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Pterostilbene and 3′-hydroxypterostilbene are effective apoptosis-inducing agents in MDR and BCR-ABL-expressing leukemia cells

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Abstract

Pterostilbene and 3,5-hydroxypterostilbene are the natural 3,5-dimethoxy analogs of *trans*-resveratrol and piceatannol, two compounds which can induce apoptosis in tumor cells. In previous studies we demonstrated the importance of a 3,5-dimethoxy motif in conferring pro-apoptotic activity to stilbene based compounds so we now wanted to evaluate the ability of pterostilbene and 3,5-hydroxypterostilbene in inducing apoptosis in sensitive and resistant leukemia cells. When tested in sensitive cell lines, HL60 and HUT78, 3′-hydroxypterostilbene was 50–97 times more potent than *trans*-resveratrol in inducing apoptosis, while pterostilbene appeared barely active. However, both compounds, but not *trans*-resveratrol and piceatannol, were able to induce apoptosis in the two Fas-ligand resistant lymphoma cell lines, HUT78B1 and HUT78B3, and the multi drug-resistant leukemia cell lines HL60-R and K562-ADR (a Bcr-Abl-expressing cell line resistant to imatinib mesylate). Of note, pterostilbene-induced apoptosis was not inhibited by the pancaspase-inhibitor Z-VAD-fmk, suggesting that this compound acts through a caspase-independent pathway. On the contrary, 3′-hydroxypterostilbene seemed to trigger apoptosis through the intrinsic apoptotic

Abbreviations: SAR, structure-activity relationships; MDR, multidrug resistance; FCS, foetal calf serum; Z-LEHD-fmk, Z-Leu-Glu(Ome)-His-Asp(Ome)-fmk; Z-IETD-fmk, *N*-acetyl-Ile-Glu-Thr-Asp-fmk; Z-VAD-fmk, acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone; DMSO, dimethylsulphoxide; PHA-LCM, phytohemagglutinin-leucocyte culture medium; CFU-GM, colony forming units-granulocyte macrophage; PBS, phosphate buffered saline; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; Fas-L, Fas-ligand; DISC, death-inducing signalling complex

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pathway: indeed, it caused a marked disruption of the mitochondrial membrane potential $\Delta\Psi$ and its apoptotic effects were inhibited by Z-VAD-fmk and the caspase-9-inhibitor Z-LEHD-fmk. Moreover, pterostilbene and 3′-hydroxypterostilbene, when used at concentrations that elicit significant apoptotic effects in tumor cell lines, did not show any cytotoxicity in normal hemopoietic stem cells. In conclusion, our data show that pterostilbene and particularly 3′-hydroxypterostilbene are interesting antitumor natural compounds that may be useful in the treatment of resistant hematological malignancies, including imatinib, non-responsive neoplasms.

Keywords: Stylbenes; Leukemia; Multidrug resistance; Apoptosis; BCR-ABL

1. Introduction

Stilbene-based compounds have over the years attracted the attention of many researchers due to their wide ranging biological activities. One of the most relevant and extensively studied stilbenes is resveratrol (*trans*-3,4′,5-trihydroxystilbene, Fig. 1), a phytoalexin present in grapes and other foods, which is capable of acting as a cancer chemopreventive agent (Burns, Yokota, Ashihara, Lean, & Crozier, 2002; Soleas, Diamandis, & Goldberg, 1997). Indeed, several in vitro and in vivo studies have shown that resveratrol inhibits cellular events associated with cancer initiation, promotion, and progression (Jang et al., 1997). Moreover, resveratrol has powerful growth inhibitory effects on various cancer cell lines, including: leukemia, colonic cancer, breast and prostate cancer cells (Gautam, Xu, Dumaguin, Janakiraman, & Chapman, 2000; Mitchell, Zhu, & Young, 1999; Schneider et al., 2000). Recently, resveratrol has also been shown to induce apoptosis in different cancer cell lines (Clement, Hirpara, Chawdhury, & Pervaiz, 1998; Huang, Ma, Goranson, & Dong, 1999; Surh et al., 1999), although the mechanism by which this occurs remains a controversial issue (Tsan, White, Maheshwari, Bremner, & Sacco, 2000; Dorrie, Gerauer, Wachter, & Zunino, 2001).

Resveratrol is bioavailable following oral administration and it remains intact in a wide range of target organs (Vitrac et al., 2003). Interesting results have been obtained with resveratrol in mice with the highly

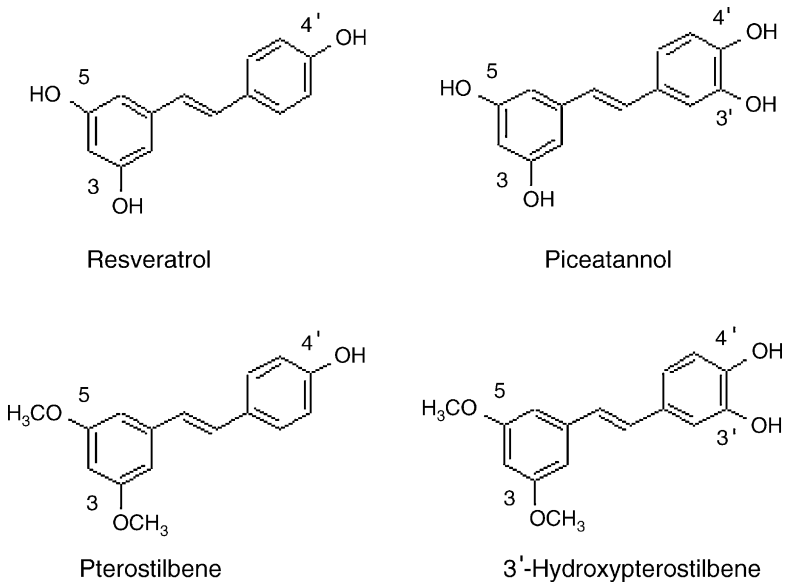

Fig. 1. Chemical structure of *trans*-resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene.

metastatic Lewis lung carcinoma. Here, resveratrol, in doses of 2.5 and 10 mg/kg, significantly reduced tumor volume (42%), tumor weight (44%) and metastasis (56%) (Kimura & Okuda, 2001). Several studies have also indicated that resveratrol is particularly active in continuous leukemic cells and it is capable of suppressing the colony-forming cell proliferation of fresh AML marrow cells from patients with AML in a dose-dependent fashion (Tsan, White, Maheshwari, & Chikkappa, 2002; Gautam et al., 2000; Surh et al., 1999).

Piceatannol and pterostilbene are two *trans*-resveratrol-like natural stilbenes which have recently aroused interest due to their chemopreventive and anticancer properties. Piceatannol has been isolated, together with resveratrol, in grapes and wine. As *trans*-resveratrol, piceatannol displays cytotoxic activity in acute leukemia and lymphoma cells and antiproliferative activity in colorectal cancer cell lines (Wolter, Clausnitzer, Akoglu, & Stein, 2002). Piceatannol differs from resveratrol by possessing an additional aromatic hydroxy group (Fig. 1) and a recent study has shown that resveratrol is metabolized to piceatannol by the cytocrome P450 enzyme CYP1B1, the latter which is overexpressed in a wide variety of human tumors (Potter et al., 2002). Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) (Fig. 1) was first isolated from red sandalwood (pterocarpus santalinus) and, together with resveratrol, it has been identified in the grape berries of Vitis Vinifera, which is the most important species grown worldwide for grape and wine production. Pterostilbene as been demonstrated to have a cancer chemopreventive activity similar to that of resveratrol and it is cytotoxic for a number of in vitro cancer cell lines (Rimando et al., 2002). Recently, 3′-hydroxypterostilbene (*trans*-3,5-dimethoxy-3′,4′-hydroxystilbene) (Fig. 1), a new natural pterostilbene analogue, has been isolated from whole specimens of the herb Sphaerophysa salsula, a shrub widely distributed in central Asia and northwest China (Ma, Li, Li, & Wang, 2002).

