Stilbenes as Multidrug Resistance Modulators and Apoptosis Inducers in Human Adenocarcinoma Cells

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Abstract. *Background: The search for promising modulators of cancer multidrug resistance (MDR) that are able to reduce the activity of P-glycoprotein, thus restoring the cytotoxicity of anticancer drugs, is ongoing. The identification of compounds that overcome the apoptosis deficiency that frequently accompanies MDR is also of great therapeutic importance. Materials and Methods: Four stilbenes, resveratrol, piceatannol and its two derivatives, were tested for their MDR-modulating and apoptosis-inducing activity in drug-sensitive (LoVo) and doxorubicin-resistant human adenocarcinoma cell line (LoVo/Dx) by means of flow cytometry and fluorescence microscopy. Results: Trans-3,5,3',4'-tetramethoxystilbene (PicMet) was identified as a promising modulator that efficiently increased accumulation of both rhodamine 123 and doxorubicin inside resistant cells. It also increased sensitivity of LoVo/Dx cells to doxorubicin. Resveratrol and trans-3,5,3',4'-tetracetoxystilbene (PicAcet) were identified as apoptosis inducers in LoVo/Dx cells. Conclusion: The stilbene structure may constitute a promising chemical scaffold for the synthesis of potential MDR modulators.*

The intrinsic or chemotherapy-induced resistance of cancer cells to anticancer agents, multidrug resistance (MDR), is one of the most serious causes of frequent therapy failure seen in neoplastic diseases. The mechanisms of MDR are diverse, most commonly resulting from the overexpression of P-glycoprotein (P-gp) or other multidrug transporters, as well as from impaired response of resistant cells to molecular signals,

leading to apoptosis. Multidrug transporters, such as P-gp, multidrug-resistance associated proteins (MRPs) and breast cancer resistance protein (BCRP) are transmembrane proteins of vast substrate specificity, acting as ATP-fueled efflux pumps for anticancer drugs (1, 2). The initial idea that simultaneous application of an anticancer drug together with a substance reducing MDR (an MDR modulator) might potentially be beneficial to patients provided the rationale for the ongoing search for MDR-modulating compounds. Despite concentrated efforts and the investigation of thousands of compounds, none of them has found its way to the clinical practice to date (3). Therefore, it is of utmost importance to continue the search in order to discover new useful modulators. Additionally, the ability of the modulators to influence different steps of the apoptosis process may be of therapeutic importance because impaired response to pro-apoptotic signals is often a characteristic of resistant cancer cells (4, 5).

The stilbenes resveratrol (*trans*-3,5,4'-trihydroxystilbene) and piceatannol (*trans*-3,5,3',4'-tetrahydroxystilbene) are plant-derived compounds that display a wide range of biological activities. Resveratrol has been reported to be an anti-inflammatory, neuroprotective, chemopreventive and pro-apoptotic agent (6-8). Moreover, it prevents platelet aggregation, affects tumor initiation, promotion and progression, inhibits angiogenesis and regulates cell cycle and gene expression. Biological activities of piceatannol are similar, including pro-apoptotic, anti-inflammatory, anti-oxidant and anti-cancer activities (9). Previous studies have investigated the interaction of resveratrol and piceatannol with model membranes, demonstrating their preferential interaction with headgroup region of lipid bilayer (10). It has also been shown that piceatannol and its two derivatives, PicMet (*trans*-3,5,3',4'-tetramethoxystilbene) and PicAcet (*trans*-3,5,3',4'-tetracetoxystilbene), inhibit multidrug transporter MRP1 in human erythrocytes (11). Additionally, PicMet has been identified as an inhibitor of voltage-gated potassium channels Kv1.3 (12). The present study tested

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resveratrol, piceatannol, PicMet and PicAcet (for structures see Figure 1) as MDR modulators and inducers of apoptosis in doxorubicin-resistant, P-glycoprotein-expressing, human adenocarcinoma cells, LoVo/Dx.

