Novel resveratrol analogs induce apoptosis and cause cell cycle arrest in HT29 human colon cancer cells: Inhibition of ribonucleotide reductase activity

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Received November 19, 2007; Accepted February 20, 2008

Abstract. Resveratrol (3,4',5-trihydroxy-*trans*-stilbene; RV), an ingredient of wine, exhibits a broad spectrum of antiproliferative effects against human cancer cells. In order to enhance these effects, we modified the molecule by introducing additional methoxyl and hydroxyl groups. The resulting novel RV analogs, M5 (3,4',5-trimethoxy-*trans*-stilbene), M5A (3,3',4,5'-tetramethoxy-*trans*-stilbene) and M8 (3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene) were investigated in HT29 human colon cancer cells. Cytotoxicity was evaluated by clonogenic assays and the induction of apoptosis was determined using a specific Hoechst/propidium iodide double staining method. Cell cycle distribution was evaluated by FACS. The influence of M8 on the concentration of deoxyribonucleoside triphosphates (dNTPs), the products of ribonucleotide reductase (RR), was determined by high-performance liquid chromatography. M5 and M5A caused a dose-dependent induction of apoptosis and led to remarkable changes of the cell cycle distribution. After treatment with M5, growth arrest occurred mainly in the G2-M phase, whereas incubation with M5A resulted in arrest in the G0-G1 phase of the cell cycle. Incubation of HT29 cells with M8 produced a significant imbalance of intracellular dNTP pools, being synonymous with the inhibition of RR activity. The dATP pools were abolished, whereas the dCTP and dTTP pools increased. Due to these promising results, the investigated RV analogs deserve further preclinical and *in vivo* testing.

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Key words: colon cancer, resveratrol, 3,4',5-trimethoxy-*trans*-stilbene, 3,3',4,5'-tetramethoxy-*trans*-stilbene, 3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene, apoptosis, cell cycle arrest, ribonucleotide reductase

Introduction

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene; RV; Fig. 1) is a polyphenol and has been classified as a phytoalexin as it is synthesized in spermatophytes in response to injury, UV irradiation and fungal attack (1). It was first isolated in 1940 as an ingredient of the roots of white hellebore (*Veratrum grandiflorum O. Loes*) and has since been found in a wide variety of ~70 plant species, including grapes, mulberries and peanuts (2). RV exhibits a remarkable inhibitory potential in the various stages of tumor development. The antitumor activity of RV was first shown by its ability to reduce the incidence of the carcinogen-induced development of cancers in experimental animals (3). Subsequently, RV has been shown to exert numerous effects that may block tumor development at several discrete stages during the multigenic process of carcinogenesis (4), involving interactions between RV and manifold targets (5,6). These targets include kinases (7,8), steroid hormone receptors (9-11), reactive oxygen species (6,12), ribonucleotide reductase (13,14) and DNA polymerases (15). RV causes arrest at the S/G2 phase transition of the cell cycle (16) and is capable of inducing differentiation and apoptosis in a multitude of human tumor cell lines, such as leukemia, colon, breast, prostate and esophageal cells (11,17-21). RV has been identified as an effective inhibitor of ribonucleotide reductase (RR) which catalyzes the rate-limiting step of *de novo* DNA synthesis, namely the reduction of ribonucleotides into the corresponding deoxyribonucleoside triphosphates (dNTPs) (13,14). This was substantiated by its effect on the significant decrease of the four intracellular dNTP concentrations, the products of RR. RV treatment had a differential effect on dNTP pools, with dATP and dGTP concentrations showing the greatest

decrease (22). The RV inhibition of ribonucleotide reductase is based on its ability to scavenge the tyrosyl radical on the R2 subunit of ribonucleotide reductase, which is essential for its function (14). Other clinically established inhibitors of RR, such as hydroxyurea or fludarabine are applied successfully in combination chemotherapy regimens for the treatment of leukemia.

The importance of all these targets for cancer development is well-known and therefore RV can beneficially contribute to cancer prevention. Since the identification of RV health benefits are largely owed to its high abundance in certain plants and foods, the discovery of further naturally occurring stilbenes, as well as chemically modified analogs that are superior to RV in their cancer chemopreventive properties, may be expected.