Regarding our research, we considered the two natural compounds pterostilbene and 3′-hydroxypterostilbene as potentially interesting. We recently synthesized and tested a library of compounds based on *trans*-resveratrol and we demonstrated, through a study of structure–activity relationships (SAR), the importance of a 3,5-dimethoxy motif in conferring pro-apoptotic activity on leukemia cells (Roberti et al., 2003). As natural compounds, pterostilbene and 3′-hydroxypterostilbene may embody privileged structures, in accordance with Evans' definition (Breinbauer, Vetter, & Waldmann, 2002). In this present paper, we investigated the activity of pterostilbene and 3′-hydroxypterostilbene on different sensitive and drug-resistant leukemia and lymphoma cell lines. Considering the importance of the 3,5-dimethoxy motif at the A phenyl ring and to further investigate its role, the cellular effects of pterostilbene and 3′-hydroxypterostilbene were compared with those of their corresponding natural 3,5-hydroxy analogues, resveratrol and piceatannol respectively.

The main difference observed between the 3,5-dihydroxy compounds (*trans*-resveratrol and piceatannol) and the 3,5-dimethoxy compounds (pterostilbene and 3′-hydroxypterostilbene) was that the latter were particularly active as apoptotic agents on cells expressing the MDR (multidrug resistant) phenotype or the antiapoptotic oncogene Bcr-Abl and in lymphoma cell lines affected by a mutation of the Fas gene and resistant to apoptosis induced by *trans*-resveratrol and piceatannol. We also observed differences regarding the apoptotic pathways activated by pterostilbene, 3′-hydroxypterostilbene, *trans*-resveratrol and piceatannol, and regarding the effects of those compounds on the cell cycle. 3′-Hydroxypterostilbene was markedly more active than the other compounds, displaying a low toxicity on normal hemopoietic stem cells. The data presented in this study indicate that 3′-hydroxypterostilbene is an interesting natural compound which may be useful in treating different types of hematological malignancies.

2. Materials and methods

2.1. Cells

Seven different cell lines were used in this study: two human myeloid leukemia cell lines, HL60 and K562; the human T lymphoma cell line, HUT78; two multidrug resistant human myeloid cell line expressing the P-glycoprotein, HL60-R and K562-ADR; two Fas-L resistant cell line, HUT78B1 and HUT78B3. HL60-R and K562-ADR were selected by continuous exposure of HL60 and K562 cell lines

to increasing concentrations of daunorubicin and adriamycin respectively; HUT78B1 and HUT78B3 cells were selected from the HUT78 cell line by continuous exposure to the Fas-agonistic monoclonal antibody CH11. Both K562 and K562-ADR express the antiapoptotic oncogene Bcr-Abl.

2.2. Cell culture

Continuous neoplastic cells (HL60, HL60-R, K562, K562-ADR, HUT78, HUT78B1 and HUT78B3) were grown in RPMI 1640 (Gibco Grand Island, NY, USA) containing 10% FCS (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 2 mM L-glutamine (Sigma Chemical Co, St. Louis, MO) in a 5% CO_2 atmosphere at 37 °C.

2.3. Chemicals

The caspase-inhibitors Z-LEHD-fmk (Z-Leu-Glu (Ome)-His-Asp(Ome)-fmk), Z-IETD-fmk (*N*-acetyl-Ile-Glu-Thr-Asp-fmk), and Z-VAD-fmk (acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone) were purchased from Alexis Biochemicals (Laufelfingen, Switzerland). The anti-Fas antagonistic monoclonal antibody ZB4 was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Cationic lipophilic fluorochrome $DiOC_6$ was purchased from Sigma. All other reagents were of analytical grade.

2.4. Synthesis of stilbene-derivatives

The preparation of pterostilbene and 3′-hydroxy-pterostilbene was effected as previously described (Roberti et al., 2003).

2.5. Drug preparation

Each stilbene-derivative was dissolved in dimethyl-sulphoxide (DMSO) in a stock solution at a concentration of 20 mM, stored at −20 °C and protected from the light. In each experiment DMSO never exceeded 0.2% and this percentage did not interfere with cell growth.

2.6. Cytotoxicity assays

To evaluate the number of live and dead neoplastic cells, the cells were stained with trypan blue and counted on a hemocytometer. To determine the growth inhibitory activity of the drugs tested, 2×10^5 cells were plated into 25 mm wells (Costar, Cambridge, UK) in 1 ml of complete medium and treated with different concentrations of each drug. After 48 h of incubation, the number of viable cells was determined and expressed as the percentage of control proliferation.

2.7. Clonal assays

To evaluate the cytotoxic effects of natural stilbenes on normal hemopoietic progenitor cells, a clonal assay for CFU-GM (colony-forming units-granulocyte macrophage) was performed. Bone marrow mononucleated cells were obtained from bone marrow aspirates of five normal volunteers. Bone marrow (3–5 ml) was diluted in RPMI 1640, layered over a Ficoll-Hypaque gradient (density, 1.077), centrifuged at $400 \times g$ for 30 min, and the interface mononuclear cells were collected. The interface cells were washed three times in PBS, counted, and resuspended at a concentration of 1×10^5 in MEM containing 0.9% methylcellulose, 30% FCS, 10^{-4} M beta-mercaptoethanol, 5% medium conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM) in 15 mm plastic dishes. After 7 days of culture at 37 °C in an environment of 5% CO_2 and 100% humidity, the number of CFU-GM, was evaluated.

2.8. Morphological evaluation of apoptosis and necrosis

Drug-induced apoptosis and necrosis was determined morphologically after labeling with acridine orange and ethidium bromide. Cell (2×10^5) was centrifuged ($300 \times g$) and the pellet was resuspended in 25 μl of the dye mixture. Ten microliters of the mixture was examined in oil immersion with a 100× objective using a fluorescence microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of the ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by the perinuclear condensation of chromatin, stained by acridine orange (100 μg/ml) or ethidium bromide (100 μg/ml), respectively, and by the formation of apoptotic bodies. The percentage of apoptotic cells was determined after counting at least 300 cells.

2.9. Flow cytometry analysis of cell cycle and apoptosis

Cells were washed once in ice-cold PBS and resuspended at 1×10^6 ml in a hypotonic fluorochrome solution containing propidium iodide (Sigma) 50 μg/ml in 0.1% sodium citrate plus 0.03% (v/v) nonidet P-40 (Sigma). After 30 min of incubation, the fluorescence of each sample was analyzed as single-parameter frequency histograms by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The distribution of cells in the cell cycle was analyzed with the ModFit LT program (Verity Software House, Inc.) and apoptosis was determined by evaluating the percentage of hypoploid nuclei accumulated in the sub-G0-G1 peak after labeling with propidium iodide.

2.10. Determination of apoptosis by Annexine-V

Cells (1×10^6) were washed with PBS and centrifuged at $200 \times g$ for 5 min. Cell pellet was suspended in 100 μl of a staining solution, containing FITC-conjugated Annexine-V and propidium iodide (Annexine-V-Fluos Staining Kit, Roche Molecular Biochemicals, Mannheim, Germany) and incubated for 15 min at 20 °C. The percentage of Annexine-V-positive cells was evaluated by flow cytometry (Becton Dickinson).

2.11. Evaluation of apoptosis inhibition after exposure to different caspase-inhibitors

Cells (2×10^5) were exposed to the caspase-inhibitors and, after 2 h, treated with each natural stilbene compound. The percentage of apoptosis was calculated after 48 h of incubation. The caspase-inhibitors used in this study were: caspase-9-inhibitor Z-LEHD-fmk; caspase-8-inhibitor Z-IETD-fmk; and the pan-caspases-inhibitor Z-VAD-fmk. All the caspase-inhibitors were used at the concentration of 50 μM.

2.12. Fas apoptotic pathway

To understand the implication of Fas in drug-induced programmed cell death, 2×10^5 cells were treated with the Fas-blocking monoclonal antibody ZB4 (2 μg/ml) or with the caspase-8-inhibitor Z-IETD-fmk, as described above. After 2 h of incubation, the cells were exposed to each stilbene compound.

2.13. Mitochondrial apoptotic pathway

The mitochondrial membrane potential ($\Delta\Psi$) was measured by flow cytometry after staining the cells with the cationic lipophilic fluorochrome $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide). Cells, adjusted to a density of 0.5×10^6 ml^{-1}, were incubated at 37 °C for 30 min in the presence of $DiOC_6$ (40 nM). The analysis of fluorochrome incorporation was performed using a Becton Dickinson cytofluorometer.

