both androgen-independent and -dependent mechanisms, raising the question whether resveratrol action in androgen-dependent cells was mediated through AR. Therefore, we next analyzed whether resveratrol acted as an antagonist for AR-mediated DHT-responsive growth proliferation in LNCaP cells. Cells were treated with

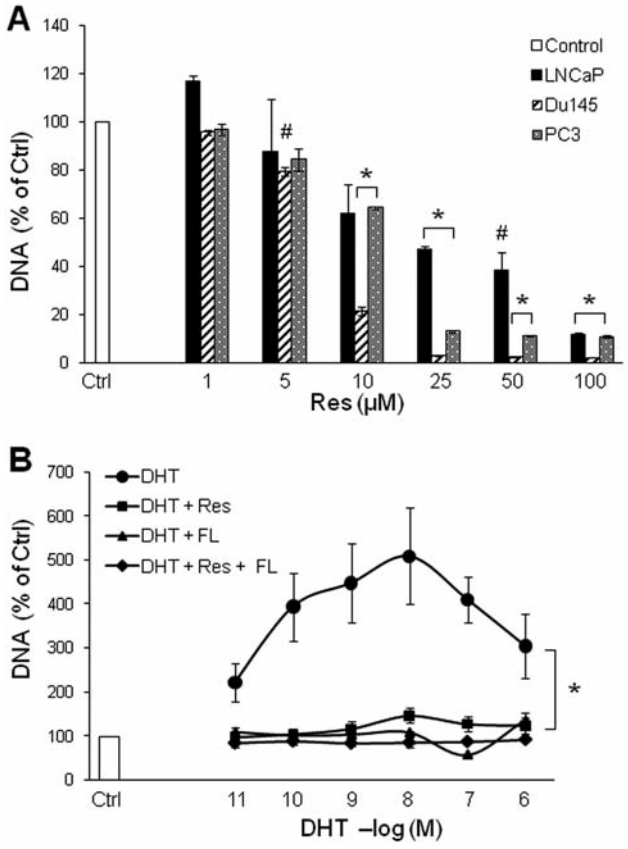

Figure 1. *Effects of resveratrol on growth of prostate cancer cells. A: Resveratrol inhibits growth of LNCaP, Du145 and PC3 cells. Cells were grown in media containing different doses of resveratrol for 6 days. The DNA concentrations are plotted as a percentage that of untreated cells (Ctrl). Data are presented as the mean±SE of three independent experiments in which each treatment (data point) was performed in triplicates. Statistically significant at #p<0.01 and *p<0.001 compared to Ctrl. B: Resveratrol blocks androgen-promoted growth of LNCaP cells. Cells were grown in media containing different doses of dihydrotestosterone (DHT) with /without 50 μM resveratrol (Res) and/or 10^{-4}M flutamide (FL) for 6 days. DNA concentrations are plotted as a percentage that of untreated cells (Ctrl). Data are shown as mean±SE of three independent experiments in which each treatment (data point) was performed in triplicates. DHT-treatment statistically significantly differs from Ctrl and combination treatments (*p<0.05).*