In this context, we prepared a series of hydroxylated and methoxylated RV analogs with the aim of identifying new agents with potential clinical relevance and establishing a structure- and anticancer-activity relationship. The antitumor effects of M5 (3,4',5-trimethoxy-*trans*-stilbene), M5A (3,3',4,5'-tetramethoxy-*trans*-stilbene), and M8 (3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene) are presented herein. The activity of these new compounds was tested in HT29 human colon cancer cells. Their cytotoxicity was evaluated by clonogenic assays and the induction of apoptosis was determined by employing a specific Hoechst/propidium iodide double staining method. Cell cycle distribution after exposure to M5 and M5A was investigated by FACS analysis. Since we showed earlier that M8 causes a significant imbalance of intracellular dNTP concentrations in HL-60 cells (23), we now investigated whether M8 had comparable effects on the dNTP pools in HT29 cells.

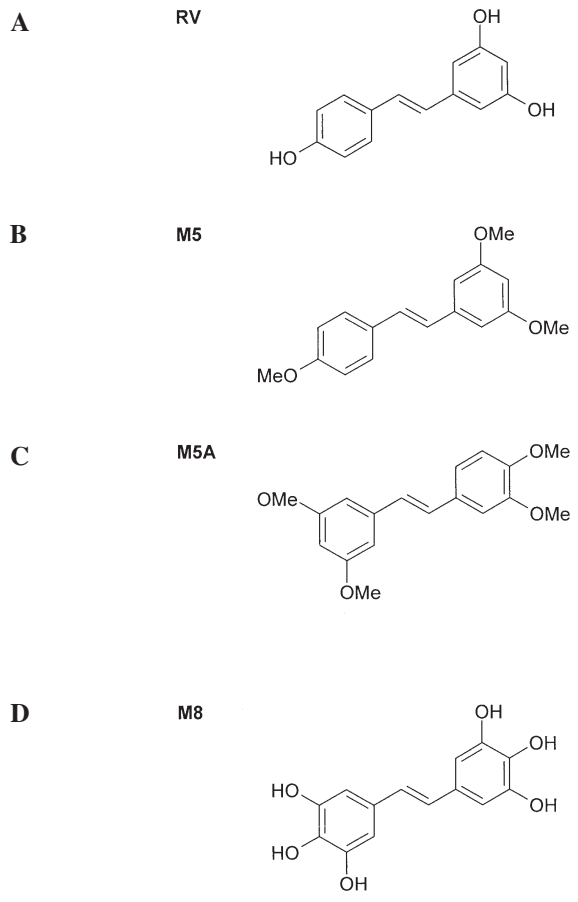

Figure 1. The structural formulas of RV, M5, M5A and M8 including the nomenclature, molecular weight and empirical formula. (A) 3,4',5-Trihydroxy-*trans*-stilbene $C_{14}H_{12}O_3$, MW = 228.25. (B) 3,4',5-Trimethoxy-*trans*-stilbene $C_{17}H_{18}O_3$, MW = 270.33. (C) 3,3',4,5'-Tetramethoxy-*trans*-stilbene $C_{18}H_{20}O_4$, MW = 300.36. (D) 3,3',4,4',5,5'-Hexahydroxy-*trans*-stilbene $C_{14}H_{12}O_6$, MW = 276.25.

Materials and methods

Chemicals and supplies. M5 (3,4',5-trimethoxy-*trans*-stilbene), M5A (3,3',4,5'-tetramethoxy-*trans*-stilbene) and M8 (3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene) were synthesized as previously described (24) and provided by the Department of Clinical Pharmacy and Diagnostics, Faculty of Life Sciences, University of Vienna, Austria. The structural formulas are shown in Fig. 1. RV and the other chemicals and reagents were commercially available (Sigma-Aldrich, Vienna, Austria) and of the highest purity.

Cell culture. The HT29 human colon cancer cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2. The media and supplements were obtained from Life Technologies (Paisley, Scotland, UK). Cell counts were determined using a micro cellcounter CC-108 (Sysmex, Kobe, Japan). Cells in the logarithmic phase of growth were used for the experiments described below.

Clonogenic assay. Cells ($1x10^3$/well) were plated in 24-well plates and allowed to attach overnight at 37˚C in a humidified 5% CO_2 atmosphere. After 24 h, the medium was removed and replaced by a 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 min. Colonies of >50 cells were counted using an inverted microscope at 40-fold magnification. All of the experiments were performed in triplicate and repeated three times.