Materials and Methods

Materials. Resveratrol (*trans*-3,5,4'-trihydroxystilbene) and piceatannol (*trans*-3,5,3',4'-tetrahydroxystilbene) were purchased from Sigma (Poznan, Poland). The derivatives *trans*-3,5,3',4'-tetramethoxystilbene (PicMet) and *trans*-3,5,3',4'-tetracetoxystilbene (PicAcet) were prepared through methylation and acetylation reactions of piceatannol, which was isolated from *Euphorbia lagascae* seeds, as described previously (11,13). Doxorubicin, rhodamine 123, camptothecin, propidium iodide (PI) and sulforhodamine B (SRB) were the products of Sigma (Poznan, Poland). Annexin-V, AlexaFluor® 488 conjugate was obtained from Molecular Probes (Eugene, OR, USA). Other reagents used were of analytical grade. Rhodamine 123 and doxorubicin were dissolved in water. All other compounds were dissolved in dimethyl sulfoxide (DMSO).

Cell culture. Human colorectal adenocarcinoma cell line, LoVo, and its resistant subline, LoVo/Dx, obtained by prolonged exposure to doxorubicin (14), were cultivated in Ham's F12 medium (Cytogen, Lodz, Poland), supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA), L-glutamine and antibiotics at 37°C and 5% CO_2. Doxorubicin (at 100 ng/ml concentration) was constantly present in the culture medium of the LoVo/Dx cells. The drug was withdrawn a week before experiments. Cells were detached from the culture flasks by treatment with Non-enzymatic Cell Dissociation Solution (Sigma, Poznan, Poland).

Cell viability assay. Cells were seeded (30,000 cells/well) onto 96-well plates in 75 μl of medium and allowed to attach (60 minutes, 37°C). Then, 75 μl of medium containing an appropriate concentration of the studied compounds was added and the cells were incubated for another 72 hours. The SRB assay was performed according to the method of Skehan *et al*. (15). Absorbance at 492 nm was read and the percentage of cell survival was calculated as: (A_{492} of treated cells/A_{492} of control cells) ×100%. The control cells were treated with pure F12 medium only. The influence of DMSO (maximal concentration in samples 0.3%) on the cells was also monitored. Experiments were performed in triplicate.

Apoptosis induction by modulators. Cells were seeded (700,000 cells/well) onto 6-well plates and cultivated for 24 h. Next 100 μM of the modulator was added and the cells were grown for a further 48 h. Apoptosis inducer camptothecin was used as a positive control. For analysis, 100,000 cells were collected, washed and incubated with Annexin-V, AlexaFluor® conjugate (15 minutes, room temperature, buffer: 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Propidium iodide (final concentration 7.5 nM) was added to the cells two minutes before measurement. The fluorescence of the cell population was measured by flow cytometry using a Beckton Dickinson (Sunnyvale, CA, USA) FACSCalibur instrument equipped with 488 nm argon laser. Fluorescence was recorded with a 530/30 nm bandpass filter for AlexaFluor® 488 and 585/42 nm bandpass filter for PI. A total of 5,000 events were registered and subsequently analyzed by quadrant statistics with the

use of Cell Quest® software (Beckton Dickinson) for the presence of viable (annexin-V- and PI-negative), apoptotic (annexin-V-positive and PI-negative) and necrotic (annexin-V- and PI-positive) cells.

Accumulation of rhodamine 123 by cancer cells. LoVo or LoVo/Dx cells (300,000 cells/ml in serum- and phenol red-free medium) were incubated with the appropriate concentration of the modulators (15 mins, room temperature). Next, rhodamine 123 was added (final concentration 2 μM) and the incubation was continued for 60 mins at 37°C. Then cells were washed and resuspended in PBS for analysis. The fluorescence of the cell population was measured by flow cytometry using a Beckton Dickinson FACSCalibur instrument equipped with a 488 nm argon laser. Fluorescence was recorded *via* a 530/30 nm band pass filter. A total of 5,000 events were registered and analyzed with the use of Cell Quest® software (Beckton Dickinson). The influence of DMSO (maximal concentration in samples 0.8%) on the cells was also monitored. Fluorescence intensity ratio (FIR) was calculated from the following equation on the basis of measured fluorescence values (FL).

$$FIR = \frac{(FL_{LoVoDx\ treated})/(FL_{LoVoDx\ control})}{(FL_{LoVo\ treated})/(FL_{LoVo\ control})}$$

Control samples were treated with medium only (no modulator).