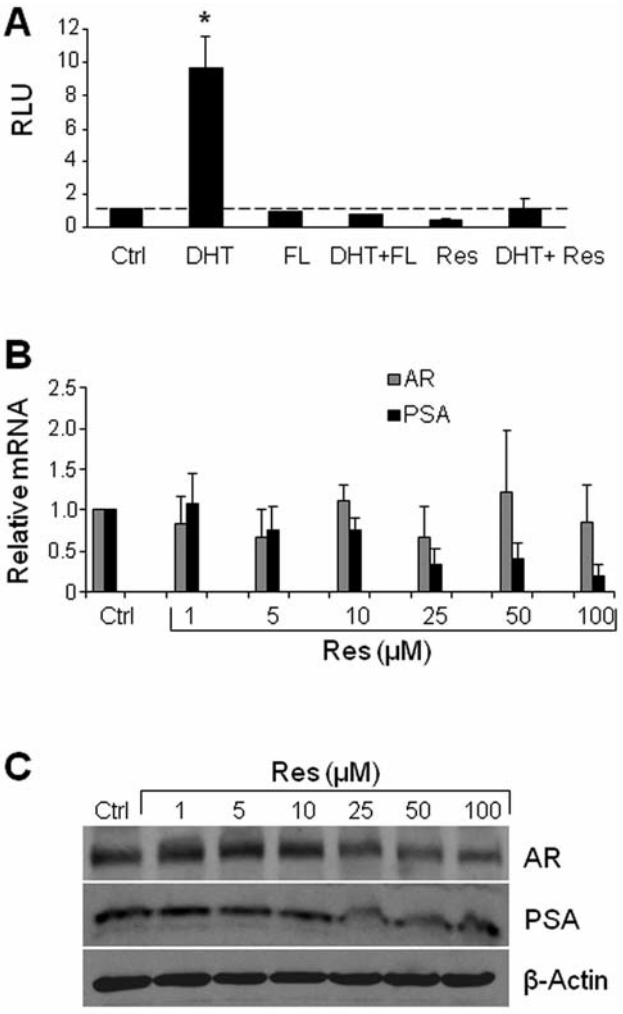

Figure 2. *Effects of resveratrol on androgen receptor and prostate-specific antigen. A: Resveratrol inhibits dihydrotestosterone-mediated transcriptional activation of AR in LNCaP cells. Cells were transiently co-transfected with pGL3-ARE-Luc and pRL-tk-Luc and treated with 10-8M DHT, 10-4M flutamide (FL), 50 μM resveratrol (Res) or their combinations for 24 h. Relative luciferase units (RLU) are presented by the ratio of Firefly to Renilla luciferase activity. The RLU of control untreated cells was set as 1, and changes on luciferase activity of treated cells are expressed as a percentage that of the Ctrl. Experiments were repeated three times in triplicates, and values are indicated as means±SD. DHT-treatment differs statistically significantly from Ctrl and all other treatments (*p<0.05). B: Resveratrol inhibits AR and PSA mRNA levels in LNCaP cells. Total RNA was isolated from cells following treatment with ethanol (Ctrl) and different doses of resveratrol for 24 h, and real-time PCR was performed and analyzed using the ΔΔCt method as described in the Materials and Methods. The expression of the control was set to 1, and changes in mRNA levels of treated cells are expressed as a percentage that of the Ctrl. Data shown are the mean±SD of at least four independent experiments performed in duplicates. C: Resveratrol down-regulates AR and PSA protein levels in a dose-dependent manner in LNCaP cells. Cell lysates were isolated from cells following treatment with ethanol (Ctrl) and different doses of resveratrol for 24 h, and Western blot was performed as described in the Materials and Methods. β-Actin was used as a loading control. Representative blots of three independent experiments with similar results are shown.*

combination of a fixed concentration of resveratrol (50 μM) and different concentrations of DHT (10^{-11} – 10^{-6} M) for 6 days. Resveratrol successfully and significantly blocked the DHT-promoted growth proliferation of LNCaP cells to the same extent as 10^{-4} M antiandrogen flutamide (Figure 1B). The effects of the combination of resveratrol and flutamide were similar to those of each compound alone.

Resveratrol inhibits AR transactivation and AR and PSA expression at mRNA and protein levels in LNCaP cells. Considering that resveratrol-mediated growth inhibition in