3. Results

3.1. Cell growth inhibition

Cells were exposed to different concentrations of *trans*-resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene. The number of living cells was calculated over a period of 48 h and expressed as a percentage of the number of cells incubated in the medium alone (Fig. 2). In order to compare the antiproliferative activity of the drugs tested, IC50 (a drug concentration which is capable of inhibiting 50% cell growth) was also calculated (Table 1). As shown in Fig. 2, the compounds induced a dose-dependent cell growth inhibition in all cell lines tested. However their activity differed on the basis of the cell line used (Table 1). In sensitive HL60, HUT78 and K562 cells, the IC50 of *trans*-resveratrol, piceatannol and pterostilbene ranged from 5 to 42 μM. 3′-Hydroxypterostilbene was markedly more active than the other compounds, displaying an IC50 of 0.8 μM in HL60 and K562 cell lines, and 0.6 μM in HUT78 cells (Table 1). *trans*-Resveratrol and piceatannol were less active on MDR HL60-R and K562-ADR cells, compared to the corresponding, sensitive parental cells (Fig. 2, Table 1). In contrast, pterostilbene and 3′-hydroxypterostilbene showed a dose response curve and an IC50 in HL60-R and K562-ADR cells, which were similar to those observed in sensitive cells (Fig. 2, Table 1).

3.2. Apoptosis

The percentage of apoptotic cells was calculated morphologically, as described in Section 2. The per-

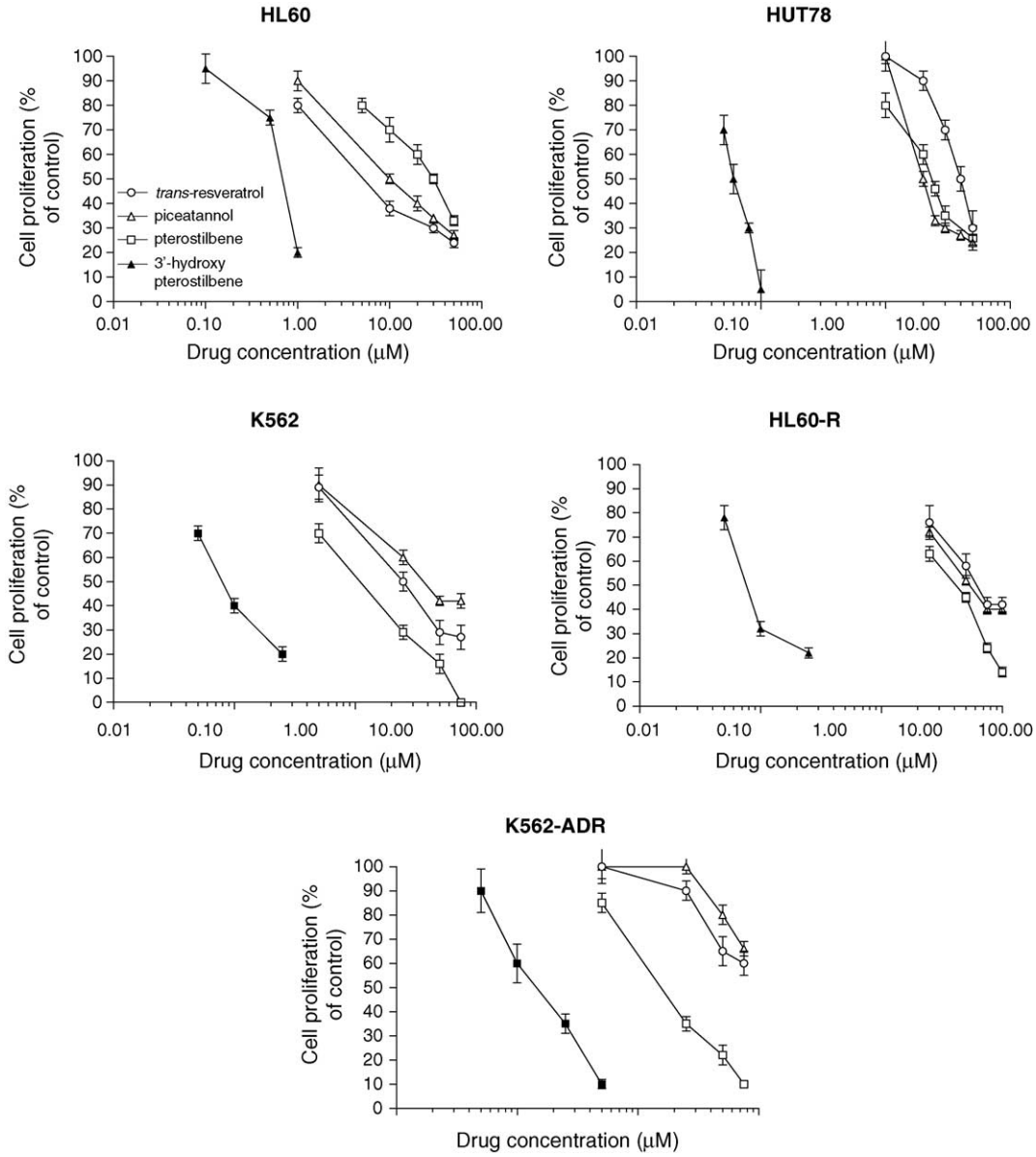

Fig. 2. Antiproliferative activity of *trans*-resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene on HL60, HUT78, K562, HL60-R and K562-ADR cell lines. Cells were grown for 48 h in presence (or absence) of different concentrations of stilbenes. The number of cells was expressed as percentage of the control. Error bars represent standard error of assays from five independent experiments.

centage of apoptotic cells was confirmed by flow cytometry, after staining the cells with FITC-conjugated Annexin-V plus PI, and by calculating the percentage of cells in the sub-G0-G1 phase of the cell cycle by flow cytometry, after staining the cells with propidium iodide. AC50 (the drug concentration capable of inducing apoptosis in 50% of cells) was calculated in order to compare the apoptotic activity of each compound (Table 1).

trans-Resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene-induced apoptosis in a dose- and cell line-dependent manner. *trans*-Resveratrol and

piceatannol induced apoptosis in HL60 and HUT78 cells when used at concentrations higher than 30 μM for 48 h; however, these compounds were barely active on K562 cells (Fig. 3). This was not surprising, considering that K562 is a Bcr-Abl-expressing cell line which is resistant to different apoptosis-inducing stimuli, including anticancer agents (Amarante-Mendes et al., 1998; Martins et al., 1997; McGahon et al., 1994, 1997). Pterostilbene was not found to be a powerful inductor of apoptosis in HL60 and HUT78 cells (AC50:

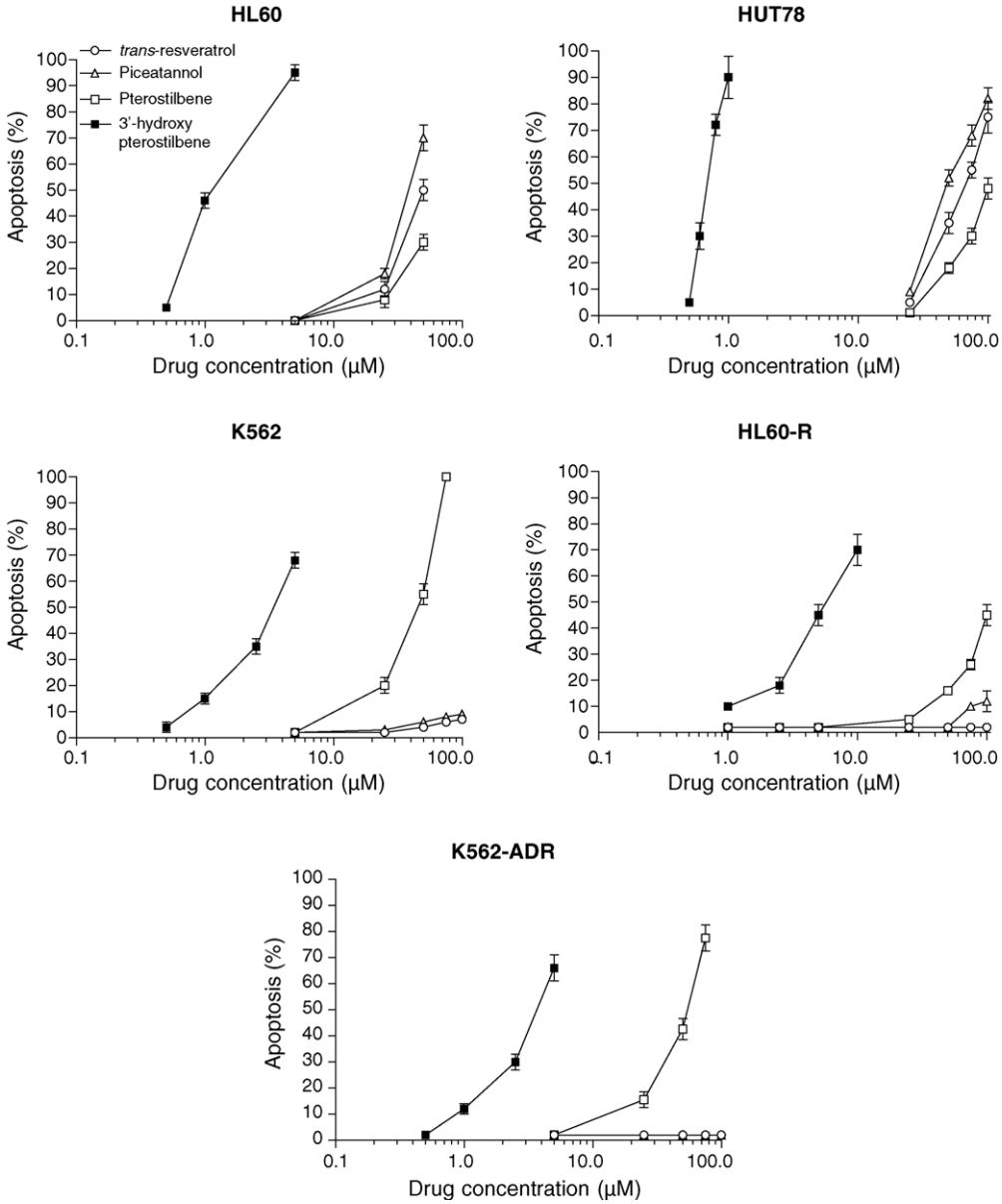

Fig. 3. Percentage of apoptotic cells induced by *trans*-resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene on HL60, HUT78, K562, HL60-R and K562-ADR cell lines after 48 h of drug exposure. Error bars represent standard error of assays from five independent experiments.

Table 1
IC50 and AC50 values of *trans*-resveratrol, piceatanol, pterostilbene and 3′-hydroxypterostilbene in sensitive HL60 and HUT78 cells, in Bcr-Abl-expressing K562 cells, in MDR HL60-R cells and in MDR and Bcr-Abl-expressing K562-ADR cells

Compounds	HL60		HUT78		K562		HL60-R		K562-ADR	
	IC50	AC50	IC50	AC50	IC50	AC50	IC50	AC50	IC50	AC50
trans-Resveratrol	5 ± 2	50 ± 6	42 ± 6	68 ± 8	28 ± 6	230 ± 11	60 ± 6	>300	123 ± 12	>300
Piceatannol	10 ± 3	42 ± 5	20 ± 6	47 ± 6	22 ± 4	220 ± 8	50 ± 7	>300	126 ± 8	>300
Pterostilbene	35 ± 7	75 ± 5	24 ± 3	98 ± 10	10 ± 3	45 ± 7	40 ± 3	85 ± 11	12 ± 2	50 ± 4
3′-Hydroxy-pterostilbene	0.8 ± 0.1	1 ± 0.2	0.6 ± 0.08	0.7 ± 0.04	0.8 ± 0.05	3 ± 0.2	0.9 ± 0.1	5 ± 0.4	1.2 ± 0.1	3.5 ± 0.2

75 and 98 μM respectively) but in K562 cells it showed an AC50 of only 45 μM. 3′-Hydroxypterostilbene was markedly more active than the other compounds tested. Indeed, in HL60 it was approximately 40–50 times more active than piceatannol and *trans*-resveratrol. In HUT78 cells, 3′-hydroxypterostilbene produced an AC50 which was 97 times lower than that of *trans*-resveratrol and 67 times lower than that of piceatannol; in K562 cells, 3′-hydroxypterostilbene was 15 times more active than pterostilbene (Table 1). *trans*-Resveratrol and piceatannol were barely active as apoptosis-inducing agents on MDR HL60-R cells, while pterostilbene gave a value of AC50 on HL60-R, which is similar to that obtained in HL60 cells (Fig. 3, Table 1). In addition, 3′-hydroxypterostilbene was markedly active on HL60-R cells, showing an AC50 of only 5 μM (Fig. 3, Table 1).

Of interest were the effects of pterostilbene and 3′-hydroxypterostilbene on K562-ADR cells. This cell line is more resistant than parental K562 cells to the apoptotic activity of anticancer drugs, such as metotrexate, cisplatin and cytarabine (drugs active in P-glycoprotein expressing cells), or to the apoptotic activity of daunorubicin in combination with verapamil (Fig. 4). As shown in Fig. 3 and Table 1, K562-ADR cells were resistant to *trans*-resveratrol and piceatannol apoptosis-inducing activity. We observed that imatinib mesylate (Gleevec), a specific Bcr-Abl tyrosine kinase inhibitor currently widely used in treating Bcr-Abl-positive chronic and acute leukemias, was able

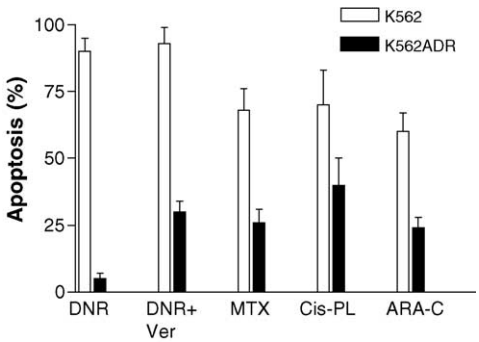

Fig. 4. Percentage of apoptotic cells induced by 0.5 μg/ml daunorubicin (DNR), 0.5 μg/ml daunorubicin + 5 μg/ml verapamil (DNR + Ver), 1 μg/ml metotrexate (MTX), 10 μg/ml cisplatin (Cis-PL) and 20 μg/ml cytarabine (ARA-C) on K562 and K562-ADR cells. Apoptosis was calculated after 92 h of treatment. Error bars represent standard error of assays from four independent experiments.

