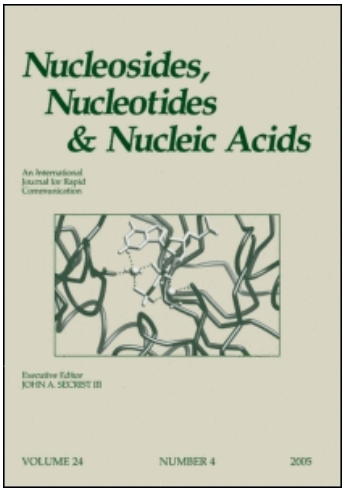

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Saiko, P. , Horvath, Z. , Murias, M. , Handler, N. , Jaeger, W. , Erker, T. , Fritzer-Szekeres, M. and Szekeres, T.(2006) 'Antitumor Effects of 3,3',4,4',5,5'-Hexahydroxystilbene in hl-60 Human Promyelocytic Leukemia Cells', Nucleosides, Nucleotides and Nucleic Acids, 25: 9, 1013 — 1017
To link to this Article: DOI: 10.1080/15257770600890624
URL: http://dx.doi.org/10.1080/15257770600890624

Nucleosides, Nucleotides, and Nucleic Acids, 25:1013–1017, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770600890624

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ANTITUMOR EFFECTS OF 3,3′,4,4′,5,5′-HEXAHYDROXYSTILBENE IN HL-60 HUMAN PROMYELOCYTIC LEUKEMIA CELLS

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□ *Resveratrol (3,4′,5-trihydroxystilbene, RV) exerts remarkable cytostatic and cytotoxic effects against a multitude of human cancer cell lines. Since the introduction of additional hydroxyl groups was supposed to increase the biological activity of RV, we have synthesized a number of polyhydroxylated stilbene analogues as potential antitumor agents. In this study, the activity of 3,3′,4,4′,5,5′-hexahydroxystilbene (M8) was investigated in HL-60 human promyelocytic leukemia cells. Employing a growth inhibition assay, incubation with M8 and RV resulted in IC_{50} values of 6.25 and 12 μM, respectively. Using a specific Hoechst/propidium iodide double staining method, we found that M8 was able to induce apoptosis in concentrations significantly lower than those of RV. In addition, M8 arrested cells in the S phase and totally depleted cells in the G2-M phase of the cell cycle (143% and 0% of control after treatment with 12.5 μM M8, respectively). We therefore believe that this promising agent deserves further preclinical and in vivo testing.*

Keywords Polyhydroxylated stilbenes; Arabinofuranosylcytosine; Apoptosis; Cell cycle; Synergistic combination effects

INTRODUCTION

Resveratrol (RV), a naturally occurring stilbene derivative found mainly in the skin of grapes and red wine, has been drawn into the spotlight of interest because of its manifold biochemical effects. RV yields cytostatic and cytotoxic effects against a multitude of human cancer cell lines and induces

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apoptosis via the activation of caspases as well as through the release of mitochondrial cytochrome c.[1] In addition, RV also has been shown to inhibit DNA polymerase and to arrest cells in the S and G2 phases of the cell cycle. The effects of RV partially are attributed to its polyhydroxyphenolic structure. In an attempt to identify further potent metabolites of RV, our group synthesized a number of polyhydroxylated analogues (M1–M10).

In the present study, the biochemical and apoptotic effects of RV and M8 were tested in HL-60 human promyelocytic leukemia cells. Employing a growth inhibition assay, M8 and RV were compared for their cytotoxicity. The induction of apoptosis was studied using a specific Hoechst/propidium iodide double staining method.[2] Following treatment with RV or M8, the cell cycle distribution of HL-60 cells was investigated by flow cytometry.

METHODS

Chemicals

Ara-C was purchased from Sigma, Vienna, Austria. M8 was synthesized and provided by the Department of Pharmaceutical Chemistry, University of Vienna.[3] All other reagents used were of highest purity available.

Growth Inhibition Assay

HL-60 cells (0.1×10^6 per ml) were incubated with various concentrations of RV or M8 for 72 hours. Cell viability was determined by trypan blue exclusion.

Hoechst Dye 33258 and Propidium Iodide Double Staining

The Hoechst staining was performed according to the method described by Grusch coworkers.[2] HL-60 cells (0.1×10^6 per ml) were exposed to increasing concentrations of M8 or RV for 24 hours. Subsequently, Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) were added directly to the cells to final concentrations of 5 μg/ml and 2 μg/ml, respectively. After 1 h of incubation at 37°C, cells were examined by fluorescence microscopy (Zeiss Axiovert 35) equipped with appropriate DAPI filters for Hoechst 33258 and PI. Cells were photographed with an Olympus camera using Kodak Ektachrome P1600 films (Kodak, Rochester, NY, USA).