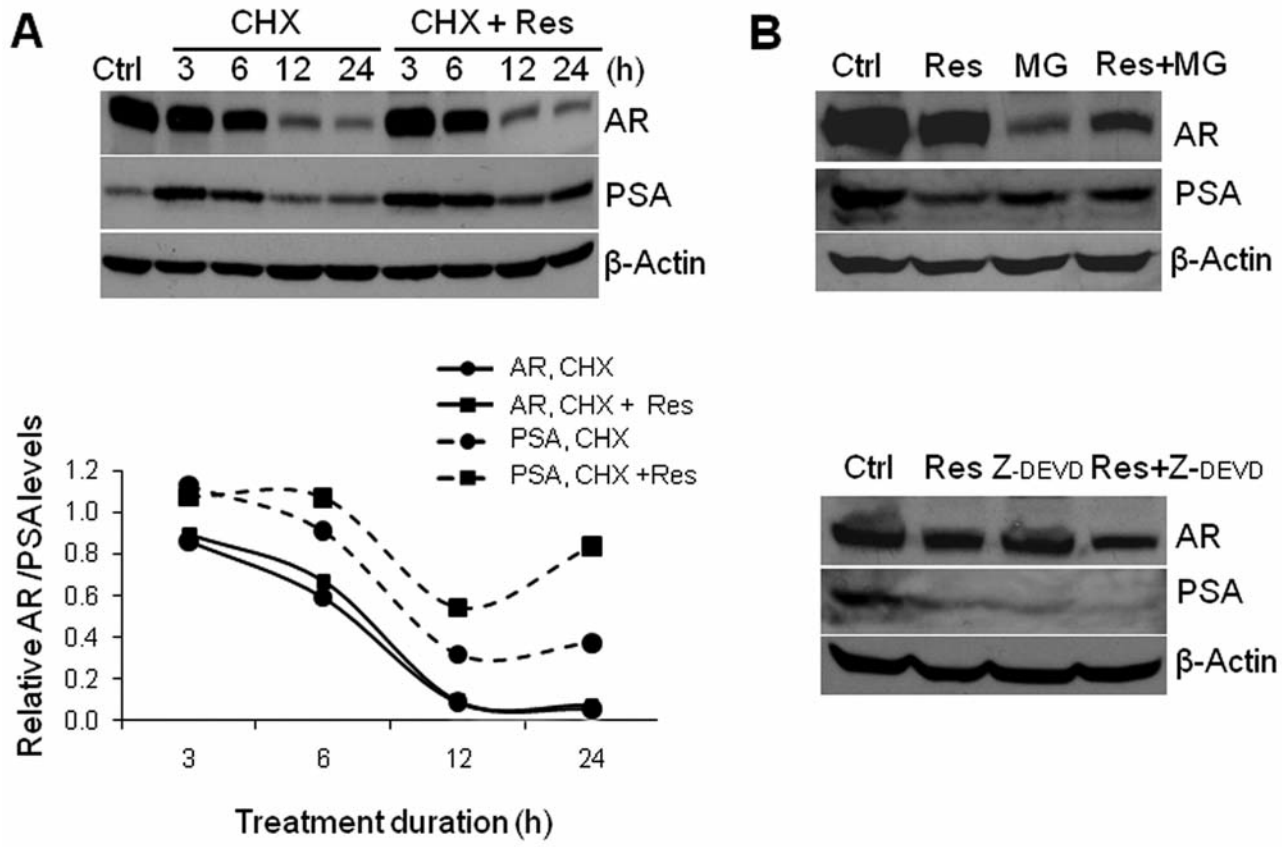

Figure 3. *A: Effects of cycloheximide and resveratrol on AR and PSA synthesis inhibition. Upper panel: LNCaP cells were treated with 50 μM CHX alone or in combination with Res (50 μM) for different times. Cell lysates were subject to Western blot. Lower panel: Graphical representation of the changes in AR and PSA protein synthesis levels after treatment with CHX and CHX + Res. B: Effects of proteasome inhibitor MG132 and caspase-3 inhibitor Z-DEVD-FMK on Res-induced AR and PSA degradation. Upper panel: LNCaP cells were treated with 50 μM Res and 10 μM MG132 (MG) or combinations for total of 24 h with MG pretreatment. Cell lysates were subject to Western blot. Lower panel: LNCaP cells were treated with 50 μM Res and 10 μM Z-DEVD-FMK (Z-DEVD) or combinations for total of 24 h with Z-DEVD pretreatment. Cell lysates were subject to Western blot as described in the Materials and Methods. Each experiment was repeated at least three times with similar results. Representative blots are shown.*

