

Novel Resveratrol Derivatives Induce Apoptosis and Cause Cell Cycle Arrest in Prostate Cancer Cell Lines

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Abstract. *Background:* Resveratrol (RV), a naturally occurring phytoalexin, exerts manifold biological effects against a variety of human tumor cell lines. In this study, the cytotoxic and biological effects of novel RV derivatives were investigated in prostate cancer cells. *Materials and Methods:* Cytotoxicity of the compounds was assessed by clonogenic assays in PC-3, LNCaP and DU-145 human prostate cancer cell lines. Induction of apoptosis was studied by Hoechst-propidium-iodide double staining. Cell cycle phase distribution of prostate cancer cells was analyzed using flow cytometry. *Results:* Methoxy- and hydroxy-substituted RV derivatives exerted cytotoxic effects against all three cell lines. The most potent compounds, 3,3',4,4',5,5'-hexahydroxy-stilbene and 3,4,4',5-tetramethoxy-stilbene, induced apoptosis at concentrations lower than RV and caused cell cycle arrest in the cell lines investigated. *Conclusion:* Introducing additional hydroxy- and methoxymoiety to the stilbene ring of RV is capable of enhancing its cytotoxic and pro-apoptotic effects in hormone-responsive and non-responsive prostate cancer cells.

Resveratrol (3,4',5-trihydroxy-stilbene, RV) is a naturally occurring phytoalexin found in the skin of grapes and red wine. It protects plants against fungal infection and is present

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in concentrations of up to 10 μ M in red wines and to a much lesser extent in white wines (1). RV possesses chemopreventive and cytostatic properties against a variety of human tumor cell lines, including several prostate cancer cell lines (2-6). There has been intense research on the biochemical effects of RV. RV inhibits tumor initiation, promotion, and progression in both solid and hematological tumor cells by causing an arrest in the S- and G2-phases of the cell cycle (7-9). Several studies have revealed that RV is capable of inducing apoptosis and differentiation in many tumor cell lines, such as human leukemia, breast cancer and esophageal cancer cells. Apoptosis can occur *via* Fas (CD95)-dependent or -independent, mitochondrial mechanisms or through activation of caspases and cleavage of poly(ADP-ribose)polymerase (PARP) (10-14).

Beyond these effects, RV also exhibits estrogenic effects mediated through the activation of estrogen receptors. Bowers *et al.* have shown that it acts as a mixed agonist/antagonist for estrogen receptors (ER) α and β . They revealed that RV binds ER α and ER β with comparable affinity, but both with lower affinity than estradiol. One possible explanation for the lower affinity of RV binding to ER β compared with other phytoestrogens is its structural similarity with diethylstilbestrol, which binds to ER β with lower affinity than ER α (15).

These properties render RV and related compounds very promising candidates for the treatment of hormone-sensitive tumors, such as prostate cancer.

In prostate cancer cell lines, the pleiotropic effects of RV have also been investigated in detail. RV has been shown to inhibit proliferation of several hormone-responsive and non-responsive prostate cancer cell lines (5, 16). It also induced apoptosis and cell cycle arrest in these cell lines (17, 18).

It is believed that some of the cytotoxic effects of RV are due to its unique polyphenolic structure. Morris *et al.* have

demonstrated that the presence of hydroxyl groups is required in order to exert pro-apoptotic and anti-tumor effects in prostate cancer cell lines (19). Therefore, an increase in the number of hydroxyl groups could enhance its biochemical effects. Piceatannol, a naturally occurring hydroxyl-substituted metabolite of RV, has already been shown to induce apoptosis in prostate cancer cell lines (20).

In this study, the effects of several synthesized polyhydroxy- and polymethoxy-substituted derivatives of RV, with an increasing number of hydroxy- and methoxy groups are reported. The most promising agents, 3,3',4,4',5,5'-hexahydroxy-stilbene (M8) and 3,4,4',5-tetramethoxy-stilbene (M4) were compared in more detail with RV.

