Invest New Drugs (2011) 29:98–109
DOI 10.1007/s10637-009-9340-7

PRECLINICAL STUDIES

Inhibition of MDR1 activity and induction of apoptosis by analogues of nifedipine and diltiazem: an in vitro analysis

Maurizio Viale · Cinzia Cordazzo · Daniela de Totero · Roberta Budriesi · Camillo Rosano · Alberto Leoni · Pierfranco Ioan · Cinzia Aiello · Michela Croce · Aldo Andreani · Mirella Rambaldi · Patrizia Russo · Alberto Chiarini · Domenico Spinelli

Received: 3 September 2009 / Accepted: 28 September 2009 / Published online: 30 October 2009

Summary We report herein the reversal of multidrug resistance-1 (MDR1) in A2780/DX3 cells by the two nifedipine-like compounds **1** and **2** that are part of a library of 1,4-dihydropyridines (1,4-DHPs) calcium-channel modulators bearing in C-4 a different substituted imidazo[2,1-*b*] thiazole system. By methylthiazol tetrazolium (MTT) assay, cytofluorimetry, and fluorescence microscopy we evaluated their ability to reverse MDR in our cell system. Moreover, together with compound **3** (the diltiazem-like 8-(4-chlorophenyl)-5-methyl-8-[(2*Z*)-pent-2-en-1-yloxy]-8*H*-[1,2,4] oxadiazolo[3,4-*c*][1,4]thiazin-3-one) we analyzed their ability to potentiate the triggering of apoptosis after exposure to doxorubicin, through the nuclear morphological analysis after 4′,6-diamidino-2-phenylindole (DAPI), the fluorescein isothiocyanate (FITC)-Annexin-V/propidium iodide (PI) staining and the caspase activity determination. Our results demonstrate that compounds **1** and **2,** at concentrations showing a very low (5%) or absent inhibition of cell proliferation, in combination with doxorubicin enhance its antiproliferative activity (from 30% to 54% IC_{50} reduction) in A2780/DX3 cells through an increase of doxorubicin intracellular accumulation. These compounds together with compound **3,** which has already been demonstrated to act as a potent inhibitor of MDR1 function, were also able to significantly potentiate the activation of the apoptosis machinery triggered by the exposure to doxorubicin. In conclusion, our results identify two new molecules structurally related to the calcium-channel blocker nifedipine, but characterized by a very low LTCC blockers activity, able to potentiate the antiproliferative and apoptotic activities of doxorubicin through an increase of its intracellular concentration likely caused by the inhibition of MDR1 function.

M. Viale (✉) · D. de Totero · C. Aiello · M. Croce
Istituto Nazionale per la Ricerca sul Cancro,
S.C. Terapia Immunologica,
L.go R. Benzi 10,
16132 Genova, Italy
e-mail: maurizio.viale@istge.it

C. Cordazzo
Dipartimento di Chimica Organica "A. Mangini",
Università degli Studi di Bologna,
Via San Giacomo 11,
40126 Bologna, Italy

R. Budriesi · A. Leoni · P. Ioan · A. Andreani · M. Rambaldi · A. Chiarini
Dipartimento di Scienze Farmaceutiche,
Università degli Studi di Bologna,
Via Belmeloro 6,
40126 Bologna, Italy

C. Rosano
Istituto Nazionale per la Ricerca sul Cancro,
S.C. Nanobiotecnologie,
L.go R. Benzi 10,
16132 Genova, Italy

P. Russo
Istituto Nazionale per la Ricerca sul Cancro,
S.S. Tumori Polmonari,
L.go R. Benzi 10,
16132 Genova, Italy

D. Spinelli (✉)
Dipartimento di Chimica "G. Ciamician",
Università degli Studi di Bologna,
Via Selmi 2,
40126 Bologna, Italy

Keywords Antitumor agents · Apoptosis · 1,4-dihydropyridines · Multidrug resistance · Pgp-170

Introduction

The resistance of many tumors to anticancer drugs has been frequently linked to the over-expression of the MDR1 gene [1–3]. This gene encodes for the ATP-dependent transporter P-glycoprotein-170 (Pgp-170) that is involved in pumping out of the cells a wide variety of neutral to slightly cationic hydrophobic xenobiotics [4, 5]. In particular, Pgp-170 is present in organs such as the placenta and the blood-brain barrier where it pumps out toxic exogenous compounds thus protecting, respectively, foetus and brain from toxic damages [6–8].