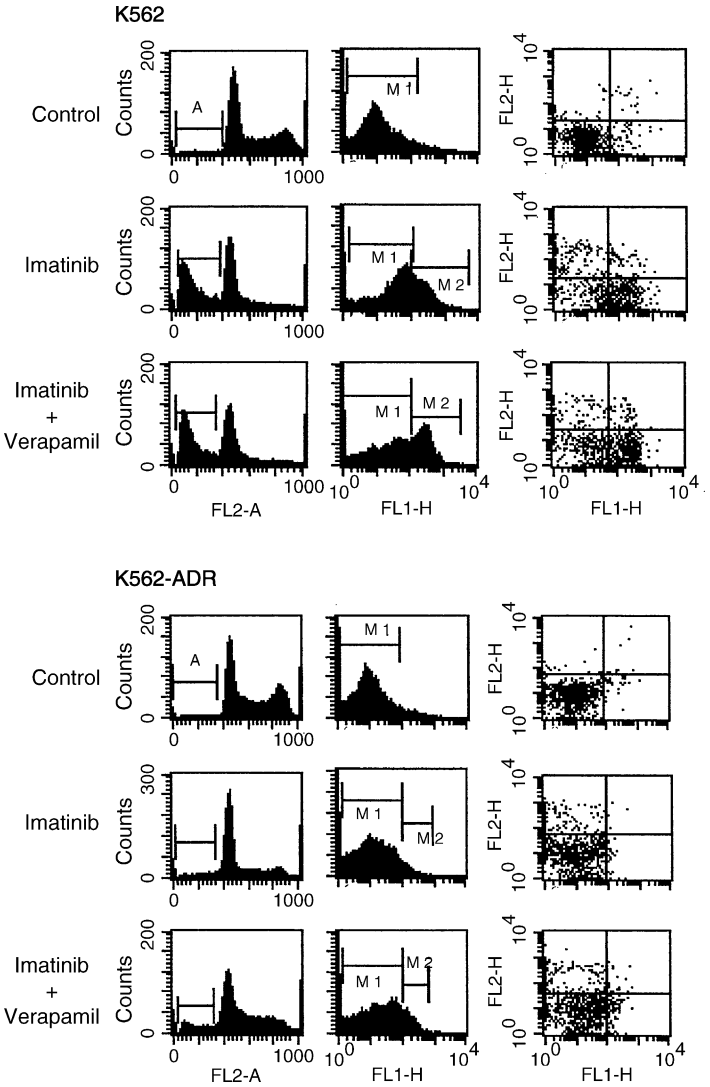

Fig. 5. Apoptosis evaluated on K562 and K562-ADR cells by flow cytometry after 48 h treatment with 0.5 μM imatinib mesylate or 0.5 μM imatinib mesylate + 5 μg/ml verapamil. Left-hand column: flow cytometric analysis of apoptotic cells evaluated after staining cells with propidium iodide (PI) and analysis of sub-G0-G1 peak (A). Central column: flow cytometric analysis of apoptotic cells after staining with annexin-V; the M1 and M2 gates demarcate annexin-V-negative and -positive populations respectively. Right-hand column: flow cytometric analysis of apoptotic cells after staining with annexin-V and propidium iodide. Cells in the bottom right quadrant of each dot plot represent the percentage of cells in early apoptosis (annexin-V-positive and PI-negative). Data are representative of three separate experiments.

to induce apoptosis in K562 cells after 48 h of drug exposure but it failed to induce apoptosis in K562-ADR cells (Fig. 5). When used in combination with verapamil, imatinib was also barely active in K562-ADR cells for the first 48 h of treatment (Fig. 5). 3′-Hydroxypterostilbene was six times less active than imatinib in K562 cells (AC50: 3 μM versus 0.5 μM respectively) but it was active in K562-ADR cells without the addition of an MDR reversing agent (Fig. 6), thereby producing in these cells an AC50, which was similar to that observed in parental K562 cells (Table 1). Although pterostilbene was markedly less active than

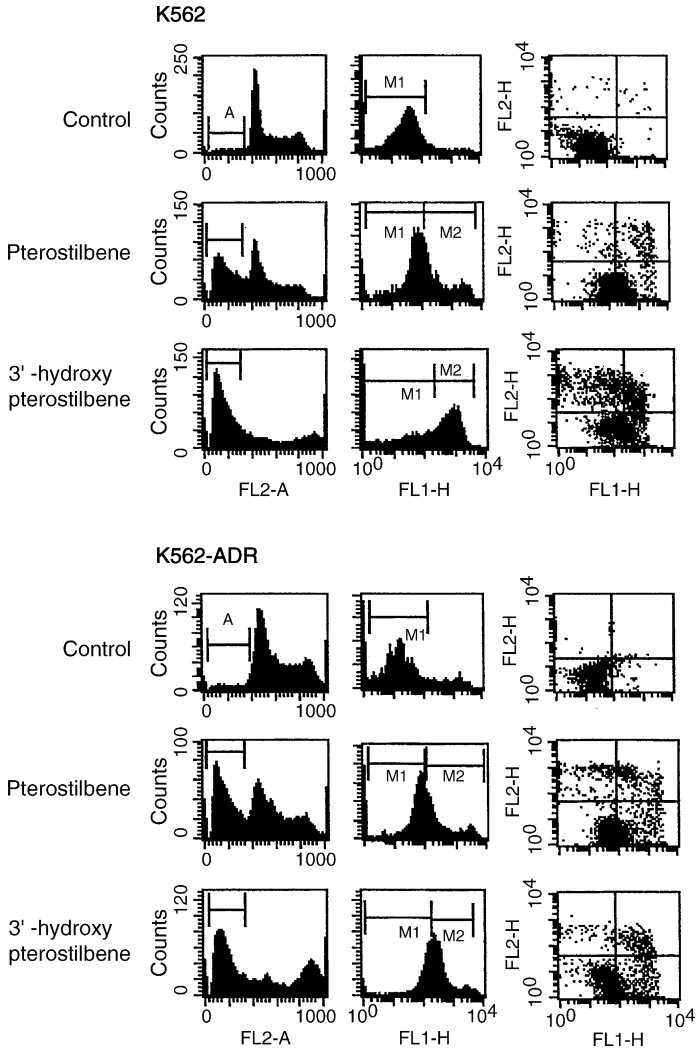

Fig. 6. Apoptosis evaluated on K562 and K562-ADR cells by flow cytometry after 48 h treatment with 50 μM pterostilbene or 3 μM 3′-hydroxypterostilbene. Left-hand column: flow cytometric analysis of apoptotic cells evaluated after staining cells with propidium iodide (PI) and analysis of sub-G0-G1 peak (A). Central column: flow cytometric analysis of apoptotic cells after staining with annexin-V; the M1 and M2 gates demarcate annexin-V-negative and -positive populations respectively. Right-hand column: flow cytometric analysis of apoptotic cells after staining with annexin-V and propidium iodide. Cells in the bottom right quadrant of each dot plot represent the percentage of cells in early apoptosis (annexin-V-positive and PI-negative). Data are representative of three separate experiments.

3′-hydroxypterostilbene, it showed similar apoptosis-inducing activity in K562 and K562-ADR cells (Fig. 6, Table 1).

3.3. Cytotoxicity on normal CFU-GM

In order to evaluate the toxicity of *trans*-resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene on normal hemopoietic cells, bone marrow cells were treated with each compound and the number of CFU-GM was evaluated after 7 days of culture (Fig. 7). All compounds caused a dose-dependent inhibition in CFU-GM forming capacities. Pterostilbene was the less toxic compound tested in this study for hemopoietic stem cells. In contrast, piceatannol showed a toxicity on CFU-GM which was similar to

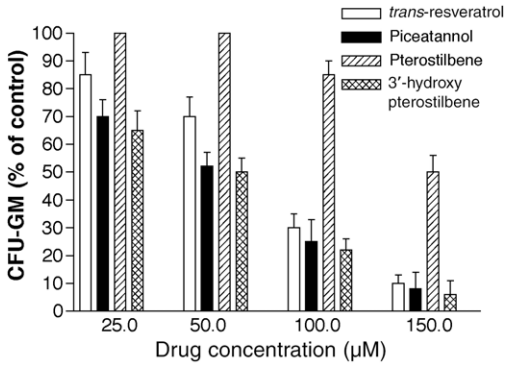

Fig. 7. Effects of *trans*-resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene on normal CFU-GM. Cells were exposed to different concentrations of each compound. The number of CFU-GM was calculated after 7 days of cell culture.

that observed in leukemia and lymphoma cell lines, while *trans*-resveratrol was slightly less toxic than piceatannol. Interesting results were obtained with 3′-hydroxypterostilbene which caused an approximate decrease of 50% in the CFU-GM number when used at a concentration of 50 μM (IC50CFU-GM = 50 μM) while, in the leukemia and lymphoma cells used in this study, the IC50 and AC50 of this compound ranged from 0.6 to 3.5 μM (Table 1).

3.4. Cell cycle analysis

To study the effects on the cell cycle, the cells were exposed for 18 h to each stilbene compound tested in this study (Fig. 8). *trans*-Resveratrol and piceatannol caused an increase of cells in the S phase, which was more evident in K562 cells than in HL60. Pterostilbene caused an increase of HL60 cells in the S phase, while in K562 no important modifications of the cell cycle was evident after 18 h. To verify whether any modifications in the cell cycle could be observed thereafter, the K562 cell cycle was analyzed after 24, 48 and 72 h of treatment with pterostilbene. As shown in Fig. 9, after a period of 48 h, pterostilbene-induced a marked increase of K562 cells in the sub-G0-G1 and a decrease in the G2-M phase; after 72 h the sub-G0-G1 peak was more evident and cells in S and G2-M phases were markedly decreased (Fig. 9).

