Evaluation of antitumor effects of two vine stalk oligomers of resveratrol on a panel of lymphoid and myeloid cell lines: Comparison with resveratrol

Catherine Barjot a, Magali Tournaire a, Chantal Castagnino b, Claire Vigorc, Joseph Vercauteren c,⁎, Jean-François Rossia,d

a CellGen SA, INSERM U847, 99 rue Puech Villa, 34197 Montpellier Cedex 5, France
b Polyphenols R&D SARL, CEEI Cap Alpha — Avenue de l’Europe, 34830 Clapiers, France
c Laboratory of Pharmacognosy, Faculty of Pharmacy 15, Avenue Charles Flahaut B.P. 14491, University of Montpellier I, 34093 Montpellier Cedex 5, France
d Department of Hematology-Oncology, Lapeyronie Hospital, 371, Avenue du Doyen Gaston Giraud, 34295 Montpellier Cedex 5, France

Received 22 May 2007; accepted 26 August 2007

Abstract

This study aims to evaluate and compare the antiproliferative and proapoptotic effects of resveratrol (trans-3,4′,5-trihydroxystilbene) with two of its naturally occurring oligomers, ɛ-viniferin (a dimer) and miyabenol C (a trimer). Proliferation assays performed on myeloid and lymphoid cell lines show that the three compounds inhibit cell growth of all cell types tested, with miyabenol C being the most efficient (IC50 ranging from 10.8 to 29.4 μM). Further analysis performed on the multiple myeloma cell line U266 shows that all compounds modify cell cycle distribution probably via actions on different targets. Whereas cells treated with resveratrol accumulate in S phase, cells treated with ɛ-viniferin and miyabenol C accumulate in G2/M and G0/G1, respectively. Miyabenol C is also the most efficient at inducing cell death in U266 cells. All compounds induce apoptosis of U266 cells via mechanisms entirely dependent on caspase activation and associated with mitochondrial membrane potential disruption. Compounds do not act directly on the mitochondrial membrane, but could induce activation of upstream caspases such as caspase 8 and/or caspase 2, depending on the compound. In no case did upstream caspase 8 activation involve Fas/FasL interaction. Taken together, these results show that ɛ-viniferin and, more importantly, miyabenol C represent potent antitumor agents that require further investigation, either alone or in combination with resveratrol.

© 2007 Elsevier Inc. All rights reserved.

Keywords: B cell malignancies; Multiple myeloma; Resveratrol; ɛ-viniferin; Miyabenol C

Introduction

Resveratrol (trans-3,4′,5-trihydroxystilbene) is a naturally occurring phenolic compound with powerful anticancer activity. Present in various plants (common grape vine (Vitis vinifera), peanuts, most berries and rhubarb), resveratrol has evoked considerable interest for its ability to act as a chemopreventative and chemotherapeutic agent. By affecting a broad range of intracellular mediators involved in tumor development, resveratrol inhibits the three major steps of carcinogenesis: initiation, promotion and progression (Jang et al., 1997; Subbaramaiah et al., 1998; Aggarwal et al., 2004; Signorelli and Ghidoni, 2005; Delmas et al., 2006).

In vitro studies on a wide variety of human tumor cells show that resveratrol inhibits proliferation and induces apoptosis and is particularly effective in cells of myeloid and lymphoid origin (Clement et al., 1998; Ragione et al., 1998; Surh et al., 1999; Bernhard et al., 2000; Tsan et al., 2000; Dorrie et al., 2001; Wieder et al., 2001; Billard et al., 2002; Ferry-Dumazet et al., 2002; Roman et al., 2002; Estrov et al., 2003; Jazirehi and Bonavida, 2004; Luzi et al., 2004; Shimizu et al., 2006).