Doxorubicin localization in cancer cells. The cells were seeded (15,000 cells/well) onto an 8-well μ-Slide microscopy chamber (Ibidi, Munich, Germany). After 48 h a fresh volume of F12 medium was added containing 50 μM of doxorubicin (plus 100 μM of the modulator in treated samples) and cells were incubated for 60 mins at 37°C. Cells were washed with PBS, and then with serum- and phenol red-free F12 medium. Images were collected with a Nikon Eclipse TE2000-E microscope (Nikon Instruments, Amstelveen, the Netherlands), equipped with a PlanFluor 40× (0.60) objective. Fluorescence was excited in a range 528-553 nm, and collected in a range 578-633 nm.

Results

Assessment of cytotoxicity in human adenocarcinoma cells was the first step of the study on biological activity of resveratrol, piceatannol and its two newly-synthesized derivatives, PicMet and PicAcet. The results are presented in Figure 2A and 2B for the drug-sensitive LoVo and the doxorubicin-resistant LoVo/Dx cells, respectively. Resveratrol, followed by PicMet, were the most cytotoxic compounds. Only approximately 35% of cells survived 72 h-incubation with 100 μM of either resveratrol or PicMet and this number was almost the same for LoVo and LoVo/Dx sublines. On the other hand, piceatannol and PicAcet were more cytotoxic to sensitive LoVo cells than to resistant LoVo/Dx cells. At the highest concentration tested, piceatannol killed approximately 40% of LoVo cells and only 20% of LoVo/Dx cells. Cytotoxicity of PicAcet to LoVo cells was very similar (*c.a.* 40% of cells killed), whereas this compound was non-toxic to LoVo/Dx cells.
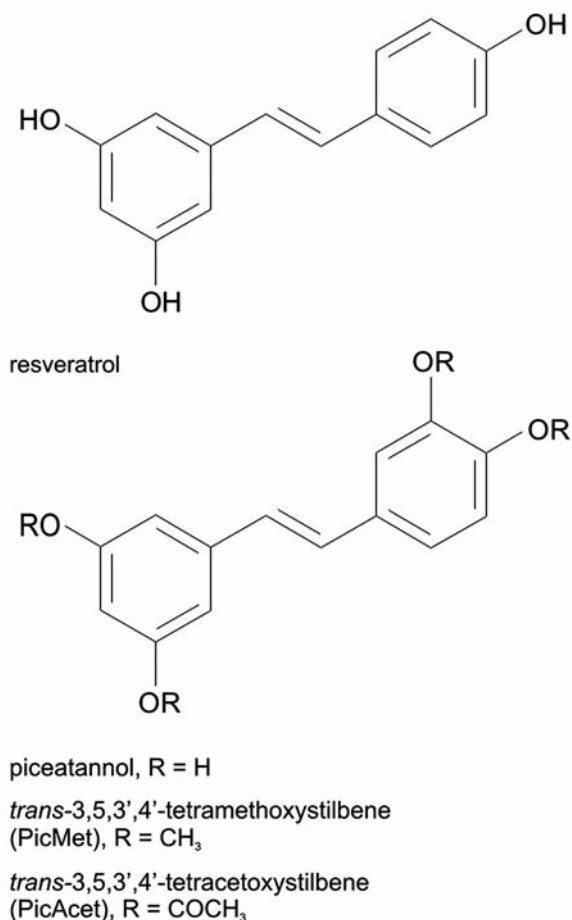
Figure 1. *Chemical structures of stilbenes.*

resveratrol

piceatannol, R = H

trans-3,5,3',4'-tetramethoxystilbene
(PicMet), R = CH₃

trans-3,5,3',4'-tetracetoxystilbene
(PicAcet), R = COCH₃

Figure 2. *Cytotoxicity of resveratrol (■), piceatannol (●), PicMet (○) and PicAcet (□) in LoVo (A) and Lovo/Dx (B) cells. Means of three experiments are presented.*