Hoechst dye 33258 and propidium iodide double staining. Hoechst staining was performed according to the method described by Grusch and coworkers (25). Cells ($0.1x10^6$/ml) were seeded in 25 cm^2 Nunc tissue culture flasks, allowed to adhere overnight and exposed to increasing concentrations of drugs for 72 h. Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma) were added directly to the cells at final concentrations of 5 and 2 μg/ml, respectively. After 60 min of incubation at 37˚C, cells were examined on a Leica DMR XA fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters for Hoechst 33258 and PI. This method is used to distinguish between early and late apoptosis, and necrosis. Cells were judged according to their morphology and the integrity of their

cell membranes, which can easily be seen after propidium iodide staining. Cells were counted under the microscope and the number of apoptotic cells was given as a percentage value.

Cell cycle distribution analysis. Cells (0.4x10^6/ml) were seeded in 25 cm^2 Nunc tissue culture flasks, allowed to adhere overnight and incubated with increasing concentrations of drugs at 37˚C under cell culture conditions. After 24 h, cells were harvested and suspended in 5 ml cold PBS, centrifuged, re-suspended and fixed in 3 ml cold ethanol (70%) for 30 min at 4˚C. After two washing steps in cold PBS, RNase A and propidium iodide were added at a final concentration of 50 μg/ml each and incubated at 4˚C for 60 min before measurement. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA).

Determination of deoxyribonucleoside triphosphates (dNTPs). Cells were seeded in 175 cm^2 tissue culture flasks (4x10^7/flask) for 24 h to ensure attachment and then incubated with increasing concentrations of M8 for another 24 h. The cells were then centrifuged at 1800 g for 5 min, re-suspended in 100 μl of PBS and extracted with 10 μl of trichloracetic acid (90%). The lysate was allowed to rest on ice for 30 min and neutralized by the addition of 1.5 volumes of freon containing 0.5 mol/l tri-noctylamin. Concentrations of dNTPs were then determined using the method described by Garrett and Santi (26). Aliquots (120 μl) of the sample were analyzed using a Merck 'La Chrom' high-performance liquid chromatography (HPLC) system (Merck, Darmstadt, Germany) equipped with D-7000 interface, L-7100 pump, L-7200 autosampler and L-7400 UV detector. Detection time was set at 80 min, with the detector operating on 280 nm for 40 min and then switched to 260 nm for another 40 min. Samples were eluted with a 3.2 M ammonium phosphate buffer (pH 3.6, adjusted by the addition of 3.2 mM H_3PO_4) containing 20 M acetonitrile using a 4.6x250 mm Partisil 10 SAX column (Whatman Ltd., Kent, UK). Separation was performed at a constant ambient temperature and a flow rate of 2 ml/min. The concentration of each dNTP was calculated as a percentage of the total area under the curve for each sample.

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

Results

Effect of drugs on the growth of HT29 cell colonies. Cells were treated with increasing concentrations of drugs and colonies were counted after 7 days of incubation as described in Materials and methods. M5, M5A and M8 inhibited the growth of HT29 cell colonies with IC_{50} values of 1.25, 15 and 8 μM, respectively, whereas RV yielded an IC_{50} value of 7 μM. The results are shown in Fig. 2.

Induction of apoptosis in HT29 cells. HT29 cells were incubated with increasing concentrations of M5 or M5A for 72 h. The cells were then double stained with Hoechst 33258 and propidium iodide as described in Materials and methods. After the incubation period, the morphology of HL-60 cells showed nuclear condensation and fragmentation (early apoptosis) as well as signs of late apoptosis with membrane damage and incorporation of propidium iodide. As shown in Fig. 3, the induction of apoptosis was dose-dependent with up to 100% of the cells showing hallmarks of apoptosis at 20 μM M5. Treatment with 30 μM M5A resulted in 95% apoptotic cells (Fig. 4).