Many tumors of epithelial origin display a high cell-membrane concentration of Pgp-170 that may be involved in some forms of intrinsic resistance. On the other hand, different anticancer drugs having unrelated structural characteristics and mechanisms of action may actively select cells with high MDR1 copy number thus causing the onset of an acquired drug resistance characterized by the unresponsiveness to anthracyclines, taxanes, *Vinca* alkaloids, podophyllotoxins, or camptothecins [9, 10].

Many ways to overcome MDR have been pursued in the two last decades, in particular the inhibition of Pgp-170 activity has been considered as a central field of research and many novel compounds have been studied for their ability to block reversibly or irreversibly the pump function of Pgp-170 [11]. The modified derivatives of the L-type calcium channel (LTCC) blockers like nifedipine or diltiazem, represent a group of compounds endowed with significant features in terms of inhibition of the Pgp-170 activity. Nevertheless until now such compounds [12, 13] displayed scarce efficacy in clinical tests due to their low binding affinity, at doses necessary to enable the inhibition of Pgp-170, while causing the onset of toxic phenomena at higher doses due to the inhibition of metabolism and excretion of anticancer drugs [14, 15].

In particular, dihydropyridine calcium antagonists such as nifedipine, amlodipine, nimodipine, and others, are commonly utilized in adults and pediatric patients as antihypertensive drugs, having also shown in some cases interesting properties as fibrinolytic agents [16, 17]. Among these, nifedipine has been tested at different doses in clinical trials as an MDR reversing agent without reaching objective responses and observing the expected dose-limiting toxicity due to the cardiovascular activity of this Ca^{++} antagonist [18, 19]. About the other compounds, in general, they have not reached the experimentation in clinical trials. Presently only amlodipine, associated and delivered together topotecan by stealthy liposomes [20, 21] seems to possess interesting characteristics in preclinical studies, thus having some objective chances to overcome the MDR phenomena. Nevertheless, also in this case it is not possible to foresee the possible toxicity of this in humans, since this drug is also a potent Ca^{++} antagonist and might therefore display potential cardiovascular dose-limiting toxicities.

In this context some time ago we started a series of experiments to study the ability of different diltiazem-like compounds, some of which have already demonstrated good cardiodepressant activities [22, 23], to compete for excretion with the typical target of Pgp-170 activity, doxorubicin, causing its accumulation into multidrug-resistant A2780/DX3 cells [24].

Here we are analyzing the reversal of MDR1 activity by two nifedipine-like compounds **1** and **2** (Fig. 1), which are again related to LTCC blockers, but characterized by a very low LTCC blockers activity (see after) and then would not show negative cardiovascular effects during the administration. These two substances are a part of a small library of 1,4-DHPs and were synthesized and tested for their cardiovascular profile on isolated guinea pig tissues [25]. All of the 33 examined compounds contained a dimethyl 2,6-dimethyl-4-[imidazo[2,1-*b*][1,3]thiazol-5-yl]-1,4-dihydropyridine-3,5-dicarboxylate scaffold possibly methylated at C-4 and/or C-5 of the thiazole ring as well variously decorated with halogens, alkyls, trifluoromethyl group, and differently substituted aryl groups at C-6 of the imidazole ring.