Additionally, the apoptosis-inducing activity of the stilbenes was studied on doxorubicin-resistant and drug-sensitive human adenocarcinoma cells by flow cytometry. Double staining with annexin-V, AlexaFluor® conjugate and propidium iodide was employed to detect phosphatidylserine exposure on the cell surface, *i.e.* the loss of membrane asymmetry that is a characteristic event during early apoptosis. Cell population was analyzed and the percentages of viable (annexin-V- and PI-negative), apoptotic (annexin-V- positive and PI-negative) and necrotic (annexin-V- and PI-positive) cells was calculated (Table I). In LoVo cells, all studied compounds, apart from piceatannol, induced apoptosis. In resistant cells, two of the studied stilbenes, PicAcet and resveratrol, were moderately active, whereas apoptosis-inducing activity of piceatannol and PicMet was negligible.

A flow cytometric assay based on rhodamine 123 accumulation by cancer cells was used to investigate MDR reversal activity of the studied stilbenes (Table I). 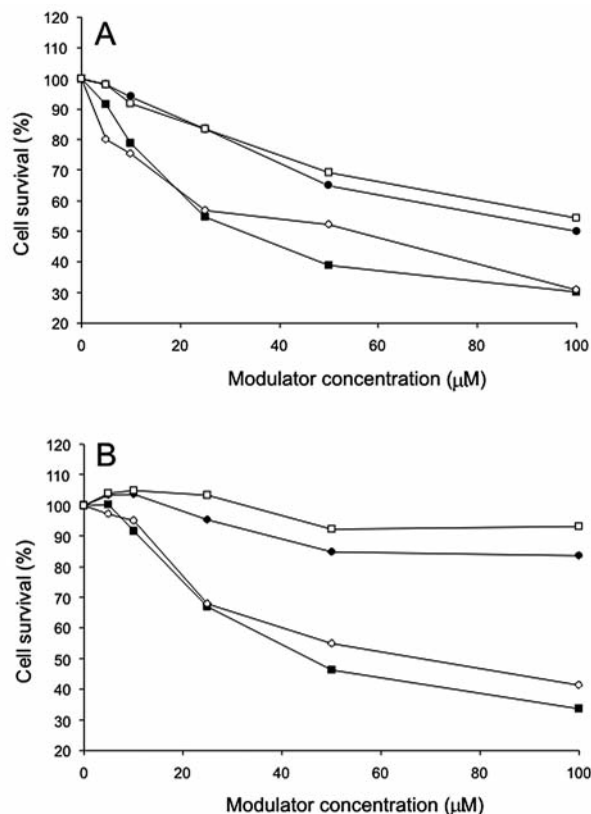When no modulator was applied the drug-sensitive LoVo cells accumulated significantly more fluorescent probe, rhodamine 123, than the resistant LoVo/Dx cells (typically mean fluorescence intensity of LoVo cells population was 6-7 times higher than for LoVo/Dx cells). PicMet was found to be the most potent MDR modulator among the studied compounds, whereas the activity of piceatannol and resveratrol was very weak. Interestingly, FIR values obtained for PicAcet were lower than 1. This resulted from stronger influence of PicAcet on rhodamine 123 accumulation by LoVo cells than by LoVo/Dx cells.

Intracellular accumulation of the anticancer drug doxorubicin was studied by fluorescence microscopy. As shown in Figure 3A and 3B, doxorubicin accumulated preferentially inside drug-sensitive LoVo cells and localized mainly inside nuclei of the cells. Resistant LoVo/Dx cells accumulated much less of the drug, as proven by very weak fluorescent signals observed inside them. Treatment of adenocarcinoma cells with 100 μM of PicMet, the strongest MDR modulator detected in the previous experiments, seriously affected doxorubicin localization pattern observed in both sublines (Figure 3C and 3D for LoVo and LoVo/Dx,

Table I. *Effect of stilbenes on the reversal of multidrug resistance in LoVo/Dx cells and apoptosis induction in LoVo/Dx and LoVo cells.*

Compound	Concentration (μM)	FIR	LoVo/Dx		LoVo	
			Apoptosis (%)	Necrosis (%)	Apoptosis (%)	Necrosis (%)
Control annexin-V+PI+			5.95	9.69	6.65	9.31
Camptothecin	15		17.56	17.96	17.00	28.31
Resveratrol	10	1.430				
	50	1.079				
	100	1.157	12.36	20.80	16.65	19.63
Piceatannol	10	1.178				
	50	1.123				
	100	1.503	9.85	8.81	7.96	14.61
PicMet	10	1.319				
	50	1.622				
	100	4.828	9.31	19.04	18.99	24.98
PicAcet	10	0.858				
	50	0.545				
	100	0.476	12.65	11.98	11.30	15.28

respectively). The change was most dramatic in case of LoVo/Dx cells in which a significant increase in intracellular fluorescence intensity was recorded, whereas only slight enhancement of the signal was seen in LoVo cells.