Cell cycle distribution in HT29 cells. HT29 cells were prepared as described in Materials and methods and incubated with increasing concentrations of M5 or M5A for 24 h. After treatment with 20 μM M5, the growth arrest occurred mainly in the G2-M phase, increasing the cell population from 18.5 to 43.5%. Simultaneously, cells in the G0-G1 phase decreased from 57.5 to 29.5%. In contrast, the growth arrest after treatment with 100 μM M5A occurred mainly in the G0-G1

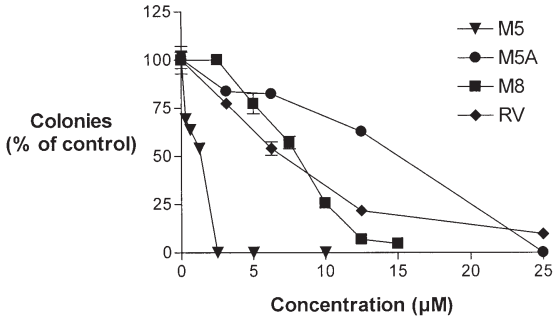

Figure 2. The growth inhibition of HT29 cell colonies after treatment with RV, M5, M5A and M8 for 7 days. Cells (1x10^3/well) were plated in 24-well plates and allowed to attach overnight at 37˚C in a humidified 5% CO_2 atmosphere. After 24 h, the medium was removed and replaced by a fresh medium containing 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 min. Colonies of >50 cells were counted using an inverted microscope at 40-fold magnification.

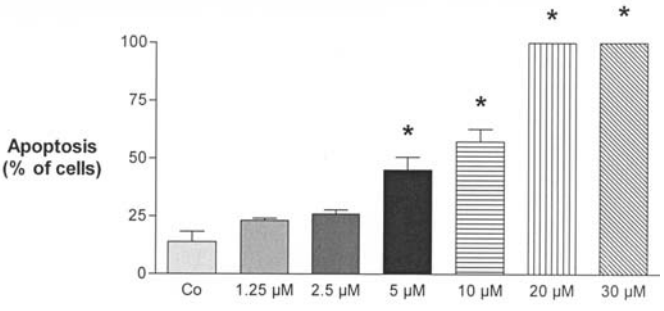

Figure 3. The induction of apoptosis in HT29 cells after incubation with M5 for 72 h. Cells (0.1x10^6/ml) were seeded in 25 cm^2 Nunc tissue culture flasks, allowed to adhere overnight and exposed to increasing concentrations of drugs for 72 h. Hoechst 33258 and propidium iodide were added directly to the cells at final concentrations of 5 and 2 μg/ml, respectively. After 60 min of incubation at 37˚C, the cells were examined on a Leica DMR XA fluorescence microscope equipped with appropriate filters for Hoechst 33258 and PI. The cells were counted under the microscope and the number of apoptotic cells was given as a percentage value. *p<0.05, values significantly different from the control.

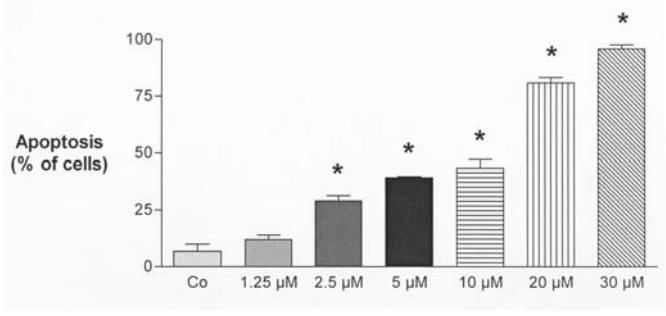

Figure 4. The induction of apoptosis in HT29 cells after incubation with M5A for 72 h. Cells ($0.1x10^6$/ml) were seeded in 25 cm^2 Nunc tissue culture flasks, allowed to adhere overnight and exposed to increasing concentrations of drugs for 72 h. Hoechst 33258 and propidium iodide were added directly to the cells at final concentrations of 5 and 2 μg/ml, respectively. After 60 min of incubation at 37˚C, cells were examined on a Leica DMR XA fluorescence microscope equipped with appropriate filters for Hoechst 33258 and PI. The cells were counted under the microscope and the number of apoptotic cells was given as a percentage value. *p<0.05, values significantly different from the control.