On going from the previously examined diltiazem-like compounds [24] to **1** and **2** we are again taking into consideration molecules related to LTCC blockers, but we are shifting towards different chemical systems showing a completely different chemical structure with respect to the

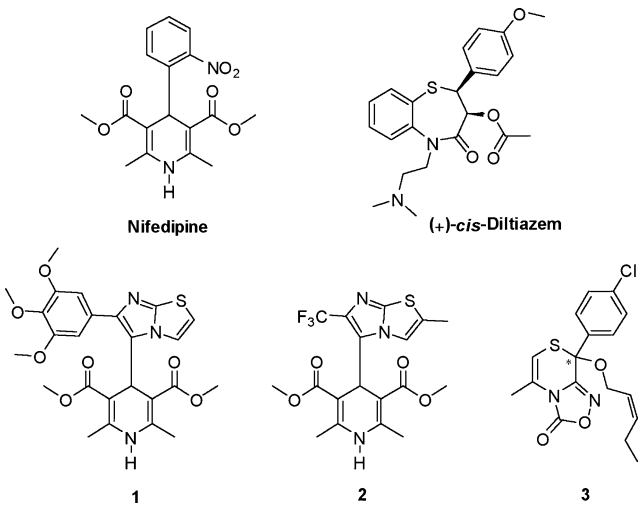

Fig. 1 Chemical structures of nifedipine, diltiazem, nifedipine- and diltiazem-like compounds **1**, **2**, and **3**

series of the diltiazem-like ones. As a matter of fact, we are moving from 8-aryl-8-hydroxy-5-R'-8*H*-[1,4]thiazino [3,4-*c*]-[1,2,4]oxadiazol-3-ones and/or their acetals [24] towards dimethyl 2,6-dimethyl-4-[imidazo[2,1-*b*][1,3] thiazol-5-yl]-1,4-dihydropyridine-3,5-dicarboxylate opportunely substituted.

Our aim was the identification of new molecules able to inhibit, if only partially, the Pgp-170 activity because of their binding affinity to the interaction site. The new examined molecules, for example, show a larger hydrophilicity and contain atoms (such as the pyridine-nitrogen) able to give interactions completely different with respect to the compounds previously examined [24].

In general, compounds **1** and **2** showed a marked selectivity for the cardiac over the vascular tissue. As a matter of fact, none of them possesses a vasorelaxant activity on the K^+-depolarized aortic strips greater than 50%, whereas they showed a relaxation activity on K^+-depolarized guinea pig ileum longitudinal smooth muscle between 51 and 97% [25]. In particular, our compounds **1** and **2** displayed a very low intrinsic activity on all examined parameters of cardiac functionality, therefore we selected them as possible interesting candidates to analyze the results of their interaction with doxorubicin on the Pgp-170 membrane transporter in doxorubicin sensitive A2780 and MDR1 resistant A2780/DX3 cells.

In addition, we analyzed the ability of the two nifedipine-like compounds to increase the doxorubicin-driven apoptosis in MDR1 resistant A2780/DX3 cells. The potentiation of doxorubicin-driven apoptosis was also tested with the diltiazem-like compound **3** [23] (Fig. 1) that in our previous work [24] demonstrated a high potency in terms of inhibition of the MDR1 activity.

Materials and methods

Chemicals and cells

The clinical form of doxorubicin from Ebewe Italia (Rome, Italy) was used for experiments and diluted in normal saline to the opportune concentrations. All of the examined compounds were firstly dissolved in 100% dimethylsulfoxide (DMSO) and then diluted in fetal calf serum (final concentration DMSO 2%). Nifedipine (purity≥98%, Sigma, St. Louis, MO, USA) was similarly diluted.

Sensitive to doxorubicin A2780 cells were maintained in culture in RPMI 1640 medium in the presence of 10% fetal calf serum, 1% glutamine, and 1% penicillin-streptomycin (complete medium), while resistant to doxorubicin A2780/DX3 cells (provided by Dr. YM Rustum and obtained by exposure to increasing concentrations of doxorubicin) were maintained in complete medium containing 100 nM doxorubicin. Two-three days before experiments doxorubicin was removed from the medium.