Different results were obtained with the use of 3′-hydroxypterostilbene. Indeed, 3′-hydroxypterostilbene-induced a marked recruitment of both HL60 and K562 cells in the G2-M phase. Morphological assays performed with a fluorescence microscope, after staining with acridine orange and ethidium bromide, showed a percentage of approximately 80% of mitotic cells in the samples treated with 3′-hydroxypterostilbene; this indicated that the recruitment in the G2-M phase of the cell cycle is prevalently an M recruitment (data not shown). After periods of 48 and 72 h, the percentage of cells in G2-M decreased while the apoptotic sub-G0-G1 peak markedly increased (Fig. 9). Results similar to those obtained in HL60 were obtained in HUT78 cells with all stilbenes tested in this study (data not shown).

3.5. Effects of natural stilbenes on the Fas/Fas-L apoptotic pathway

To evaluate the implication of the Fas/Fas-L pathway in apoptosis induced by natural stilbenes tested in this study, the Fas and Fas-ligand (Fas-L) expressing HUT78 cells (Tolomeo et al., 1998) were exposed to the anti-Fas antagonistic monoclonal antibody ZB4 or to the caspase-8-inhibitor Z-IETD-fmk and, after 2 h, were treated with *trans*-resveratrol, piceatannol, pterostilbene or 3′-hydroxypterostilbene, used at the corresponding AC50. As shown in Fig. 10A, ZB4 failed to block apoptosis induced by natural stilbenes; in contrast, the caspase-8-inhibitor Z-IETD-fmk was able to inhibit in part apoptosis induced by *trans*-resveratrol and piceatannol but not apoptosis induced by pterostilbene and 3′-hydroxypterostilbene.

In order to improve our understanding of the role of the Fas pathway, we evaluated the apoptotic activity of these compounds on two Fas-L resistant cell lines: HUT78B1 and HUT78B3. These cells express Fas on the cell surface membrane but they are unable to activate the death-inducing signalling complex (DISC) since affected by a deletion-insertion in the intron 7/exon 8 region of the Fas gene. This mutation affects the phenotype in a dominant negative fashion, i.e., even in the presence of the normal receptor (Cascino, Papoff, De Maria, Testi, & Ruberti, 1996). Both *trans*-resveratrol and piceatannol were not capable of inducing apoptosis in these cell lines while pterostilbene and 3′-hydroxypterostilbene-induced a percentage of apoptosis, which was similar to that induced in the parental Fas-L sensitive cell line HUT78 (Fig. 10B).

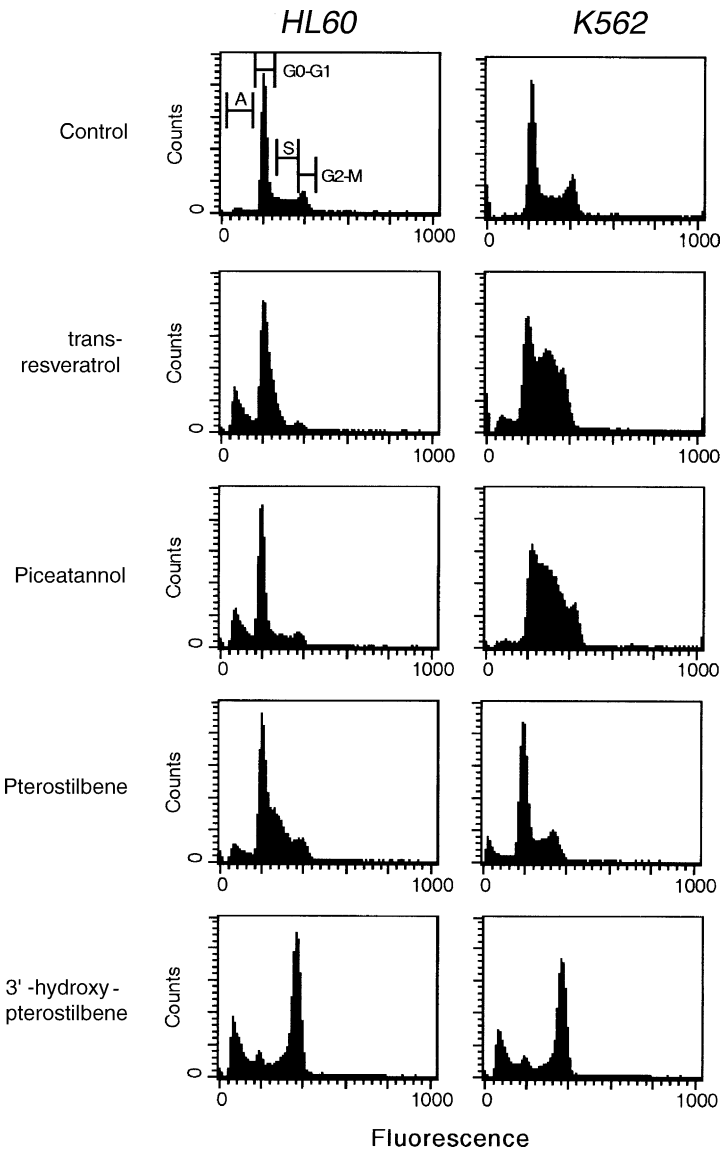

Fig. 8. Cell cycle distribution of HL60 and K562 cells evaluated by flow cytometry after 18 h of drug-exposure. *trans*-Resveratrol and piceatannol were used at the concentration of 50 μM in HL60 cells and 100 μM in K562 cells; pterostilbene at the concentration of 70 μM in HL60 cells and 40 μM in K562 cells; 3′-hydroxypterostilbene at the concentration of 1 μM in HL60 cells and 3 μM in K562 cells.

3.6. Effects of natural stilbenes on the mitochondrial apoptotic pathway

To evaluate the implication of the mitochondrial apoptotic pathway (intrinsic apoptotic pathway) in apoptosis induced by natural stilbenes under investigation in this study, the mitochondrial $\Delta\Psi$ dissipation was measured by flow cytometry after exposing the cells to $DiOC_6$. The polarization status of the mitochondrial membrane is determined by an electrochemical gradient ($\Delta\Psi$). Activation of the intrinsic (mitochondrial) apoptotic pathway can be assessed indirectly by determining whether the $\Delta\Psi$ is reduced. In this procedure, cells were incubated with a lipophilic cation fluorochrome, such as $DiOC_6$, which accumulates in the mitochondrial matrix, driven by the $\Delta\Psi$. A reduction in

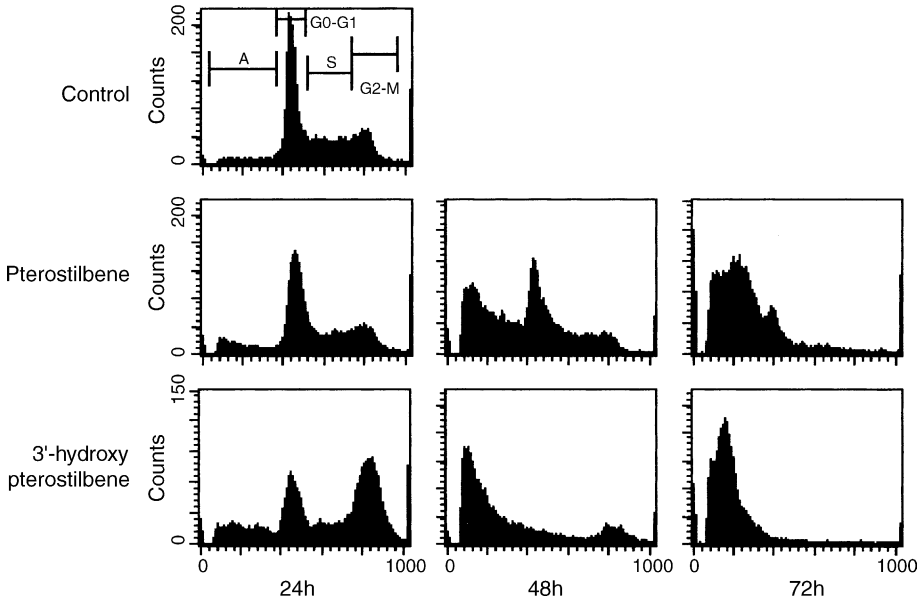

Fig. 9. Cell cycle distribution of K562 cells after 24, 48 and 72 h of drug exposure to 40 μM pterostilbene and 5 μM 3′-hydroxypterostilbene.

fluorescence intensity, as measured by cytofluorimetry, indicated a $\Delta\Psi$ dissipation and, therefore, the activation of the intrinsic apoptosis pathway. As shown in Fig. 11A, *trans*-resveratrol, piceatannol and, particularly, 3′-hydroxypterostilbene were capable of disrupting the mitochondrial membrane potential. This effect was less evident in cells treated with pterostilbene.