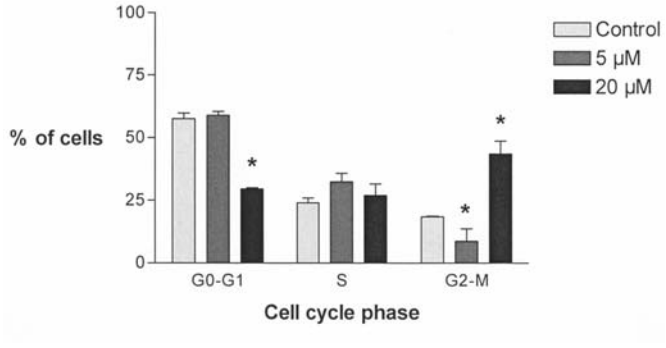

Figure 5. The cell cycle distribution in HT29 cells after incubation with M5 for 24 h. Cells ($0.4x10^6$/ml) were seeded in 25 cm^2 Nunc tissue culture flasks, allowed to adhere overnight and incubated with increasing concentrations of drugs at 37˚C under cell culture conditions. After 24 h, the cells were harvested and suspended in 5 ml cold PBS, centrifuged, re-suspended and fixed in 3 ml cold ethanol (70%) for 30 min at 4˚C. After two washing steps in cold PBS, RNase A and propidium iodide were added at a final concentration of 50 μg/ml each and incubated at 4˚C for 60 min before measurement. The cells were analyzed on a FACSCalibur flow cytometer (and cell cycle distribution was calculated with ModFit LT software). *p<0.05, values significantly different from the control.

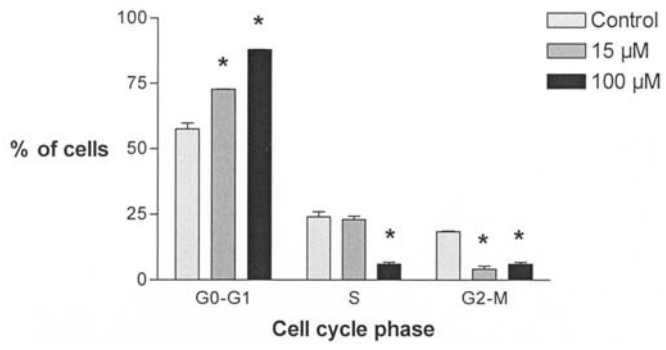

Figure 6. The cell cycle distribution in HT29 cells after incubation with M5A for 24 h. Cells ($0.4x10^6$/ml) were seeded in 25 cm^2 Nunc tissue culture flasks, allowed to adhere overnight and incubated with increasing concentrations of drugs at 37˚C under cell culture conditions. After 24 h, the cells were harvested and suspended in 5 ml cold PBS, centrifuged, re-suspended and fixed in 3 ml cold ethanol (70%) for 30 min at 4˚C. After two washing steps in cold PBS, RNase A and propidium iodide were added at a final concentration of 50 μg/ml each and incubated at 4˚C for 60 min before measurement. Cells were analyzed on a FACSCalibur flow cytometer (and cell cycle distribution was calculated with ModFit LT software). *p<0.05, values significantly different from the control.

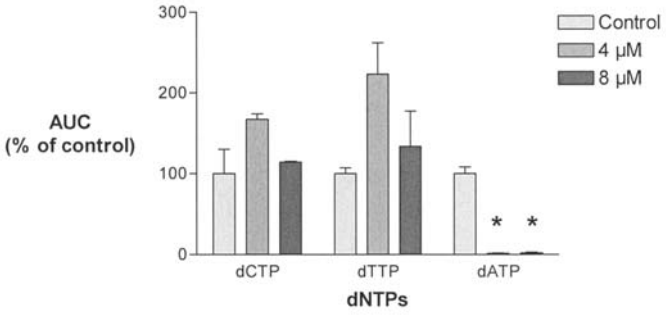

Figure 7. The concentration of dNTP pools in HT29 cells after treatment with M8 for 24 h. Cells were seeded in 175 cm^2 tissue culture flasks ($4x10^7$/flask) for 24 h to ensure attachment and then incubated with increasing concentrations of M8 for another 24 h. Afterwards, $5x10^7$ cells were separated for the extraction of dNTPs. Concentrations of dNTPs were then determined using the method described by Garrett and Santi (26) and were calculated as a percent of the total area under the curve for each sample. *p<0.05, values significantly different from the control.

phase, increasing the cell population from 57.5 to 88%. Simultaneously, the cells in the G2-M phase decreased from 18.5 to 4%. The results are shown in Figs. 5 and 6.