Materials and Methods

Chemicals and supplies. Hydroxy- and methoxy-substituted RV derivatives were synthesized as described previously and provided by the Institute of Pharmaceutical Chemistry, University of Vienna, Austria (21). The structure and denomination of the derivatives is shown in Table I. The RV and all other chemicals used were purchased from Sigma-Aldrich GmbH (Vienna, Austria), and were of the highest purity available.

Cell culture. The testosterone responsive and non-responsive prostate cancer cell lines PC-3, LNCaP and DU-145 were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum and with 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO₂. All media and supplements were obtained from Gibco Life Technologies Ltd. (Paisley, Scotland, Great Britain). The cell counts were determined using a CC-108 microcellcounter (Sysmex, Kobe, Japan). Cells in a logarithmic phase of growth were used for all the studies.

Clonogenic assay. Cells were plated in 24-well plates at a concentration of 3000 cells per well and allowed to attach overnight at 37°C in a humidified 5% CO₂ atmosphere. After 24 hours, the medium was removed and replaced by fresh medium containing the designated concentration of the drugs. The plates were then incubated for 7 days. Subsequently, the medium was carefully removed from the wells and the plates were stained with 0.5% crystal violet solution for 5 minutes. Colonies of more than 50 cells were counted using an inverted microscope at 30-fold magnification. All experiments were performed in triplicate.

Hoechst dye 33258 and propidium iodide double staining. The PC-3, LNCaP and DU-145 prostate cancer cells (0.1x10 per ml) were seeded in 25 cm² tissue culture flasks, allowed to adhere overnight and exposed to increasing concentrations of drugs for 24 hours. Afterwards, Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma) were added directly to the cells to final concentrations of 5 µg/ml and 2 µg/ml, respectively. The cells were judged according to their morphology and the integrity of their cell membranes, which were examined by fluorescence microscopy (Zeiss Axiowert 35, equipped with appropriate DAPI filters for Hoechst 33258 and PI). This method allows distinction between early apoptosis, late apoptosis, and necrosis. The cells

Table I. IC₅₀-values (µM) of resveratrol (RV) and methoxy- and hydroxyl-substituted derivatives as measured by clonogenic assay* after 7 days of incubation.

Compound	Cell line		
	PC-3	LNCaP	DU-145
Resveratrol (3,4',5-trihydroxy-stilbene)	16	5	10
M1 (3,3',5,5'-tetramethoxy-stilbene)	35	21	25
M2 (3,3',4,5,5'-pentamethoxy-stilbene)	21	>100	68
M3 (3,3',4,4',5,5'-hexamethoxy-stilbene)	5.5	37	12.5
M4 (3,4,4',5-tetramethoxy-stilbene)	3	0.4	0.4
M5 (3,4',5-trimethoxy-stilbene)	3	6.25	6
M5A (3',4,5,5'-tetramethoxy-stilbene)	35	6.25	30
M6 (3,3',5,5'-tetrahydroxy-stilbene)	16	84	17
M7 (3,3',4,5,5'-pentahydroxy-stilbene)	5	7.5	23
M8 (3,3',4,4',5,5'-hexahydroxy-stilbene)	2.5	3.4	25
M9 (3,4,4',5-tetrahydroxy-stilbene)	8	9.5	30
M10 (3',4,5,5'-tetrahydroxy-stilbene)	18	33	58

*Cells were seeded in 24-well plates and allowed to attach overnight. The next day, the compounds were added in the designated concentrations. After 7 days of incubation, plates were stained with 0.5% crystal violet solution and colonies >50 cells were counted using an inverted microscope.

were photographed with an Olympus camera using Kodak Ektachrome P1600 films (Kodak, Rochester, NY, USA). The cells were differentiated into viable, apoptotic and necrotic categories. In the untreated control cells, 1.3%, 12.3% and 0.4% underwent apoptosis for the PC-3, LNCaP and DU-145 cells, respectively.