Studies aiming to define the apoptotic pathways triggered by resveratrol suggest a mitochondrial-dependent pathway. Resveratrol-induced apoptosis is generally associated with mitochondrial membrane disruption, followed by activation of caspase 9 which, in turn, activates effector caspase 3 (Surh et al., 1999; Bernhard et al., 2000; Tsan et al., 2000; Dorrie et al., 2001; Wieder et al., 2001; Billard et al., 2002; Ferry-Dumazet et al., 2002; Roman et al., 2002; Estrov et al., 2003; Jazirehi and Bonavida, 2004; Luzi et al., 2004; Shimizu et al., 2006).
An important question is whether resveratrol evokes its effects through direct actions on mitochondria or via components upstream of mitochondrial membrane depolarization. Although one study has showed that resveratrol-induced apoptosis is mediated by FasL/Fas interaction (Clement et al., 1998), most report that the apoptosis is not dependent on Fas signaling or any other known death receptor pathway (Bernhard et al., 2000; Tsan et al., 2000; Dorrie et al., 2001; Tinhofer et al., 2001; Wieder et al., 2001). Resveratrol may act directly on the mitochondrial membrane by inhibiting the activity of the mitochondrial F0/F1 ATPase (Dorrie et al., 2001; Tinhofer et al., 2001).

Multiple myeloma (MM) is a B cell neoplasia characterized primarily by the accumulation of clonal malignant plasma cells in the bone marrow. The disease leads to severe bone complications and is ultimately fatal. As tumor cells proliferate they stimulate surrounding cells to produce factors that promote further proliferation and enhance bone resorption by stimulating osteoclast activity and inhibiting bone rebuilding (Bruno et al., 2005; Hideshima et al., 2005). Thus the disease enhances the mechanisms underlying its own development. At present, MM is incurable.

One challenge in treating MM is to find drugs that not only target the tumor cells, but also prevent the surrounding cells from participating in the signaling cascades that promote tumor cell survival and bone resorption. In this regard, resveratrol appeared to be a good candidate. Despite its ability to induce apoptosis of MM tumor cells, in vitro studies showed that resveratrol also enhances osteoblast differentiation and prevents osteoclast formation in response to activating factors resulting from the interaction between myeloma cells and stroma cells (Boissy et al., 2005).

To better understand the anticancer properties of resveratrol, we compared its effect with those of two naturally occurring resveratrol oligomers: $\varepsilon$-viniferin and miyabenol C. These phenolics are all stilbenes; $\varepsilon$-viniferin is a resveratrol dimer and miyabenol C a trimer (Fig. 1). The oligomers are not commercially available and have therefore been studied much less than the monomer. Moreover, the trimer is difficult to work with, as it is not abundant, and the structure of each newly isolated batch must be confirmed by spectroscopic and physicochemical means, including measurement of specific rotation.

To assess the potential usefulness of these three stilbenoids in hematology, we tested their effects on various cells of myeloid and lymphoid origin. We focused our attention on cell cycle modifications and components of apoptotic pathways in the multiple myeloma cell line U266, which was chosen because some of its characteristics (p53 mutated/Fas resistant, IL-6 production) are known to be involved in resistance to anticancer drugs (Chauhan et al., 2000).

Materials and methods

Chemicals

The three stilbenoids, resveratrol ($trans$-$3,4',5$-trihydroxystilbene), $\varepsilon$-viniferin and miyabenol C, were prepared from the stalks of *Vitis vinifera* L. Dried, finely powdered stalks were extracted with a water/acetone mixture (3/2:v/v) at room temperature, concentrated under reduced pressure at a temperature below 50 °C, and the aqueous phase was defatted with petroleum ether. The mother liquor was reextracted three times