Chemistry

The melting points are uncorrected. Analyses (C, H, N) were within 0.4% of the theoretical values. TLC was performed on Bakerflex plates (Silica gel IB2-F); the eluent was a mixture of petroleum ether 60–80°C/acetone in various proportions. Kieselgel 60 (VWR, Milano, Italy) was used for flash chromatography. The IR spectra were recorded in nujol on a Nicolet Avatar 320 E.S.P.; ν_{max} is expressed in cm^{-1}. The ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini (300 MHz); the chemical shift (referenced to solvent signal) is expressed in δ (ppm) and *J* in Hz. Abbreviations: th = thiazole, ar = aromatic, py = pyridine, ex = H linked to N which exchanged with D_2O. ESI-MS were registered on a micromass ZMD Waters instrument (30 V, 3.2 kV). HRMS were recorded on a Thermo Finnigan Mat95XP apparatus. The purity degree of the tested compounds (**1**–**3**) was ≥97%.

Synthesis of dimethyl 2,6-dimethyl-4-[6-(3,4,5-trimethoxyphenyl)imidazo[2,1-b][1,3]thiazol-5-yl]-1,4-dihydropyridine-3,5-dicarboxylate **1** and of dimethyl 2,6-dimethyl-4-[2-methyl-6-trifluoromethylimidazo[2,1-b][1,3]thiazol-5-yl]-1,4-dihydropyridine-3,5-dicarboxylate **2**

The compounds **1** and **2** were synthesized by means of the versatile reaction developed by Hantzsch [26].

Methylacetoacetate (2 mM) and 30% NH_4OH (4 mM) were added to a stirred solution of 6-(3,4,5-trimethoxyphenyl) imidazo[2,1-*b*]thiazole-5-carbaldehyde [25] or of 2-methyl-6-trifluoromethylimidazo[2,1-*b*]thiazole-5-carbaldehyde [27] (1 mM) dissolved in isopropyl alcohol (50 ml). The reaction mixture was refluxed for 1–4 days (according to a TLC test acetone/petroleum ether 55–85°C, 1:9 v/v, 2:8 v/v) and added of methylacetoacetate (4 mM) and 30% NH_4OH (2 mM) every 12 h. After cooling, the mixture was evaporated to dryness under reduced pressure. The derivatives were purified by flash chromatography, eluent acetone/petroleum ether, 40–60°C from 1.9 to 4.6 (v/v) with a yield of 10% for **1** and of 18% for **2**.

Data for **1** $C_{25}H_{27}N_3O_7S$ MW 513.567, mp 194–196°C, IR: 3400–3100, 1671, 1250, 1206, 1015. ^1H NMR ([D_6]DMSO): 2.18 (6H, s, CH_3), 3.24 (6H, s, $COOCH_3$), 3.70 (3H, s, OCH_3), 3.81 (6H, s, OCH_3), 5.73 (1H, s, py), 7.18 (2H, s, ar), 7.23 (1H, d, th, *J*=4.5), 7.27 (1H, d, th, *J*=4.5), 8.99 (1H, s, NH, ex D_2O). ^{13}C NMR ([D_6]DMSO): 18.65, 32.21, 51.23, 56.69, 60.91, 98.94, 106.36, 113.81, 119.62, 128.60, 132.23, 137.44, 142.93, 146.15, 147.75, 153.16, 168.03. ESI-MS m/z: 514 $[M+H]^+$, 536 $[M+Na]^+$. HRMS m/z found 513.1567 (calcd. 513.1570). EI-MS m/z (%) 513

(3.2), 454 (26.70), 291 (18.73), 290 (100), 275 (96), 247 (21.38), 232 (23.64), 223 (19.67), 217 (18.93), 192 (31.51), 69 (26.17).