Cell cycle phase distribution. The PC-3, LNCaP and DU-145 prostate cancer cells were seeded in culture flasks and incubated with various concentrations of drugs for 72 hours and then fixed in ethanol (70%). Afterwards, RNase A and propidium iodide were added to a final concentration of 50 µg/ml each and incubated at 4°C for 60 minutes before measurement. The cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA).

Statistical analysis. Dose-response curves, tables and statistical significance were calculated and graphically represented employing Prism 4.03 software (GraphPad, San Diego, CA, USA).

Results

Clonogenic assay. After 7 days of incubation, RV yielded IC₅₀-values of 16 µM, 5 µM and 10 µM in PC-3, LNCaP, and DU-145 cells, respectively. Out of all the methoxy-substituted derivatives, M4 demonstrated the greatest cytotoxic activity. In the three cell lines investigated, M4 exhibited IC₅₀-values significantly lower than RV, 3 µM in the PC-3 cells and 0.4 µM in the LNCaP and DU-145 prostate cancer cell lines.

Since some of the antitumor effects of RV are attributed to its unique polyphenolic structure, it was expected that the

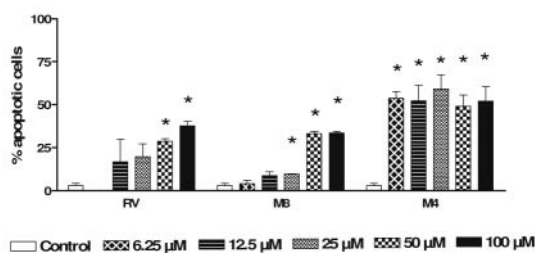


Figure 1. Induction of apoptosis by RV, M8 and M4 in PC-3 prostate cancer cells. Cells were incubated with increasing concentrations of drugs for 24 hours, and then double stained with Hoechst 33258 and propidium iodide. Cells were examined for morphological changes associated with apoptosis. Data are means and SEM of at least two determinations. Values significantly ($p < 0.05$) different from control are marked with an asterisk (*).

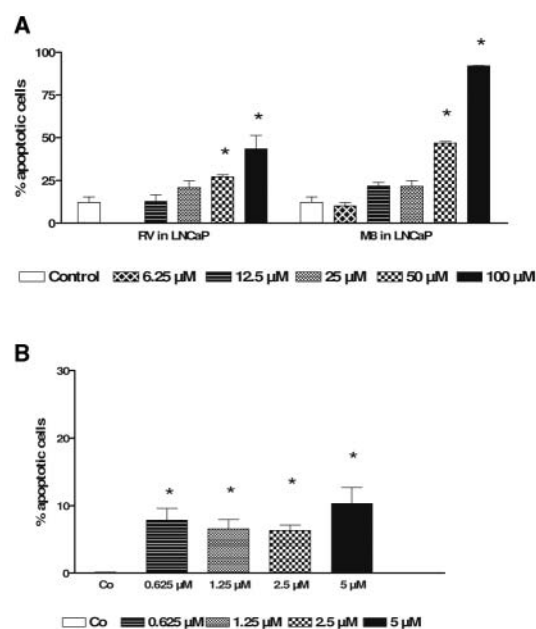


Figure 2. Induction of apoptosis by RV and M8 (A) and M4 (B) in LNCaP prostate cancer cells. Cells were incubated with increasing concentrations of drugs for 24 hours, and then double stained with Hoechst 33258 and propidium iodide. Cells were examined for morphological changes associated with apoptosis. Data are means and SEM of at least two determinations. Values significantly ($p < 0.05$) different from control are marked with an asterisk (*).

addition of further hydroxyl moieties would enhance its cytotoxic potential. Indeed, the hexahydroxy-substituted derivative of RV, M8 was found more potent in two out of the three prostate cancer cell lines investigated, reaching IC_{50} -values of 2.5 μ M and 3.4 μ M in the PC-3 and LNCaP cells, respectively. The DU-145 cells were less sensitive to M8 treatment, displaying an IC_{50} -value of 25 μ M after 7 days of incubation. The IC_{50} -values of all the compounds investigated are summarized in Table I.