Fig. 1. Chemical structures of the three stilbenoids studied: resveratrol ($trans$-$3, 4',5$-trihydroxystilbene), $\varepsilon$-viniferin, miyabenol C.
with ethyl acetate, the solutions were pooled and evaporated in vacuo. The residue was dissolved in a small amount of water and freeze-dried to obtain a crude solid extract (1.3%), which was analyzed by centrifugal partition chromatography (CPC) as described earlier (Delaunay et al., 2002) using the solvent hexane–ethyl acetate–ethanol–water (4/5/3/3:v/v/v/v). This gave five fractions. Fractions I and III contained the monomer resveratrol and the dimer ε-viniferin respectively, whereas fraction II contained a mixture of them. The trimer miyabenol C was identified in fraction IV. The four fractions were submitted to preparative HPLC on a reverse C18 phase column (internal diameter: 22 mm), using gradients of methanol in water as eluents to obtain pure compounds: resveratrol (24.6%), ε-viniferin (19.6%) and miyabenol C (0.5%). Structure elucidation of the three purified stilbenoids was based on physico-chemical and spectroscopic means. UV/Vis spectra were recorded using a JASCO V530 spectrometer and FT-IR spectra with a Bomem MB100 spectrometer. Optical rotation was measured on a JASCO DIP1010 polarimeter equipped with a sodium lamp (589 nm). 1D and 2D reverse high field nuclear magnetic resonance (NMR) spectra were obtained at 303 K using either a Bruker AVANCE-500 or 300 NMR spectrometer and ElectroSpray Ionization Mass Spectra on a LCQ Advantage, Thermo Finnigan.

Analysis showed the two oligomers matched literature data (Li et al., 1996; Kurihara et al., 1991) and the monomer matched a commercial resveratrol sample (Sigma-Aldrich, St Quentin Fallavier, France). The molecular weights are 228 for resveratrol (CAS Number: 501-36-0; molecular formula C14H12O3), 454 for ε-viniferin (molecular formula C28H22O6) and 680 for miyabenol C (molecular formula C42H32O9). Each purified stilboid was dissolved in culture medium with 2% EtOH added to a concentration of 200 μg/ml and was kept at −20 °C as a frozen stock solution.

Cell culture

Myeloma-derived cell lines U266 and RPMI-8226, U937 (histiocytic lymphoma-derived promonocytic cell line), K562 (chronic myelogenous leukemia-derived myeloid cell line) and Jurkat (acute T cell leukemia-derived lymphoid cell line) were obtained from American Tissue Type Culture Collection (ATCC; Rockville, MD).

All cell lines were cultured at 37 °C, 5% CO2 in RPMI 1640 supplemented with 10% fetal serum, 2 mM L-Gln, 100 IU/ml penicillin and 100 μg/ml streptomycin (Cambrex BioScience, Emerainville, France).

Proliferation assays, determination of IC50

Cells were seeded in 96-well microtiter plates at 10^4 cells/well and cultured with increasing concentrations of compounds or EtOH (control cells), for two or three days (depending on the cell line). During the last 8 h, cells were pulsed with 0.5 μCi/ well of tritiated thymidine (Amersham Pharmacia Biotech, Orsay, France) and harvested onto glass microfiber papers with a multiple automated cell harvester (Fisher/OSI, Elancourt, France). Thymidine uptake was counted with a β counter (Perkin Elmer, LAS, France).

The IC50 is the concentration of a compound leading to 50% as many counts per minute (cpm) as that in control cells. EtOH controls correspond to wells where all reagents except the tested compounds were added to the culture medium. This means that control cells were exposed to 2% EtOH, further diluted as for the tested compounds.

Each experiment was performed in triplicate.

Cell cycle analysis

Cell cycle analysis was performed as described in Brons et al. (1990). Cells were seeded in 24-well plates at 6.25 × 10^4 cells/well (the same number of cells per volume as in the proliferation assay) and grown in culture medium containing the same amount of EtOH as in the resveratrol solution (control) or with compounds at their IC50 concentrations for one and two days. After incubation, cells were washed in phosphate-buffered saline (PBS) and pellets were kept at 4 °C for 10 min. Cells were then resuspended in 300 μl of propidium iodide staining buffer (0.1% w/v sodium citrate, 25 μg/ml PI, 0.01% Tween20, 100 μg/ml RNase in H2O; Sigma-Aldrich, St Quentin Fallavier, France) and kept at 4 °C for 24 h. Relative DNA content was measured by fluorescence-activated cell sorting (FACS) on FACscan flow cytometer (Beckton Dickinson, San Jose, CA). Data analysis was performed using CellQuest software. A minimum of 20,000 events were collected for each sample.