The influence of PicMet on cytotoxicity of doxorubicin to adenocarcinoma cells was also studied. As can be seen in Figure 4 (●) LoVo cells were several times more sensitive to this anticancer drug than LoVo/Dx cells. Next, it was checked if PicMet at 5 μM concentration would be able to increase drug sensitivity of LoVo/Dx cells. PicMet at this concentration was previously found to be relatively non-toxic to both cell lines (Figure 2). The results of the experiment showed that in the presence of PicMet, the cytotoxicity of doxorubicin to adenocarcinoma cells was increased, and this effect was much more pronounced in case of resistant LoVo/Dx cells (Figure 4, ○). It can therefore be concluded that PicMet was an effective MDR modulator in resistant human adenocarcinoma cells when used at concentration as low as 5 μM.

Discussion

Human colorectal adenocarcinoma cell line LoVo and its doxorubicin resistant subline LoVo/Dx were chosen as a model system for the study of putative MDR-modulating and apoptosis-inducing activity of the four stilbene derivatives. The expression of five main MDR-associated transporters (P-gp, MRP1, BCRP, MDR3 protein, and lung resistance-related protein (LRP)) in these cells has been studied previously both on mRNA and protein level (16). In comparison with LoVo cells, the resistant LoVo/Dx cells were characterized by the elevated expression of P-gp,

MDR3 and to a weaker extent MRP1. However, the weak expression of the above mentioned proteins was also detected in the sensitive cells.

Resveratrol and PicMet were found to be more cytotoxic to sensitive and resistant human adenocarcinoma cells than piceatannol and PicAcet. This was in accordance with the predicted octanol:water partition coefficient (logP) values of the studied stilbenes (10, 11) as more hydrophobic compounds were more toxic to the cells. Interestingly, the toxicity of resveratrol and PicMet was similar to both LoVo and LoVo/Dx cells, while piceatannol and PicAcet were much more toxic to the sensitive cells. This might suggest that resistant LoVo/Dx cells possessed some detoxification mechanisms for both latter compounds but not for resveratrol and PicMet. The introduction of one or more methoxy groups into the stilbene structure was previously observed to increase the cytotoxicity of stilbene derivatives. For example, resveratrol derivatives *trans*-3,5-dimethoxy-4'-hydroxystilbene was found to be more toxic to mouse macrophages than its parent compound (17), and *trans*-3,4',5-trimethoxystilbene was more toxic than resveratrol to both sensitive and resistant breast cancer cells (18), colon (19) and prostate cancer cells (20). Analogously, PicMet was significantly more toxic to human adenocarcinoma cells than piceatannol.