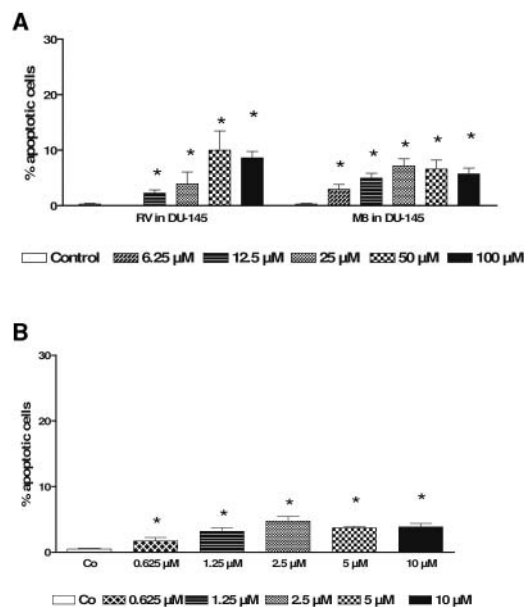


Figure 3. Induction of apoptosis by RV and M8 (A) and M4 (B) in DU-145 prostate cancer cells. Cells were incubated with increasing concentrations of drugs for 24 hours, and then double stained with Hoechst 33258 and propidium iodide. Cells were examined for morphological changes associated with apoptosis. Data are means and SEM of at least two determinations. Values significantly ($p < 0.05$) different from control are marked with an asterisk (*).

Induction of apoptosis. In the PC-3 cells, RV, M8 and M4 were all capable of inducing apoptosis as shown by the Hoechst/propidium iodide double staining method. Whereas RV only led to significant proportions of apoptotic cells at a concentration of 50 μ M or above, M8 was capable of inducing apoptosis at a concentration of 25 μ M. The proapoptotic effects of M4 greatly exceeded those of RV or even M8 in the PC-3 cells, inducing apoptosis at a concentration as low as 6.25 μ M. This effect remained constant with an increase of concentration, largely independent of the dose applied. At all the concentrations tested, M4 induced apoptosis in at least 49.3% of cells. The results are shown in Figure 1.

In the LNCaP cells, M8 was also a more potent inducer of apoptosis than RV, as depicted in Figure 2A. Whereas 50 μ M and 100 μ M RV induced apoptosis in 27.2% and 46.9% of cells, these values for M8 were significantly higher, 43.7% and 91.9%, respectively. Again, M4 exerted a constant proapoptotic effect at all the concentrations tested (Figure 2B).

In the DU-145 cells, the percentage of apoptotic cells remained significantly lower than in the two preceding cell lines. M8 still proved to be more effective than RV at inducing apoptosis in lower concentrations up to 25 μ M. However, in higher concentrations, the percentage of apoptotic cells after RV treatment exceeded that of the cells