Analysis of variations of mitochondrial membrane potential (Δψ) and apoptosis

For both types of experiments, cells were seeded in 96-well microtiter plates at 10^5 cells/well and incubated for one or two days with compounds at DC50, i.e. the concentration that PI incorporation experiments had shown led to 50% cell death after four days of culture (data not shown). Cells were then washed twice with PBS.

To analyze the variations of mitochondrial membrane potential, Δψ, cell pellets were incubated with 200 μl of 50 mM 3,3′dihexyloxacarbocyanine iodide (DiOC6(3) (Molecular Probes; purchased from Invitrogen, Cergy Pontoise, France) at 37 °C for 15 min. Cells were then analyzed by FACS at 501 nm.

To determine the percentage of cells undergoing apoptosis, cell pellets were incubated according to the manufacturer’s protocol with FITC-conjugated annexin V (Roche Diagnosis, Meylan, France) and propidium iodide (Sigma-Aldrich, St Quentin Fallavier, France) and analyzed by FACS. In both cases, 20,000 events were collected for each sample.

Inhibition of caspase activation

Pan-caspase inhibitor Z-VAD-FMK (Calbiochem product) was purchased from Laboservices, Montpellier, France and caspase-2 (Z-VDVAD-FMK) and caspase-8 (Z-IETD-FMK) inhibitors from R&D systems (Lille). All caspase inhibitors were stored as stock solutions in dimethylsulfoxide (DMSO;
Sigma-Aldrich, St Quentin Fallavier, France) according to the manufacturer’s instructions. Caspase inhibitors were used at a final concentration of 100 μM.

Cells were seeded in 96-well microtiter plates at 10^5 cells/well and preincubated with caspase inhibitors for 2 h (see Fig. 2). Compounds or culture medium (control) was added and incubation continued for two days. Fresh inhibitor was added after the first 24 h. Compounds were used at a final concentration corresponding to their DC50.

Cells were processed for changes in mitochondrial membrane potential and apoptosis according to the protocols described above. The amount of ethanol used for control corresponds to the percentage of ethanol (0.5%) contained in the resveratrol working solution, which was the highest concentration used.

Inhibition of Fas apoptotic pathway

Fas-blocking monoclonal antibody ZB4 (Coulter Immunotech, Marseille, France) was used at a final concentration of 1 μg/ml. Cells were seeded in 96-well microtiter plates at 10^5 cells/well and preincubated in culture medium with (experimental wells) or without (controls) Fas-blocking antibody for 1 h at 37 °C. Compounds were then added with or without Fas-blocking monoclonal antibody and cells were incubated for two days with addition of fresh Fas-blocking antibody after the first 24 h. Compounds were used at a final concentration corresponding to their DC50. Apoptosis was measured according to the protocols described above.

Statistical analysis

The significance of differences between experimental conditions was determined using the nonparametric Mann–Whitney test.

Results

Resveratrol and its oligomers inhibit proliferation of various myeloid and lymphoid cell lines

Resveratrol and its oligomers ε-viniferin and miyabenol C were tested for their antiproliferative activity on a panel of myeloid and lymphoid cell lines (Table 1). The three stilbenoids inhibit the proliferation of all tested cell lines with varying degrees of efficiency: miyabenol C had the lowest IC50 and is therefore the most efficient compound in inhibiting cell growth. While the difference between IC50 of miyabenol C and...
Resveratrol and its oligomers inhibit proliferation of various myeloid and lymphoid cell lines. Cells were cultured for two or three days (depending on the cell line) with increasing doses of compounds. Proliferation was assessed by measuring thymidine uptake as described in Materials and methods. IC50s (concentrations which inhibit 50% of cell growth) were calculated by comparison with cells grown in culture medium containing only compound solvent. Values are the means ± standard error from five independent experiments.