Cell Cycle Distribution Analysis

HL-60 cells (0.4×10^6 per ml) were incubated with various concentrations of M8 or RV under cell culture conditions. After 24 hours, cells were

harvested, washed with 5 ml cold PBS and centrifuged at 600 rpm. The pellet was resuspended and fixed in 3 ml cold ethanol (70%) for 30 minutes at 4°C. After two washing steps in cold PBS, RNAse A and propidium iodide were added to a final concentration of 50 μg/ml each and incubated at 4°C for 1 h before measurement. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated using ModFit LT software (Verity Software House, Topsham, ME, USA).

Statistical Calculations

Dose-response curves were calculated using the Prism 4.03 software package (GraphPad, San Diego, CA, USA) and statistical significance was determined by unpaired *t*-test.

RESULTS

Growth Inhibition Assay

After 72 hours of incubation, RV yielded an IC_{50} value of 12 μM, whereas exposure to M8 led to an IC_{50} value of 6.25 μM.

Induction of Apoptosis after Treatment with RV or M8

Incubation of HL-60 cells with 12.5 μM RV induced apoptosis in only 4.1% of treated cells. However, after incubation with 25, 50, and 100 μM

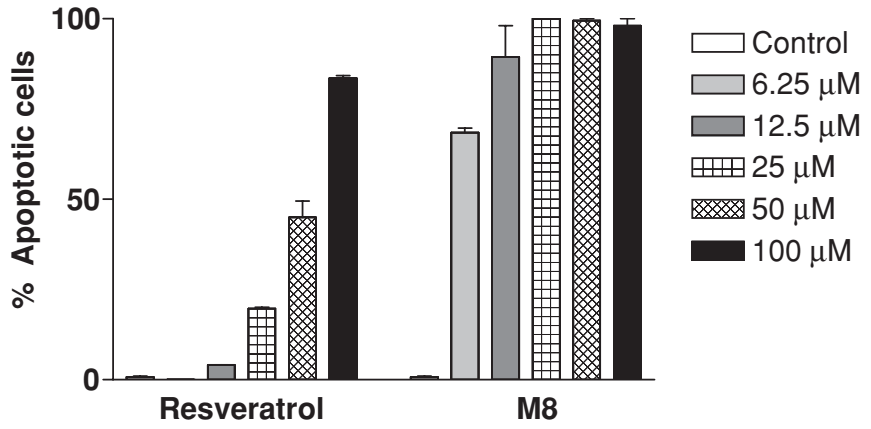

FIGURE 1 Induction of apoptosis by resveratrol and M8 in HL-60 cells. Cells were incubated with RV or M8 for 24 hours, and then double stained with Hoechst dye 33258 and propidium iodide. After the incubation period, cells were examined for morphological changes associated with apoptosis. Data are means and SEM of two determinations. All values shown are significantly different from control.

TABLE 1 Cell Cycle Distribution Patterns in HL-60 Human Promyelocytic Leukemia Cells after Treatment with M8. After 24 Hours of Incubation with M8, HL-60 Cells were Analyzed on a FACSCalibur Flow Cytometer and Cell Cycle Distribution was Calculated with ModFit LT Software. Data are Means of Two Determinations. Standard Deviations were Within 5%.

	G0/G1 (%)	S (%)	G2/M (%)
Control	42	44	14
M8 (6.25 μM)	34	48	18
M8 (12.5 μM)	37	63	0

RV, 19.3%, 44.9%, and 83.6% of the cells underwent apoptosis. M8 was shown to possess a much higher apoptosis inducing potential than RV. At M8 concentrations as low as 6.25 μM, 68.5% of cells underwent apoptosis, and after treatment with 12.5, 25, 50, and 100 μM M8, these values increased to 89.4%, 100%, 99.5%, and 98.1%, respectively. Results are depicted in Figure 1.

Effects of RV or M8 on the Cell Cycle Distribution of HL-60 cells

Following treatment with RV or M8, the cell cycle distribution of HL-60 cells was investigated by flow cytometry. Both compounds altered the cell cycle distribution, but M8 exhibited a partially different profile of effects. It arrested cells in the S phase and totally depleted cells in the G2-M phase of the cell cycle (143.2% and 0% of control after treatment with 12.5 μM M8, respectively). RV also arrested cells in the S phase, but at higher concentrations it caused an accumulation of HL-60 cells in the G2-M phase (252.4% of control values after treatment with 25 μM RV).

DISCUSSION

In this study, we investigated the activity of M8, a novel RV analogue, in HL-60 human promyelocytic leukemia cells. RV inhibited the growth of HL-60 cells with an IC_{50} value of 12 μM, whereas M8 yielded in IC_{50} of 6.25 μM. In addition, M8 induced apoptosis in concentrations significantly lower than those of RV and arrested cells in the S phase of the cell cycle. We believe that this promising agent might become an additional option for the treatment of leukemia and/or other human malignancies and, therefore, deserves further preclinical and in vivo testing.

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