Data for **2** $C_{18}H_{18}F_3N_3O_4S$ MW 429,417, 270–275°C, IR: 3400–3100, 1702, 1654, 1299, 1149. ^{1}H NMR ([D_6] DMSO): 2.25 (6H, s, CH_3), 2.43 (3H, s, CH_3), 3.44 (6H, s, COO*CH_3*), 5.50 (1H, s, py), 7.21 (1H, s, th), 9.10 (1H, s, NH, ex D_2O). ^{13}C NMR ([D_6]DMSO): 14.64, 18.75, 32.34, 51.35, 98.34, 116.38, 125.72, 128.83, 132.51, 147.21, 147.35, 167.50. ESI-MS m/z: 430 $[M+H]^+$, 452 $[M+Na]^+$. HRMS m/z found 429.0971 (calcd. 429.0970). EI-MS m/z (%) 429 (31.29), 371 (19.75), 370 (100), 224 (73.61), 206 (37.30), 165 (15.47), 95 (14.02), 81 (40.04), 69 (73.89), 57 (18.21), 55 (15.62), 41 (21.89).

MTT assay

The resistant A2780/DX3 human ovarian carcinoma cell line and its sensitive counterpart A2780 were plated at opportune densities/well into 96-well microtiter plates, centrifuged for 3 min at 275 g and then incubated for 6–8 h. In order to evaluate the concentrations of nifedipine derivatives giving 5% and 0% cell growth inhibition the compounds were administered in duplicate for a minimum of 5 concentrations (3-fold serial dilutions, maximal concentration: 100 μM, maximal volume/well: 200 μl). Nifedipine was also tested as reference compound. Three days later 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (50 μl, MTT, Sigma, St. Louis, MO, USA) solution (2 mg/ml in PBS) was added to the wells and incubated at 37°C for 4 h. Microplates were then centrifuged at 275 g for 5 min, culture medium carefully aspirated and 100% DMSO (100 μl) added. Complete and homogeneous solubilization of formazan crystals was achieved after 20 min of incubation and shaking of well contents by a multichannel pipette. A microculture plate reader 400 ATC (SLT Labinstruments, Austria) was used to measure the absorbance at 540 nm [28]. The searched concentrations and, when possible, the corresponding IC_{50}s were calculated on the basis of the analysis of single dose response curves. Each experiment was repeated 3–4 times.

In a second series of experiments cells were immediately diluted in a medium containing the opportune concentrations of the various compounds (nifedipine and nifedipine-like **1** and **2**) at the concentrations calculated by the previous MTT analysis for both A2780 and A2780/DX3 cells and giving 0% and 5% inhibition of cell proliferation. The final volume was 180 μl. Plates were then centrifuged and 6 h later doxorubicin was added at the opportune concentrations for A2780 (range 1–0.0016 μM) and A2780/DX3 (range 30–0.048 μM) cells. The added serial dilutions of doxorubicin also contained concentrations of nifedipine or nifedipine-like molecules equal to that already contained in the wells. By this kind of treatment we obtained a pre-treatment of 6 h with the experimental molecules thereafter treated also with the anticancer drug doxorubicin. After 3 days the MTT assay was applied as already described.

IC_{50}s were calculated on the basis of the analysis of single dose response curves. Each experiment was repeated 4 times.

Cytofluorimetric and microscopic study of intracellular accumulation of doxorubicin

Sensitive and resistant to doxorubicin cells were plated in 25 cm^2 flasks at 1×10^6 and 1.5×10^6 cells/flask, respectively, in 10 ml. Twenty-four hours later, when cells reached about 75–85% confluence, they were treated with nifedipine or nifedipine-like compounds using concentrations 5X that giving 5% inhibition of cell proliferation, as calculated in the MTT assay. Doxorubicin was added 2 h later in a small volume (5.8 or 29 μl) to reach the final concentrations of 2 and 10 μM in A2780 and A2780/DX3 cells, respectively. Incubation was performed for additional 2 h. Cells were then detached by trypsin at 37°C for 5 min, rapidly washed twice with cold phosphate buffered saline (PBS), and fixed for 20 min with 3.7% paraformaldehyde in PBS containing 2% sucrose. Cells were again washed twice with PBS containing 2% fetal calf serum, pelleted and concentrated in the same medium. Untreated cells and control cells treated with nifedipine and nifedipine-like compounds were also assayed. The intracellular fluorescence intensity of cells was determined by flow cytometry (FACScan, BD Biosciences, Milano, Italy) using 488 nm excitation and 575 nm bandpass filter for doxorubicin detection. Values were expressed in arbitrary units as mean fluorescence intensity (MFI).