Compared with resveratrol, the IC50 of ε-viniferin was either higher (RPMI8262 and Jurkat; \( p \leq 0.01 \)) or not statistically different (U266, K562 and U937).

Miyabenol C was therefore the most effective compound in the largest number of cell lines. These data do not show any cell line specific action for any of the compounds.

Resveratrol and its oligomers differentially affect cell cycle progression of the myeloma cell line U266

Since the tested stilbenoids blocked cell proliferation, we then analyzed their impact on cell cycle progression of the U266 myeloma cell line (Fig. 2).

Cells were incubated with compounds at their IC50 concentrations, and were then assessed for their repartition in different phases of the cell cycle after one and two days of incubation. As shown in Fig. 2, the cell cycle profile is progressively modified as incubation with the compounds increases. Importantly, the repartition of the cells in the different phases of the cell cycle was different for each compound.

**Table 1**

IC50 values (\( \mu \text{M} \)) of resveratrol, ε-viniferin and miyabenol C in various myeloid and lymphoid cell lines

<table>
<thead>
<tr>
<th></th>
<th>U266</th>
<th>RPMI8262</th>
<th>Jurkat</th>
<th>K562</th>
<th>U937</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resveratrol</td>
<td>26.3±11.9</td>
<td>42.6±1.6</td>
<td>43.2±13.7</td>
<td>28.6±7.6</td>
<td></td>
</tr>
<tr>
<td>ε-viniferin</td>
<td>45.7±5.5</td>
<td>83.5±14.6</td>
<td>39.3±3.8</td>
<td>20.4±5.8</td>
<td></td>
</tr>
<tr>
<td>Miyabenol C</td>
<td>29.4±8.5</td>
<td>18.7±5.7</td>
<td>10.8±1.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Resveratrol was not statistically significant in the RPMI8626 cell line (despite a mean value inferior to resveratrol), the differences in IC50 values were statistically significant in the four other cell lines (U266, Jurkat, K562 and U937; \( p \leq 0.01 \)).

**Fig. 3.** Resveratrol and its oligomers induce caspase-dependent apoptosis associated with membrane mitochondrial damage. U266 cells (1 × 10^6/ml) were incubated with the compounds for 48 h in the presence or absence of the broad-spectrum caspase inhibitor Z-V-AD-FMK (100 \( \mu \text{M} \)) as described in Materials and methods. Compounds were used at their DC50 concentrations (concentrations which induce 50% of cell death after four days of culture): resveratrol (203 \( \mu \text{M} \)), ε-viniferin (38 \( \mu \text{M} \)) and miyabenol C (30 \( \mu \text{M} \)). (A) Percentages of early apoptotic cells (annexin (An) positive (+)/propidium iodide (PI) negative (−) = An+/PI−) and (B) percentages of early apoptotic plus late and/or necrotic cells (An+/PI− and PI+) in cells cultured with or without Z-V-AD-FMK, as determined by FACS analysis of cells stained with FITC-conjugated annexinV and propidium iodide. Histograms show differences between compound-treated cells and their respective controls; values are means ± standard error from five independent experiments. Because the caspase inhibitor Z-V-AD-FMK itself induces a low percentage of annexin-positive cells, control cells for experiments without Z-V-AD-FMK were cells grown in culture medium containing EtOH (0.5%), whereas control cells for experiments with Z-V-AD-FMK were grown in culture medium containing EtOH (0.5%) plus Z-V-AD-FMK (100 \( \mu \text{M} \)). (C) Effects of compounds on mitochondrial membrane potential \( \Delta \psi \). After 48 h of culture, cells were stained with 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) (50 nM) and the intensity of fluorescence was evaluated by FACS. Control cells were grown in culture medium containing 0.5% EtOH only. Low DiOC6(3) staining indicates cells with disrupted \( \Delta \psi \). For this, 20,000 events were counted and data shown are from one experiment representative of four independent experiments.
phases of the cell cycle varies according to the compound used. Whereas resveratrol induces accumulation of the cells in S phase, as described in the literature, cells treated with ε-viniferin and miyabenol C accumulate in the G2/M and the G1 phases, respectively. The most pronounced effect is observed in cells treated with ε-viniferin where, compared to controls, the difference in the number of cells accumulating in G2/M is statistically significant after 24 h of treatment, as is the reduction of cells in G0/G1.