The assay, based on differential staining of viable, apoptotic and necrotic cells by AlexaFluor®-annexin V and PI was used to study the apoptosis-inducing activity of the studied stilbenes in human adenocarcinoma cells. Annexin V binds specifically to phosphatidylserine that is exposed on the surfaces of the apoptotic cells, while PI stains only necrotic cells *i.e.* those with perturbed membrane integrity. In sensitive LoVo cells all stilbenes, with the exception of
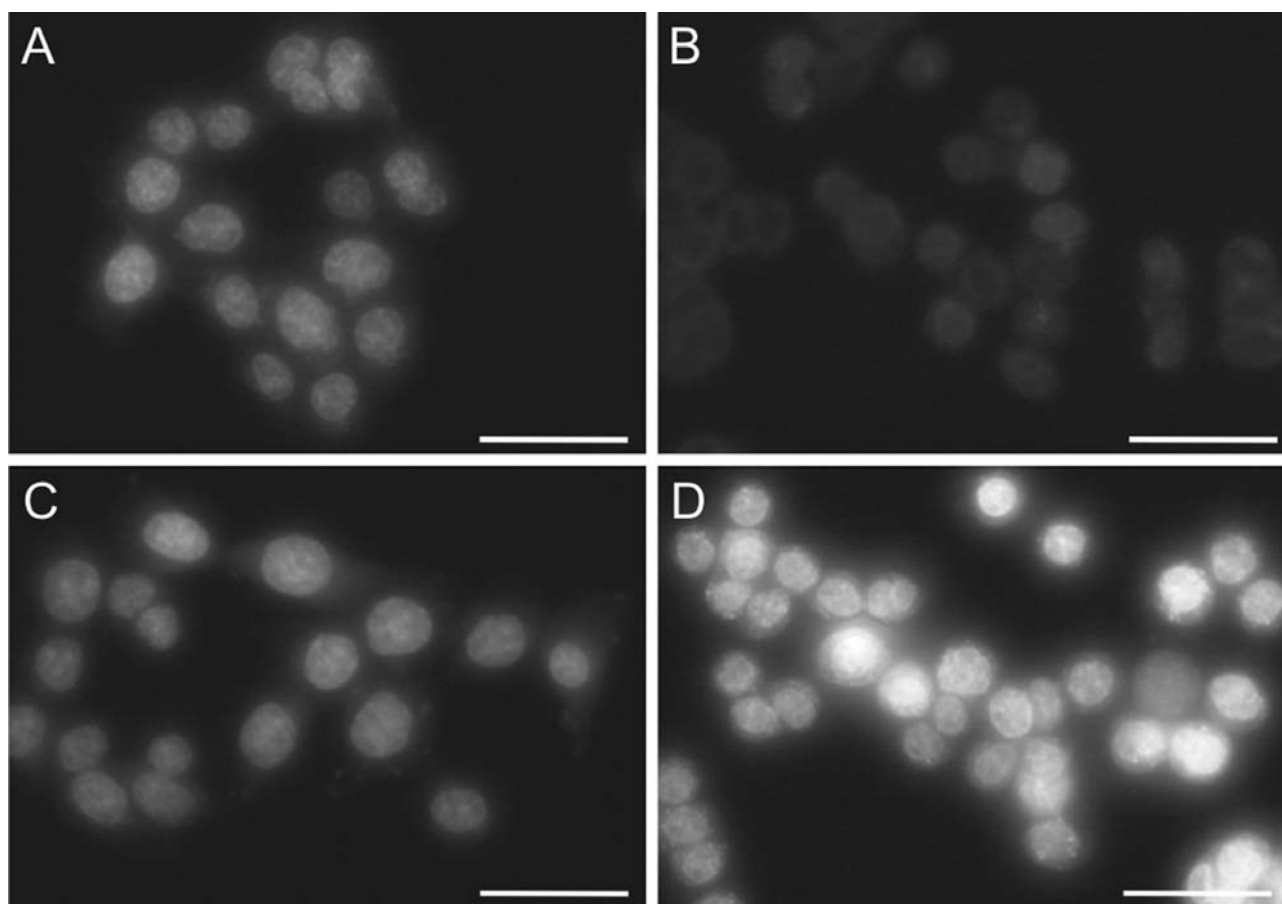
Figure 3. *LoVo (A, C) and LoVo/Dx (B, D) cells treated with (50μm) doxorubicin alone (A, B) and with 100 μM of PicMet (C, D). Scale bar is 50 μm.*

piceatannol, induced apoptosis. In doxorubicin-resistant LoVo/Dx cells, only PicAcet and resveratrol were found to be moderately active apoptosis inducers, while the activity of piceatannol and PicMet was negligible. Both resveratrol (21-23) and piceatannol (24, 25) have previously been shown to be potent apoptosis inducers in a variety of cancer cell lines. The ability to potentiate apoptosis induced by anticancer drugs has also been observed for resveratrol (26, 27). Methoxylated resveratrol derivatives have been demonstrated to induce apoptosis in sensitive (19, 28) and resistant cancer cell lines (13, 29). The activity of the stilbenes differed in various cell lines, however their apoptosis-inducing activity was usually higher in sensitive cell lines than in respective resistant sublines (29). This was also true for the pair LoVo and LoVo/Dx studied in the present work as the stilbenes were slightly stronger apoptosis inducers in sensitive than in resistant adenocarcinoma cells.