For microscopical analysis A2780 and A2780/DX3 cells were plated at 1×10^5 and 0.7×10^5 cells, respectively, in chamber slides in a final volume of 1 ml. When they reached 75–85% confluence they were treated as described for the cytofluorimetric studies with the only difference regarding doxorubicin concentration that was 10 μM for both cell lines. After being washed twice with cold PBS, cells were fixed for 20 min with 3.7% paraformaldehyde in PBS containing 2% sucrose and washed twice again in PBS containing 2% sucrose. Opportune controls (untreated cells and cells treated with nifedipine and nifedipine-like compounds) were always made. Slides were mounted with a coverslip using GelMount (Biomeda Inc., CA). Cell imaging was performed with an Axiovert 200 M epifluorescent microscope equipped with the filter set Omega XF104-2 (Brattleboro, VT USA) using the Axiovision Software (Carl Zeiss, Jena, Germany).

FITC-Annexin-V/PI assay

The induction of apoptosis was evaluated in resistant A2780/DX3 cells. Cells were plated into 75 cm^2 flasks and treated with the IC_{50} of doxorubicin and the concentrations of **1**, **2**, and **3** giving 5% inhibition of cell proliferation. After 72 h culture, floating, and adherent cells were harvested, washed twice with cold PBS containing 2% fetal calf serum and apoptosis determined by double staining with FITC-Annexin-V and PI (rh Annexin-V/FITC Kit, Bender MedSystem GmbH, Vienna, Austria). Briefly, 0.5×10^6 cells were resuspended in binding buffer 1X and stained with 5 μl of FITC-Annexin-V. After 10 min incubation 10 μl of PI were added and samples incubated for 5 min. Samples were immediately analyzed using a Flow Cytometer (FACScan, BD Biosciences, Milano, Italy) with dedicated software.

Visualization of apoptotic cells by DAPI staining

A2780/DX3 cells were plated at 4000/well into 24-well microtiter plates (flat-bottomed) and treated as described for the western blot analysis. After 72 h all cells were harvested and washed twice with cold PBS and then fixed in 70% ethanol in PBS (100 μl) and maintained at 4°C. Just before examination of fluorescence at the microscope, 5 μl of a solution of DAPI (10 μg/ml) in water were added and the percentage of apoptotic segmented nuclei/cells stained with the fluorescent dye evaluated.

Determination of caspases' activation

The activation of caspase complex was evaluated by using the CaspScreen Flow Cytometric Apoptosis Detection kit (Chemicon Int., Temecula, CA). This assay utilizes a substrate that contains two aspartate residues linked to rhodamine 110 (D2R) that become fluorescent only upon cleavage of the substrate by cellular caspases. Briefly, cells were treated as described for the western blot analysis. Once harvested and washed twice with cold PBS cells were resuspended in 0.3 ml of incubation buffer at 37°C and DTT (3 μl) plus D2R (1 μl) reagents added. After 20 min incubation in the dark samples were analysed by flow cytometry using 488 nm excitation and 530 nm bandpass filter.

Statistics

The Wilcoxon signed rank and the Mann-Whitney tests for non-parametric data were used for statistical analysis (StatView 4.5 software, Abacus Concepts Inc., Burlington, MA, USA). The resistance indexes were calculated as the ratio between the mean IC_{50} of resistant and that of sensitive wild-type cells.

Results

Nifedipine and nifedipine-like compounds and cell proliferation

The two nifedipine-like molecules **1** and **2** did not show significant inhibition of cell proliferation. For both of them the IC_{50} for A2780 and A2780/DX3 cells were much higher than 100 μM. This result is particularly significant if compared to the mean IC_{50}s calculated for doxorubicin, which were on average 0.02±0.01 and 1.51±0.16 μM for A2780 and A2780/DX3 cells (mean resistance index=75), respectively.