All compounds induce caspase-dependent apoptosis associated with disruption of normal mitochondrial membrane potential (Δψ)

For all studies related to the mechanisms underlying apoptosis, compounds were used at their DC50 concentrations, i.e. the concentration that induced 50% cell death after four days of treatment (data not shown). To analyze early events in the process of cell death, all assays were performed after two days of treatment with compounds. Measurement of the percentage of cells undergoing treatment-induced apoptosis shows that all compounds induced apoptosis of the U266 cell line (Fig. 3A). In all cases, apoptosis was entirely mediated by caspase activation since it was prevented by the use of the broad-spectrum caspase inhibitor Z-VAD-FMK. The reduction of the percentage of annexin-positive cells, including early apoptotic (An+/PI−) and late and/or necrotic cells (An+/PI+), to a level below 2%, indicates that caspase-mediated apoptosis accounts for nearly all of the cytotoxic effect of these stilbenoids (Fig. 3B).

Parallel evaluation of the percentage of cells showing a disruption of Δψ shows that stilbenoid-induced apoptosis involves the mitochondrial pathway (Fig. 3C).

The next question was whether resveratrol acts directly on mitochondria or through components upstream of mitochondrial membrane depolarization.

Compound-induced disruption of Δψ mostly depends on upstream caspase activation

Previous reports show that Δψ disruption in acute lymphoblastic leukemia-derived (ALL-derived) cell lines treated with resveratrol is independent of upstream activation of caspases and suggest that resveratrol could act directly on the mitochondrial membrane (Dorrie et al., 2001; Tinhofer et al., 2001).

To whether compound-induced disruption of Δψ depends on upstream caspases activation, we analyzed the percentage of cells with low mitochondrial potential after incubating the cells with each of the compounds with or without Z-VAD-FMK. As shown in Fig. 4, most of the stilbenoid-induced disruption of Δψ is prevented by Z-VAD-FMK. This suggests that, unlike previous observations in ALL-derived cell lines, depolarization of the mitochondrial membrane in U266 could depend on upstream caspase activation.

Compound-induced apoptosis involves activation of caspase 8 and, to a lesser extent, caspase 2

To further investigate mechanisms leading to Δψ disruption, we analyzed the compound-induced depolarization of mitochondrial membrane in the presence or absence of specific inhibitors of two caspases shown to act upstream of mitochondria damage: caspase 8 and caspase 2 (Li et al., 1998; Lassus et al., 2002; Dirsch et al., 2004; Lin et al., 2004; Mohan et al., 2006).

As shown in Fig. 5A, only resveratrol-induced Δψ disruption is clearly modified by inhibition of caspase 2. Inhibition of caspase 2 does not significantly affect mitochondrial membrane changes induced by ε-viniferin or miyabenol C. This indicates that upstream activation of caspase 2 partially accounts for resveratrol-induced Δψ disruption, whereas it does not play any significant role in mitochondrial changes induced by ε-viniferin and miyabenol C. Nevertheless, Δψ disruptions induced by all three compounds involve upstream activation of caspase 8, particularly in cells treated with miyabenol C (Fig. 5B).

Measurements of the percentage of annexin-positive cells (early and late apoptotic cells) in the presence or absence of a caspase 2 inhibitor (Fig. 5C) show that caspase 2 slightly intervenes in resveratrol and ε-viniferin-induced cell death. While caspase 2 largely mediates the cytotoxic effect of miyabenol C, it is not involved in mitochondrial membrane changes induced by this trimer (Fig. 5A). This indicates that with miyabenol C, caspase 2 activation occurs downstream of Δψ disruption.