androgen-responsive LNCaP cells can be related to modulation of AR by resveratrol, we next assessed how resveratrol affects AR transcriptional activity and whether it acts as an antagonist. LNCaP cells were transiently co-transfected with pGL-GRE-Luc and pRL-tk-Luc reporter constructs for 18 h and treated with resveratrol (50 μM), DHT (10^{-8} M) and flutamide (10^{-4} M) alone and in combinations (same concentrations as alone) for the next 24 h, after which luciferase activities were determined. AR was activated by DHT, and both resveratrol and flutamide were similarly potent in significantly blocking the DHT-induced transactivation function of AR (Figure 2A). To investigate the dose-dependent effect of resveratrol on AR and PSA expression at mRNA and protein levels, we treated cells with different doses (1-100 μM) of resveratrol for 24 h and isolated total RNA for real-time PCR and protein for Western blot. As shown in Figure 2B there was a dose-dependent down-regulation of *PSA* mRNA in resveratrol-treated cells compared to untreated cells. Data on changes of *AR* mRNA levels were less consistent. Results of Western blot showed that resveratrol inhibited AR and PSA protein levels in a dose-dependent manner at concentrations of 25 μM and greater (Figure 2C). In the cells treated with 25 or 50 μM resveratrol, the level of AR was about 74% and 67%, respectively, of that in the control untreated cells while the level of PSA was about 54% and 35% compared to control. Interestingly, these results indicate the possibility of independent mechanisms of AR and PSA regulation by resveratrol with more efficient reduction of intracellular PSA on both mRNA and protein levels.

Resveratrol-mediated down-regulation of AR and PSA involves the caspase-3 and proteasome degradation pathways, respectively. Since resveratrol had an inconsistent

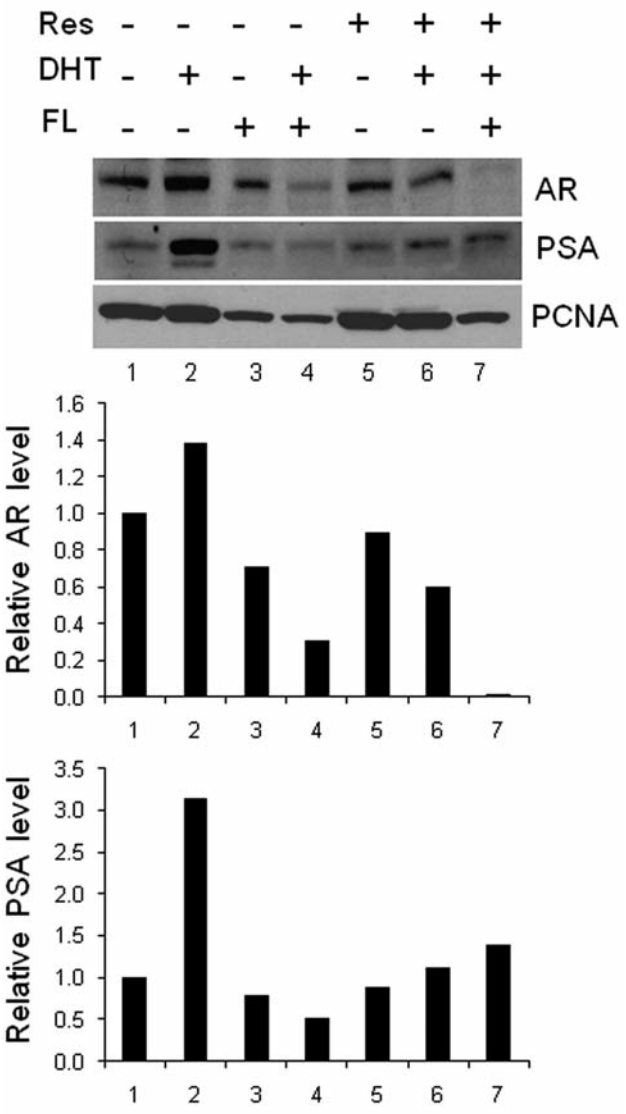

Figure 4. *Effects of combined treatment with resveratrol and flutamide on dihydrotestosterone-stimulated expression of AR and PSA proteins in LNCaP cells. Cells cultured in phenol red-free media with stripped serum were treated with 50 μM Res, 10^{-8}M DHT, 10^{-4}M FL or combinations at the same doses for 24 h. Nuclear extracts were separated on 10% SDS-PAGE. Proliferating cell nuclear antigen (PCNA) was used as a loading control but flutamide had unknown specific effect on PCNA in all three independent sets of experiments. Densitometer quantitation of bands was carried out using Image J software, and levels of AR and PSA normalized to unaffected PCNA are shown.*