On the basis of the same experiments we also calculated the concentrations of our molecules giving 0% (IC_0) and 5% (IC_5) inhibition of cell proliferation (Table 1) that were then used to perform all the experiments of co-treatment with doxorubicin.

Nifedipine and nifedipine analogues increase intracellular doxorubicin accumulation

Nifedipine and compounds **1** and **2** were analyzed for their ability to counteract the efflux of doxorubicin from A2780 and A2780/DX3 cells. In our experimental conditions nifedipine caused a percent increase accumulation of doxorubicin of 160±39% (±SE, n=7), compared to doxorubicin alone and calculated by the ratio of MFI values, while the compounds **1** and **2** caused an increase of intracellular doxorubicin content of 150±37% (n=6) and 128±28% (n=5), respectively. It should also be underlined that none of the tested substances caused a significant accumulation of doxorubicin in sensitive A2780 cells (data not shown). In Fig. 2 representative histograms of flow cytometric analysis of nifedipine and by comparison of compounds **1** and **2** are shown.

Intracellular accumulation of doxorubicin was qualitatively confirmed by microscopy analysis of A2780/DX3 cells co-treated with nifedipine and nifedipine-like com-

Table 1 Concentrations (μM) of nifedipine and nifedipine-like compounds inhibiting 0% and 5% proliferation in sensitive A2780 and resistant to doxorubicin A2780/DX3 cells

	A2780		A2780/DX3	
	0%[a]	5%	0%[a]	5%
Nifedipine	0.9	3.3	12.9	46.0
1	2.2	4.3	2.0	4.4
2	0.4	1.8	0.5	3.1

[a] The concentration (μM) giving 0% inhibition of cell growth was calculated on the basis of concentration-response curves obtained for all compounds and represents the maximal concentration we can apply without effect on cell growth