As already suggested by Fig. 5B, Fig. 5C shows that inhibition of caspase 8 decreases the percentage of annexin-positive cells, with the strongest effect in the experiments with miyabenol C. This confirms that caspase 8 mediates an important part of the cytotoxic effect of these compounds.
These results show that stilbenoid-induced cell death is mediated, at least in part, by caspase 2 and caspase 8, the latter playing a more important role in all cases.

As caspase 8 is the main known initiator of death receptor-mediated apoptosis, we wondered whether compound-induced cell death would involve the Fas pathway as described by Clement et al. in the promyelocytic cell line HL60 and the breast carcinoma cell line T47D (Clement et al., 1998). Those authors showed that resveratrol activates the Fas pathway by upregulating FasL expression. Although U266 cells were reported to be resistant to Fas-mediated apoptosis (Shima et al., 1995), this does not exclude that stilbenoids can induce U266 cell apoptosis via the Fas pathway. Indeed, Fas resistance of U266 cells has been attributed to the constitutive activation of the IL-6 signaling pathway leading to upregulation of the antiapoptotic Bcl-xL (Catlett-Falcone et al., 1999). Since resveratrol was shown to inhibit IL-6 receptor and Bcl-xL expression, it is possible that resveratrol induces upregulation of FasL leading to subsequent apoptosis of U266 cells (Jazirehi and Bonavida, 2004; own data).

**Apoptotic cell death induced by these compounds does not involve Fas/FasL interaction**

To investigate whether U266 cell death induced by our compounds was mediated by Fas signaling, we measured the percentage of annexin-positive cells following two days of treatment with compounds in the presence or absence of a Fas antagonist antibody.

The clone of the antagonist antibody (ZB4) and the blocking conditions were identical to those used by Clement et al. (1998)
and Dorrie et al. (2001). Results presented in Fig. 6 show that blocking FasL/Fas interaction does not prevent compound-induced cell death. Parallel analysis of FasL expression did not show any upregulation of FasL expression in response to the compounds (data not shown). Therefore, apoptotic cell death of U266 cells induced by resveratrol and its oligomers ε-viniferin and miyabenol C does not involve Fas/FasL interaction.

Discussion

The aim of the study was to evaluate and compare the antiproliferative and proapoptotic properties of resveratrol with two of its natural oligomers, the dimer ε-viniferin and the trimer miyabenol C.

Assays performed on a panel of myeloid and lymphoid cell lines show that the three compounds inhibit the proliferation of all tested cell lines, but miyabenol C is the most active. Cell cycle analysis performed on the myeloid U266 cells indicates that the three stilbenoids differentially modify cell cycle distribution, suggesting action on different targets.

All subsequent experiments aiming to analyze stilbenoid-induced cell death were conducted on the U266 cell line. DC50s show that miyabenol C is the most active in inducing cell death (miyabenol C = 30.3 μM > ε-viniferin = 38.5 μM > resveratrol = 207.7 μM). This is supported by the data presented in Fig. 2B, which show that when used at a molarity 6.8 times lower than resveratrol, miyabenol C induces about the same percentage of annexin-positive cells after two days of culture.

At their DC50, the three compounds induce caspase-dependent apoptosis of the U266 cell line associated with Δψ disruption. Unlike most of the other studies performed with resveratrol in different cell lines (Ferry-Dumazet et al., 2002), we find caspase-dependent apoptosis accounts for the totality of compound-induced cell death, and the mitochondrial membrane depolarization is entirely dependent on upstream caspase activation.

The Δψ disruptions induced by the three compounds involve upstream caspase 8 activation, but our data show some differences between compounds in terms of which caspase activation pathways they induce, suggesting different mechanisms of action. While caspase 8 accounts for most of the mitochondrial membrane Δψ disruption mechanism and subsequent cell death induced by miyabenol C, caspase 8 activation only accounts for part of the apoptosis induced by resveratrol and ε-viniferin.