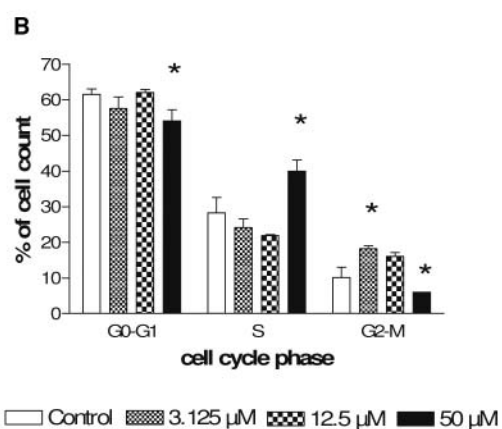
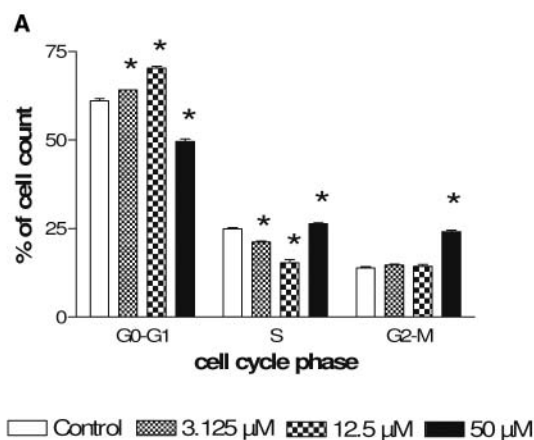


Figure 4. Cell cycle phase distribution of PC-3 (A) and LNCaP (B) prostate cancer cells after treatment with M8. Cells were incubated with increasing concentrations of the drugs for 24 hours, and then analyzed by flow cytometry. Data are means and SEM of two determinations. Values significantly ($p < 0.05$) different from control are marked with an asterisk (*).

treated by M8. In this cell line, M4 also induced apoptosis in a dose-dependent manner, reaching peak effects of 4.75% apoptotic cells after treatment with 2.5 μM M4. The results are shown in Figures 3A and 3B.

Cell cycle phase distribution. In the PC-3 cells, low-dose M8 caused cell cycle arrest in the G0/G1-phase, while cells treated with a higher concentration (50 μM) of M8 were arrested in the G2/M-phase, as shown in Figure 4A.

In contrast, the LNCaP cells were arrested in the S-phase and depleted in the G2/M-phase of the cell cycle after treatment with higher doses of M8, whereas lower concentrations of M8 arrested the LNCaP cells in the G2/M-phase (Figure 4B). M4 showed a comparable effect on the cell cycle distribution of both PC-3 and LNCaP cells, causing an arrest in the G2/M-phase of the cell cycle (Figures 5A and 5B).

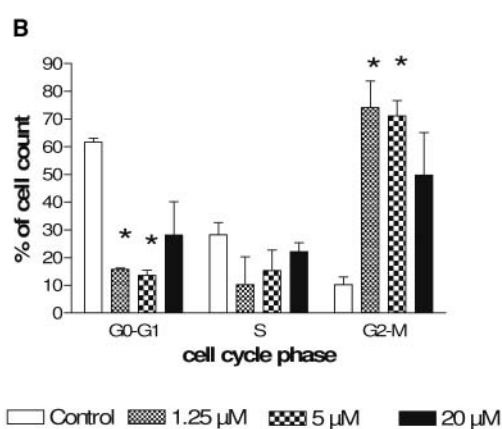
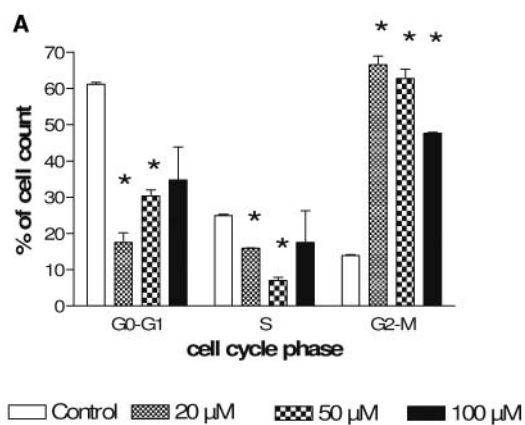


Figure 5. Cell cycle phase distribution of PC-3 (A) and LNCaP (B) prostate cancer cells after treatment with M4. Cells were incubated with increasing concentrations of the drugs for 24 hours, and then analyzed by flow cytometry. Data are means and SEM of two determinations. Values significantly ($p < 0.05$) different from control are marked with an asterisk (*).