Effect of M8 on the deoxyribonucleoside triphosphate (dNTP) pools in HT29 cells. HT29 cells were incubated with increasing concentrations of M8 for 24 h. Then dNTP pool sizes were determined using the HPLC method described in Materials and methods. M8 treatment caused a remarkable imbalance of dNTPs in HT29 cells. The incubation of cells with 4 and 8 μM M8 resulted in a significant depletion of intracellular dATP pools to 1.5 and 2% of controls, respectively. In contrast, treatment with 4 μM M8 significantly increased the dCTP and dTTP pools to 167 and 223% of the control values, respectively. The dGTP pools remained beyond the detectability of the method. The results are shown in Fig. 7.

Discussion

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene; RV) is a naturally occurring polyphenolic compound found in grapes, wine and various medical plants. Numerous studies have shown that RV exerts remarkable antioxidant, anti-inflammatory and antitumor activities and demonstrates a preventive effect for cancer. RV was proven to inhibit ribonucleotide reductase (RR) which is the rate-limiting enzyme for *de novo* DNA synthesis and therefore considered an excellent target for cancer chemotherapy. Balanced deoxynucleosidetriphosphate (dNTP) concentrations are essential for DNA synthesis, especially in rapidly proliferating tumor cells. Various inhibitors of RR, such as hydroxyurea or difluorodeoxycytidine (Gemcitabine, dFdC), are commonly used for the treatment of various human malignancies.

In contrast to the detailed knowledge of RV activities in biological systems, little is known about the effects of other polyhydroxylated stilbenes. RV undergoes cytochrome P450 catalyzed hydroxylation to piceatannol (3,3',4',5-tetrahydroxy-*trans*-stilbene; PCA) and two other unidentified mono- and dihydroxy-RV analogs. This demonstrates that a natural dietary cancer preventative agent can be converted to a compound with known chemopreventive and anticancer activity by the enzyme CYP1B1, which is overexpressed in a wide variety of human tumors.

In this context, we prepared a series of polyhydroxylated RV analogs with the aim of discovering new agents with potential clinical relevance. One of these derivatives is M8 (3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene), which proved to be a potent inhibitor of RR, resulting in a significant alteration of the dNTP pool balance in HT29 cells. Due to this effect, DNA synthesis in rapidly growing cancer cells is blocked. M8 was already found to be a potent inhibitor of RR by causing a remarkable imbalance of the dNTP pools in HL-60 cells (23), but possesses a slightly different profile regarding its effects on intracellular dNTP concentrations in HT29 cells. Incubation with 4 μM M8 caused a significant increase in intracellular dCTP and dTTP pools, whereas dATP pools were abolished. In particular, a similar depletion of dATP pool sizes was previously observed with Gemcitabine (27), a mechanism mainly contributing to the antitumor properties of this clinically established anticancer drug. The amount of dGTP remained beyond the detectability of the method.

Furthermore, a number of methoxylated RV analogs have recently been synthesized and tested, demonstrating the importance of a 3,5-dimethoxy motif in conferring pro-apoptotic activity to stilbene derivatives (28). The ability of pterostilbene and 3'-hydroxypterostilbene, natural 3,5-dimethoxy analogs of RV and PCA, in terms of inducing apoptosis in sensitive and resistant leukemia cells were also evaluated (29). In addition, pterostilbene and 3'-hydroxy-pterostilbene, when used at concentrations that exhibit significant apoptotic effects in tumor cell lines, did not show any cytotoxicity in normal hemopoietic stem cells.

Of the methoxylated RV analogs prepared by our group, M5 (3,4',5-trimethoxy-*trans*-stilbene) and M5A (3,3',4,5'-tetramethoxy-*trans*-stilbene) were evaluated in HT29 cells. Regarding the inhibition of tumor cell growth, M5 turned out to be much more potent than M5A, thereby underpinning the findings of Roberti and coworkers (28). The two compounds proved to be strong inducers of apoptosis, with M5 showing greater fractions of apoptotic cells at lower concentrations. Similarly, M5 and M5A caused significant cell cycle perturbations. M5 led to arrest mainly in the G2-M phase, while decreasing cells in the G0-G1 phase. In contrast, cell cycle arrest after treatment with M5A mainly occurred in the G0-G1 phase of the cell cycle, thereby decreasing cells in the G2-M phase.

Due to these promising results, the investigated RV analogs deserve further preclinical and *in vivo* testing.

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