Caspase 2 does not seem to be a key mediator of ε-viniferin cytotoxicity, but it is involved in resveratrol and miyabenol C-induced cell death. Moreover, whereas caspase 2 activation by miyabenol C treatment occurs downstream of mitochondrial membrane depolarization, it occurs upstream of mitochondrial Δψ disruption with resveratrol treatment. Involvement of caspase 2 activation in mitochondrial membrane disruption has already been reported for colon cancer cells treated with resveratrol (Mohan et al., 2006). These authors showed that activated caspase 2 induces conformational changes in Bax/Bak, causing translocation of Bax from the cytosol to the mitochondrial membrane, provoking Δψ disruption and subsequent release of cytochromes, apoptosis-inducing factors (AIF) and endonucleases.

The caspase activation pathway common to the three compounds is the apical activation of caspase 8. As this caspase is known as the initiating caspase of death receptor-mediated apoptosis, we investigated whether compound-induced caspase 8 activation could involve Fas/FasL interaction as shown for resveratrol by Clement et al. (1998). Contrary to their results, none of the three stilbenoids tested induced apoptosis in U266 cells by inducing FasL expression and subsequent FasL/Fas interaction. Flow cytometry analysis of FasL expression in cells treated with the three stilbenoids versus control cells further confirmed the absence of FasL upregulation (data not shown).
Whether compounds induce the activation of other known as yet unidentified death receptors must be investigated. The existence of an unidentified death receptor or a death receptor-like factor still able to interact with Fas (TNFRSF6)-associated via death domain (FADD) was suggested by Wang and his collaborators, who showed that treatment of the Jurkat cells with the resveratrol analogue, 3,4,5-trihydroxy-trans stilbene (3,4,5-THS) induced activation of caspase 8 and apoptosis by a FADD protein-dependent mechanisms without involving the known death receptor ligands Fas, TNFα and TRAIL (Wang et al., 2005).

Another explanation could be that compounds induce apoptosis of U266 cells by forming an active death-inducing signaling complex (DISC) involving Fas, FADD and procaspase 8 in the absence of any interaction with Fasl. This phenomenon has been observed in different cell lines with many cytotoxic drugs including resveratrol (Micheau et al., 1999; Delmas et al., 2003).

In conclusion, the three grape vine phenolics inhibit the proliferation of a panel of myeloid and lymphoid cell lines and induce the apoptosis of the p53 mutated/Fas resistant myeloma cell line U266. Stilbenoid-induced cell death is entirely dependent on caspase activation and involves mitochondria apoptotic events. In the same cell line, the three stilbenoids most probably induce some common, but also some different caspase activation pathways.

Of the three compounds, miyabenol C is the most effective. Moreover, we show that it is less toxic than resveratrol to normal peripheral blood mononuclear cells (PBMCs). Used at its DC50, resveratrol induces significant cell death of normal PBMCs, whereas miyabenol C, used at its DC50, does not show any toxicity toward normal PBMCs (data not shown). Therefore, miyabenol C represents a potent antimutagen for malignancies of myeloid and lymphoid origin. Given its ability to induce cell death of the myeloma cell line U266, it would be worth testing on other myeloma cell lines as well as primary cultures of malignant plasmocytes.

As mentioned in the Introduction, MM is a complex disease which is difficult to treat because of the complexity of the interplay between myeloma cells and the cells of their microenvironment as well as of the network of cytokine-induced intracellular signalling pathways.

Most of the recently developed therapies for MM patients, including the standard ones, usually trigger caspase 8 or 9 activation (Catley et al., 2005; Richardson et al., 2005). Since the three stilbenoids could trigger different apoptosis pathways, with some involving caspase 2 (generally not targeted by standard treatments) and other as yet unidentified caspases, it might be interesting to either use a combination of stilbenoids or to use stilbenoids in combination with existing treatments.

Taking into account the ability of resveratrol to interfere with the cross-talk between tumor cells and their microenvironment, this compound should certainly be part of the drug cocktail.

References