Discussion

Prostate cancer is a leading cause of death in men worldwide and its incidence has been continuously increasing in the past decade (22). Although most patients with localized prostate cancer can be cured by surgery, there is an increasing need to find effective agents to develop adjuvant chemotherapeutic approaches in order to treat metastatic disease. Naturally derived polyphenolic compounds, such as resveratrol, have been demonstrated to possess cytostatic activity against prostate cancer cells (7, 23).

Several agents developed from RV exerted cytotoxic effects on prostate cancer cells in the present study, as shown in Table I. Of the hydroxylated compounds, 3,4,5,3',4',5'-hexahydroxy-stilbene (M8) and of the methoxylated derivatives, 3,4,4',5-tetramethoxy-stilbene (M4) demonstrated the lowest IC₅₀-values in all cell lines

tested, and were chosen for further studies on the mechanism of action of these RV derivatives.

Our group had previously found, that M8 was a more effective inducer of apoptosis in HL-60 human promyelocytic leukemia cells than RV (24). In two of the prostate cancer cell lines in the present study, M8 also exceeded the apoptosis-inducing effects of RV. M4 proved to be an even more potent agent regarding the induction of apoptosis, exerting comparable effects at concentrations 10 times lower than those of RV or M8. RV and M8 induced apoptosis in a dose-dependent manner, whereas the apoptotic effects of M4 remained independent of the concentration applied. These findings suggest that different mechanisms are responsible for apoptotic cell death depending on the substituted groups on the stilbene ring of RV.

The cytotoxic effects of M8 have been characterized in HL-60 human promyelocytic leukemia cells in which M8 inhibited the activation of nuclear factor- κ B (NF- κ B) and arrested cells in the S-phase (24). In prostate cancer cells, M8 also arrested cells in the S- or G2/M-phases, depending on the doses applied.

The methoxy-substituted derivative, M4, arrested the prostate cancer cells solely in the G2/M-phase, while depleting cells in the G0/G1- and S-phases of the cell cycle. This compound has been previously described as an excellent inhibitor of microtubule formation, which could explain the results observed in prostate cancer cells (25).

Although RV has been demonstrated to activate estrogen receptors and to inhibit expression and function of the androgen receptor, in our experiments, no significant difference was recorded in cytotoxicity and apoptotic potential between hormone-responsive and non-responsive cell lines. These results indicated that other mechanisms than the antioestrogenic activity might be responsible for the observed cytotoxic effects. Mitchell *et al.* demonstrated that RV inhibits the expression of the androgen receptor (AR) and represses different classes of AR-driven genes at different levels (26). However, Hsieh and Wu showed that RV decreased prostate-specific antigen (PSA) expression in LNCaP cells independently of AR (27), thus supporting our findings.

RV exerts manifold antitumor effects (28) and is an excellent inhibitor of the enzyme ribonucleotide reductase (RR), which catalyzes the formation of dNTPs from the corresponding NTPs (29). We have previously demonstrated, that both RV and M8 depleted intracellular dNTP concentrations, thus interfering with *de novo* DNA synthesis (24, 30). Although RV also affects other key enzymes of DNA synthesis, the inhibition of RR seems to play a crucial role for the anti-cancer effects of this compound. To date, several inhibitors of RR have been tested successfully in prostate cancer cell lines and in mouse models (31, 32). Therefore, inhibition of RR by RV and its derivatives might explain the

overall efficacy of these compounds in hormone-responsive as well as in non-responsive prostate cancer cell lines.

Following characterization of the most effective compounds, their dose-dependent induction of apoptosis in PC-3 and LNCaP prostate cancer cell lines was demonstrated. Furthermore, both M8 and M4 arrested prostate cancer cells in various phases of the cell cycle. Due to these promising results, further studies investigating the *in vivo* effects of M8 and M4 in prostate cancer models are warranted.

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