As the disruption in $\Delta\Psi$ causes the release of pro-apoptotic factors (such as cytochrome c) from the mitochondria which activate, in turn, caspase-9, we evaluated the effects of natural stilbenes in the presence of the specific caspase-9-inhibitor Z-LEHD-fmk. Z-LEHD-fmk clearly inhibited apoptosis induced by *trans*-resveratrol, piceatannol and 3′-hydroxypterostilbene but it was unable to inhibit apoptosis induced by pterostilbene (Fig. 11B). Of note, apoptosis induced by pterostilbene was not inhibited by the pan-caspase-inhibitor Z-VAD-fmk which, in contrast, was able to inhibit apoptosis induced by *trans*-resveratrol, piceatannol and 3′-hydroxypterostilbene (Fig. 11C).

4. Discussion

Stilbene-based compounds have recently received wide attention due to their spectrum of biological characteristics, which includes cancer chemopreventive and chemotherapeutic properties. In this context, we attempted to evaluate the apoptotic activity of novel classes of stilbene compounds, which are structurally related to Vitamin A or resveratrol (Simoni & Tolomeo, 2001; Tolomeo & Simoni, 2002). On the basis of our previous observations regarding the importance of the 3,5-dimethoxy motif at the A phenyl ring in conferring pro-apoptotic activity (Roberti et al., 2003), the effects of two natural 3,5-dimethylated stilbene compounds – pterostilbene and its 3′-hydroxy derivative – on different sensitive and drug-resistant leukemia and lymphoma cell lines were evaluated. The data were compared with those obtained with the 3,5-dihydroxy-corresponding compounds: resveratrol and piceatannol. The results obtained in this study indicate that 3,5-dimethylated stilbene compounds are active as apoptotic inducing agents in various leukemia cells, which are resistant to apoptosis induced by several anticancer agents, including *trans*-resveratrol and piceatannol. Moreover, both pterostilbene and its 3′-hydroxy derivative were less toxic on normal hemopoietic stem cells than on leukemia and lymphoma cells.

The 3′-hydroxy derivative of pterostilbene was from 50 to 97 times more active than *trans*-resveratrol in sensitive leukemia and lymphoma cell lines and markedly

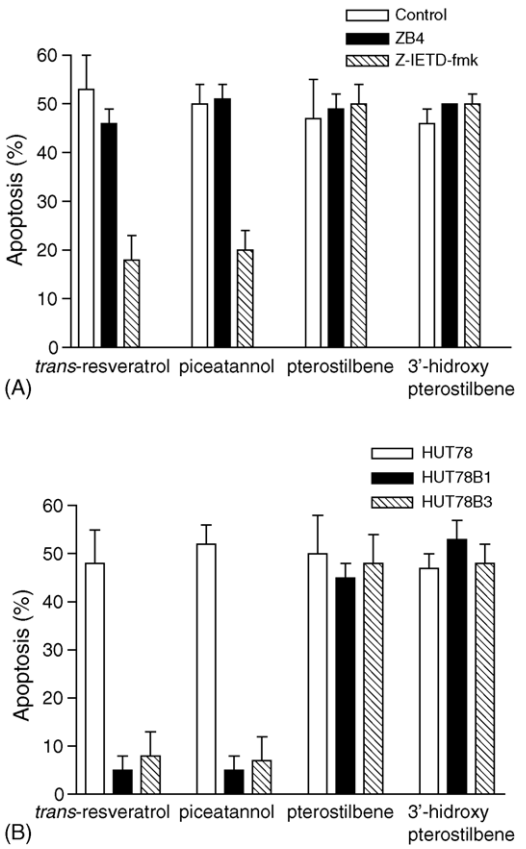

Fig. 10. (A) Effects of the anti-Fas antagonistic monoclonal antibody ZB4 and caspase-8-inhibitor Z-IETD-fmk on apoptosis induced by stilbene compounds. HUT78 cells were exposed to ZB4 and Z-IETD and after 2 h to 68 μM *trans*-resveratrol, 47 μM piceatannol, 98 μM pterostilbene or 0.7 μM 3′-hydroxypterostilbene. Apoptosis was evaluated after 48 h of treatment. (B) Apoptosis induced by stilbene compounds used at the concentrations reported in (A) in HUT78, HUT78B1 and HUT78B3 cell lines. Apoptosis was evaluated after 48 h of treatment. Error bars represent standard error of assays from five independent experiments.

more active than pterostilbene in cells expressing the MDR phenotype or in cells expressing the antiapoptotic oncogene Bcr-Abl. Moreover, 3′-hydroxypterostilbene was found to be a powerful inducer of apoptosis on K562-ADR, a cell line expressing the MDR phenotype and the Bcr-Abl oncogene, and resistant to apoptosis induced by most anticancer agents, including imatinib mesylate (Gleevec). Recently, it has been observed that cells expressing P-glycoprotein (MDR) are resistant to imatinib (Mahon et al., 2003). However, in the present study we observed that an MDR reversing agent, such as verapamil, only partially reversed the resistance to imatinib in K562-ADR cells. In contrast, 3′-hydroxypterostilbene displayed very similar activity in K562 and K562-ADR cells. Whilst we did not analyze the effects of 3′-hydroxypterostilbene on the tyrosine kinase activity of Bcr-Abl in this paper, the lack of selectivity shown by this natural stilbene on Bcr-Abl cells suggests the presence of a mechanism whose action differs, at least in part, to that of imatinib.

Pterostilbene and 3′-hydroxypterostilbene differed with respect to *trans*-resveratrol and piceatannol, not only in their pattern of activity on sensitive and resistant cells but also regarding the effects on the cell cycle and apoptotic pathways. The former were particularly evident in K562 cells in which *trans*-resveratrol and piceatannol caused a clear recruitment in the S phase, while 3′-hydroxypterostilbene in the M phase. Cells blocked in the M phase by 24 h of treatment with 3′-hydroxypterostilbene subsequently underwent apoptosis. Pterostilbene did not cause any important modifications in the cell cycle in K562 cells during the first 24 h but a marked increase in the apoptotic peak and a decrease in the percentage of cells in the G2-M and S phases were evident after 48 and 72 h of treatment.

Regarding the apoptotic pathway, the fact that the pan-caspase-inhibitor Z-VAD-fmk was capable of inhibiting apoptosis induced by *trans*-resveratrol, piceatannol and 3′-hydroxypterostilbene but not apoptosis induced by pterostilbene suggests that pterostilbene could activate apoptosis through a caspase-independent mechanism(s). Although caspase activation is considered a hallmark of apoptotic cell death, other less-defined cell death pathways have been described that appear not to require caspase activation. Recently, it has been observed that, in response to apoptotic stimuli, mitochondria can also release caspase-independent cell death effectors such as AIF and endonuclease G (Cregan, Dawson, & Slack, 2004).