inhibitive effect on AR mRNA and convincing down-regulation of AR and PSA at the protein level, we sought to examine the posttranslational regulation of AR and PSA by resveratrol. Treatment with protein synthesis inhibitor cycloheximide (50 μM) alone and in combination with resveratrol (50 μM) resulted in a similar time-dependent decrease of AR in cells, whereas changes in PSA levels indicated an apparent unexpected stabilization of PSA by resveratrol (Figure 3A). We next examined degradation pathways possibly involved in resveratrol-regulated decrease of AR and PSA. We treated cells in the presence of proteasome inhibitor MG132 and caspase-3 inhibitor Z-DEVD-FMK and found that MG132 did not restore AR levels, neither when it was used alone nor in combination with resveratrol (Figure 3B, upper panel), whereas Z-DEVD-FMK restored AR when used alone but was less effective when in combination with resveratrol (Figure 3B, lower panel). These results suggest that AR is degraded by caspase-3-dependent pathways rather than by a proteasome pathway. In contrast, PSA degradation most likely occurs through proteasome pathways since MG132 was able to partially restore resveratrol-dependent inhibition of PSA.

Effect of combination of resveratrol and flutamide on DHT-stimulated expression of AR and PSA. The next question we asked was whether resveratrol antagonizes the known effect of androgens on stimulation of AR and PSA protein levels. We treated LNCaP cells with resveratrol (50 μM), DHT (10^{-8} M) and flutamide (10^{-4} M) alone and in combinations for 24 h and examined nuclear extracts by Western blot. As expected, AR and PSA showed a dramatic increase in protein levels with DHT treatment (Figure 4, lane 2). Predictably, flutamide alone (lane 3) reduced both AR and PSA protein levels, and in combined treatment with DHT (lane 4), powerfully inhibited DHT-stimulated increase of both proteins. Importantly, not only did resveratrol alone inhibit AR and PSA but it also significantly blocked the DHT-stimulated increase (lanes 5, 6) of both AR and PSA. Combining resveratrol with flutamide proved to be most effective in reducing AR expression, which was completely abolished after simultaneous treatment with these two compounds (lane 7). The CI of combined inhibition of DHT-induced AR expression was less than 1, which indicated synergy between resveratrol and flutamide. Interestingly, once again, changes in PSA differed from changes in AR when treated with the same compounds; for PSA levels, CI indicated slight antagonism between resveratrol and flutamide.

Discussion

Given that resveratrol is a dietary compound, its use as a preventive agent seems compelling. Moreover, resveratrol has also been shown to inhibit cancer cell proliferation (13, 15-17) and angiogenesis-dependent processes such as tumor growth, invasion and metastasis (9-11, 18-20), thus presenting potential as a therapeutic agent.

AR signaling is central to the development and progression to advanced androgen-resistant PCa, in which AR is still present and acts through alternative pathways (2).

Dietary compounds such as resveratrol that are able to reduce AR expression and its signaling, *i.e.* PSA, are therefore potentially effective preventive and therapeutic agents for PCa, especially in conjunction with other agents. Combination of therapeutic agents with pharmacologically safe compounds may be highly beneficial due to reduced adverse effects related to toxicity. In the present study, we examined the effects of resveratrol alone and in combination with AR antagonist flutamide on AR signaling in PCa cells.