The ability of the stilbene derivatives to inhibit P-glycoprotein was also investigated. As has previously been

shown, the rhodamine 123 accumulation assay was indicative of P-gp transport activity, although this was not the only MDR-related transporter expressed in the studied cell lines (16). PicMet was identified as the most potent P-gp inhibitor, piceatannol and resveratrol were weak inhibitors, and PicAcet was not active in this respect. Additionally, the treatment of LoVo/Dx cells with PicMet caused a dramatic increase of doxorubicin accumulation inside the resistant cells as observed by fluorescence microscopy. PicMet was also found to be an effective MDR modulator since it increased doxorubicin cytotoxicity to resistant LoVo/Dx cells. It should be noted that PicMet was the most hydrophobic of the set of stilbenes studied in the present work (see (10, 11) for logP values). PicMet was previously shown to strongly reduce P-gp activity in mouse lymphoma cells and increase their sensitivity to doxorubicin, however piceatannol was found to be inactive in this cell line (13). Resveratrol has been identified as a moderately active P-gp inhibitor in multidrug-resistant human epidermoid carcinoma KB-C2 cells (30). Furthermore, it has also been reported to
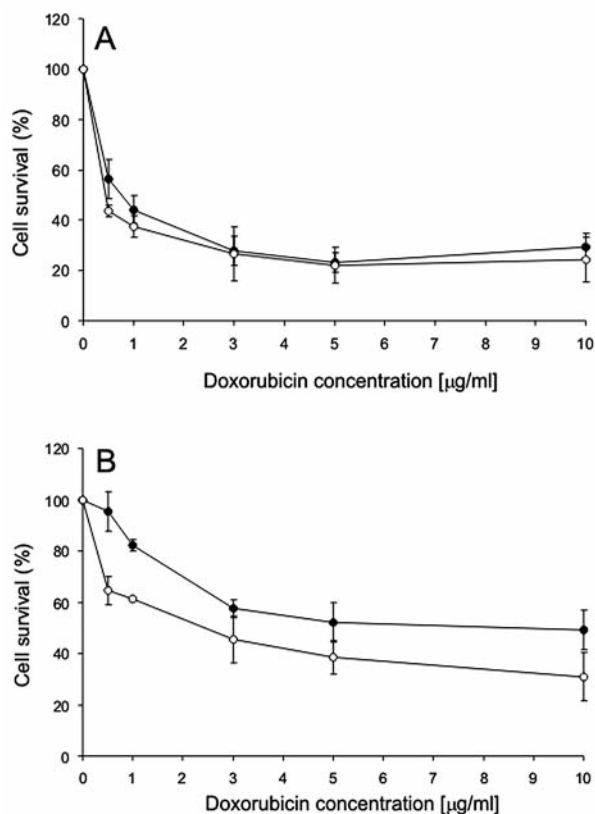
Figure 4. *Influence of PicMet on doxorubicin cytotoxicity in LoVo (A) and LoVo/Dx (B) cells. Closed symbols represent doxorubicin alone, and open symbols represent doxorubicin plus 5 μM PicMet. Means ± S.D. of three experiments are presented.*

interact with BCRP (31). In spite of its relatively weak inhibitory potency towards P-gp, resveratrol has been observed to be an effective MDR modulator in vincristine-resistant epidermoid carcinoma cell line, sensitizing the resistant cells to such anticancer drugs as vincristine, paclitaxel and doxorubicin (27). However, this activity of resveratrol was attributed rather to the ability of this compound to potentiate drug-induced apoptosis than its ability to modulate MDR-related transporter activity.

In conclusion, the above results confirm that the stilbene structure may constitute a promising chemical scaffold for the synthesis of potential MDR modulators. In the present study, PicMet was identified as a potent P-glycoprotein inhibitor, able to influence accumulation of doxorubicin inside resistant human adenocarcinoma cells and to modulate MDR in these cells.

Acknowledgements

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