3′-Hydroxypterostilbene seems to activate apoptosis prevalently through the intrinsic pathway, as shown by the ability of this stilbene to significantly disrupt the mitochondrial membrane potential and the capacity of the caspase-9-inhibitor Z-LEHD-fmk to inhibit 3′-hydroxypterostilbene-induced-apoptosis. Pterostilbene and 3′-hydroxypterostilbene were active in HUT78B1 and HUT78B3, two Fas-L resistant lymphoma cell lines affected by a deletion-insertion in the intron 7/exon 8 region of the Fas gene and resistant

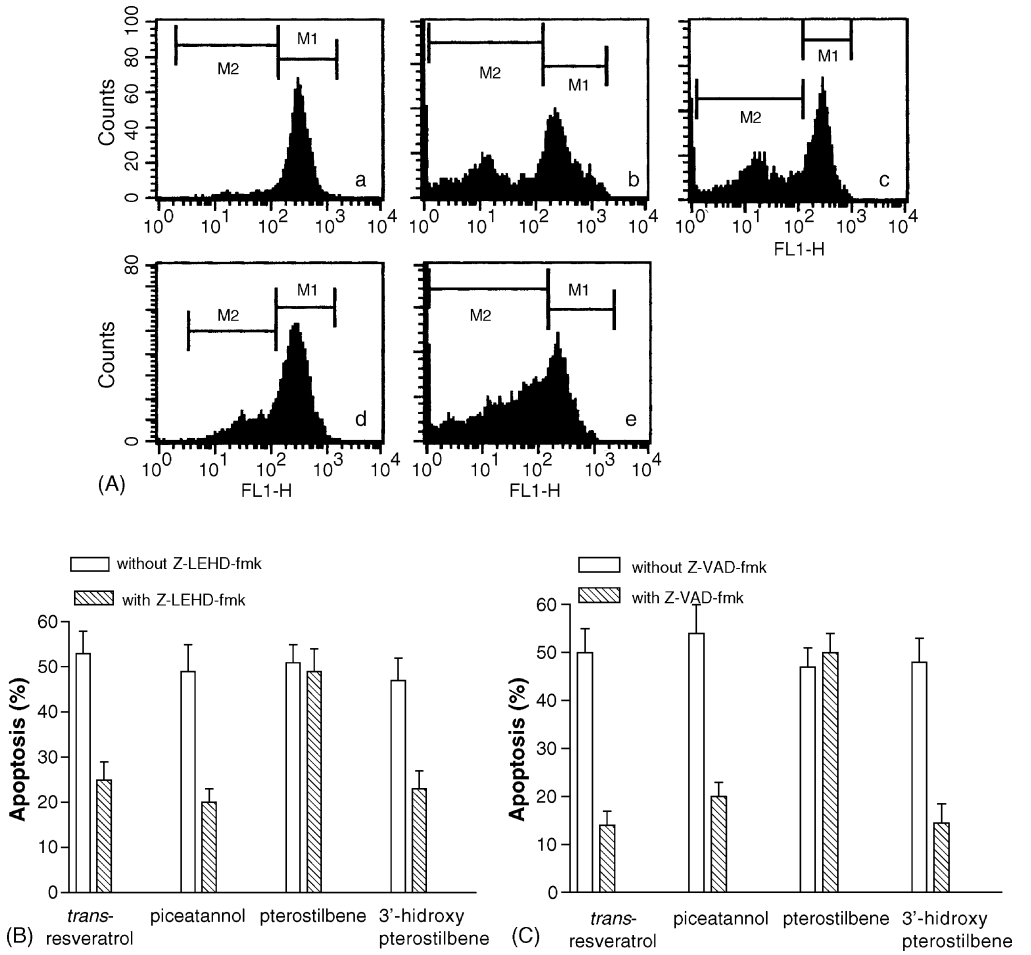

Fig. 11. (A) Effects of stilbene compounds on mitochondrial membrane potential $\Delta\Psi$. HUT78 cells were treated with 68 μM *trans*-resveratrol, 47 μM piceatannol, 98 μM pterostilbene or 0.7 μM 3′-hydroxypterostilbene and after 12 h were stained with $DiOC_6$. The intensity of fluorescence was evaluated by flow cytometry. Cells with a depolarization of mitochondrial membrane show a lower fluorescence compared with the control. The M1 and M2 gates demarcate cell population with normal $\Delta\Psi$ or with disrupted $\Delta\Psi$, respectively; (a) control, (b) *trans*-resveratrol, (c) piceatannol, (d) pterostilbene, (e) 3′-hydroxypterostilbene. (B) Effects of the caspase-9-inhibitor Z-LEHD-fmk on apoptosis induced by stilbene compounds. HUT78 cells were exposed to Z-LEHD-fmk and after 2 h to *trans*-resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene at the concentrations reported in (A). Apoptosis was evaluated after 48 h of treatment. (C) Effects of the pan-caspase-inhibitor Z-VAD-fmk on apoptosis induced by stilbene compounds. HUT78 cells were exposed to Z-LEHD-fmk and after 2 h to *trans*-resveratrol, piceatannol, pterostilbene and 3′-hydroxypterostilbene at the concentrations reported in (A). Apoptosis was evaluated after 48 h of treatment. Error bars represent standard error of assays from five independent experiments.

to apoptosis, which was induced by *trans*-resveratrol and piceatannol. This latter data, together with the evidence of the absence of the apoptosis inhibitory activity of ZB4 and Z-IETD-fmk, suggests that the extrinsic apoptotic pathway could not be implicated in apoptosis induced by pterostilbene and 3′-hydroxypterostilbene.

Some considerations can also be made regarding the apoptotic pathways activated by *trans*-resveratrol and piceatannol. The current data concerning the role of the extrinsic and intrinsic apoptotic pathways in apoptosis, induced by resveratrol (Bernhard et al., 2000; Clement et al., 1998; Dorrie et al., 2001; Tsan et al., 2000) is controversial. Initially, Clément et al. demonstrated that treatment with resveratrol enhances the Fas-L expression in HL60 cells and in T47D breast carcinoma cells, and that resveratrol-mediated cell death

is specifically Fas-signalling dependent (extrinsic apoptosis pathway). Dorrie et al. have also shown that resveratrol can induce apoptosis in both Fas-sensitive and Fas-resistant cells, thereby suggesting that the mechanism for the apoptosis induction of resveratrol is independent of the Fas-pathway, involve instead a depolarization of mitochondrial membranes and activation of caspase-9 (intrinsic apoptotic pathway). More recently, Delmas et al. have observed that resveratrol did not modulate the expression of Fas and Fas-L on the surface of cancer cells, and the inhibition of Fas/Fas-L interaction with ZB4 did not influence the apoptotic response to resveratrol. They observed that resveratrol induced the clustering of Fas (together with FADD and pro-caspase-8) and its redistribution in cholesterol and a sphingolipid-rich fraction of SW-480 cells with the formation of a death-inducing signalling complex (DISC).

In our study, ZB4 did not inhibit resveratrol- and piceatannol-induced apoptosis, while the caspase-8-inhibitor Z-IETD-fmk partially inhibited apoptosis caused by resveratrol. Moreover, *trans*-resveratrol and piceatannol were unable to induce apoptosis in HUT78B1 and HUT78B3 which are Fas-expressing cells, incapable of forming a death-inducing signalling complex (Cascino et al., 1996). In contrast to pterostilbene and 3′-hydroxypterostilbene, these data suggest that *trans*-resveratrol and piceatannol could activate apoptosis, mainly by inducing the formation of a death-inducing signalling complex. However, both *trans*-resveratrol and piceatannol caused a disruption of the mitochondrial membrane potential, and apoptosis activated by these compounds was partially inhibited by the caspase-9-inhibitor Z-LEHD-fmk. This suggests that the mitochondrial pathway could also be implicated in apoptosis, induced by *trans*-resveratrol and piceatannol.

In conclusion, the data presented in this study indicate that pterostilbene and its natural 3′-hydroxy derivative both possess interesting antileukemic properties and they may constitute effective and powerful drugs in MDR and apoptosis resistant hematological malgnancies. Although 3′-hydroxypterostilbene does not selectively act on cells expressing Bcr-Abl (a property possessed by imatinib), the high activity shown by 3′-hydroxypterostilbene on Bcr-Abl-expressing leukemia cells suggests that this compound may be useful in treating Bcr-Abl-expressing chronic and acute leukemias, and in some Bcr-Abl leukemias which are resistant to imatinib.

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