Regulation of AR by resveratrol in LNCaP cells has been reported with conflicting data (6, 21-23). Resveratrol failed to reduce AR expression in one study (23), whereas a decrease in *AR* mRNA and protein expression by resveratrol was achieved at 50 μM in another (21). We found that AR was not particularly modulated at the mRNA level by resveratrol, whereas *PSA* mRNA was significantly affected at 25 μM and higher. Interestingly, *AR* mRNA levels were not affected even by DHT treatment in LNCaP cells (24), suggesting preferred post-translation regulation of AR by different ligands. Our results on resveratrol-induced down-regulation of AR at the protein level rather than at the mRNA level are consistent with data from Harada *et al*. (25).

Our finding with cycloheximide and resveratrol indicated that protein degradation rather than protein synthesis is a possible primary mechanism for resveratrol-induced down-regulation of AR and PSA. We hypothesized that resveratrol may activate degradation of AR and PSA either through proteasome or caspase-3 proteolytic pathways, which have been reported to participate in AR degradation (26-27). Interestingly, the overall balance of AR degradation favored the caspase-3- mediated pathways, whereas the proteasome pathway seemed more probable as PSA degradation pathway. These results imply that in addition to AR-mediated PSA regulation, resveratrol may directly down-regulate AR and PSA through separate degradation mechanisms and not necessarily treat PSA as merely an AR-responsive and dependent protein. More systematic studies are required to elucidate differential post-transcriptional and post-translational mechanisms of resveratrol regulation of AR and PSA.

Our results with resveratrol clearly demonstrate that resveratrol is an AR antagonist to a similar extent as flutamide. What are the mechanisms of action for these two agents? DHT increases the AR protein level by binding to AR, stabilizing it and triggering binding to DNA (24, 28). Pure antiandrogen flutamide competes for binding to AR, and causes nuclear translocation but fails to transactivate AR due to the conformational changes that prevent AR□DNA binding (29). It has been clearly shown that flutamide binds to the ligand-binding domain of AR, whereas resveratrol does not (25). This is surprising considering that the structure of the ligand-binding domain is highly conserved between AR and estrogen receptor (ER), and it is well established that Res binds to both ERα and ERβ (30). However, whether or how resveratrol actually binds to AR is unclear. In fact, at present, no information on direct binding of resveratrol to AR is available except data on extremely weak affinity of resveratrol for AR (31-32). It is possible that resveratrol regulates AR signaling *via* other mechanisms. For example, resveratrol can induce the activation/phosphorylation of c-Jun, which directly interacts with AR, resulting in a decrease in PSA expression (33, 34). Alternatively, even if resveratrol does bind to AR, it is possible that resveratrol-bound AR is retained in the cytoplasm, making AR more susceptible to enzymatic degradation, while flutamide-bound AR translocates to the nucleus. Indeed, it was recently shown that resveratrol treatment itself did not affect subcellular localization of AR (mainly cytoplasmic) and in combination, resveratrol did not interfere with hormone-induced nuclear translocation of AR (21). Yet another mechanism of resveratrol regulation of AR function included a decrease in AR acetylation, which in turn suppressed the accumulation of AR in the nucleus due to increased nuclear export of non-acetylated AR (35). Our results support the idea that resveratrol acts through different mechanisms from flutamide, without competing for binding to AR, because in combination experiments with DHT (DHT + resveratrol + flutamide), AR expression was inhibited to a much higher degree compared to the effect of each agent alone (Figure 3A, lane 7).

In summary, we demonstrated that resveratrol inhibits growth proliferation and acts as a powerful AR antagonist in LNCaP cells, at least in part, through inhibition of AR. Used alone resveratrol reduces AR and PSA protein levels. AR levels, but not PSA levels, were reduced more effectively in combination with antiandrogen flutamide. We also showed that resveratrol can promote AR and PSA degradation separately *via* activation of different proteolytic pathways. In conclusion, we suggest that the potential of resveratrol for chemoprevention and anticancer therapy may mostly reside in its ability to sensitize tumor cells for better response to conventional hormonal therapy. These insights into the molecular mechanisms of resveratrol action may facilitate the development of resveratrol and its derivatives as therapeutics in prevention and treatment of PCa.

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