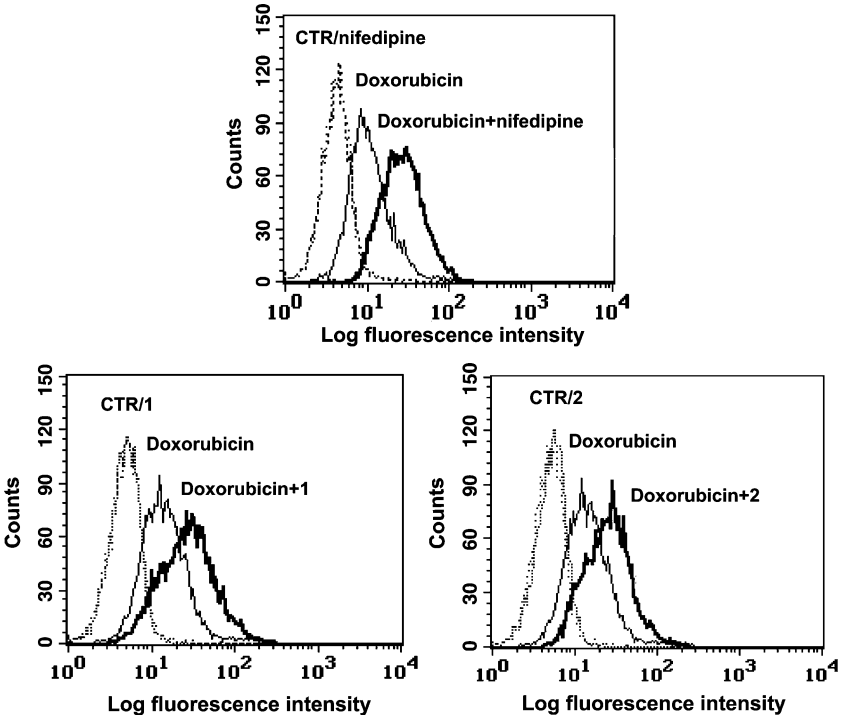

Fig. 2 Representative histograms of flow cytometric analysis of nifedipine and nifedipine-like compounds **1** and **2**. All histograms are superimposed in order to make evident the shift of doxorubicin content after combination with Pgp-170 inhibitors. CTR, untreated control. In these experiments A2780/DX3 cells were treated with the nifedipine, **1**, or **2** at concentrations 5-times higher than that giving 5% inhibition of cell proliferation. Doxorubicin (10 μM) was added 2 h later and cells incubated for other 2 h. Once detached by trypsin, washed and fixed in paraformaldehyde the doxorubicin fluorescence intensity of cells was determined by flow cytometry

pounds. Cells co-treated with compounds **1** or **2** accumulated more doxorubicin than cells receiving only doxorubicin (Fig. 3). No apparent drug accumulation was observed when sensitive A2780 cells were co-treated with nifedipine, **1**, and **2** (data not shown).

Nifedipine and nifedipine-like compounds potentiate doxorubicin inhibition of cell proliferation

When A2780 and A2780/DX3 cells were co-treated with scalar concentrations of doxorubicin and with the IC_0 and

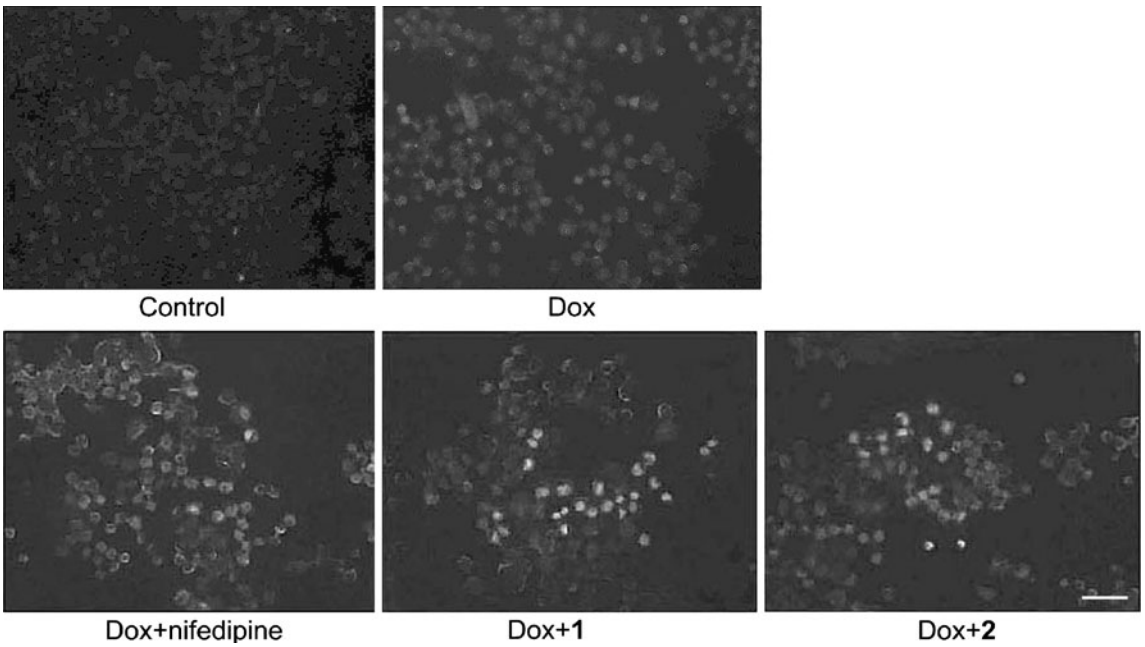

Fig. 3 Microscopy evaluation of doxorubicin accumulation in A2780/DX3 cells after co-treatment with nifedipine and nifedipine-like compounds. Cells were treated as described for the cytofluorimetric studies in the presence of doxorubicin at 10 μM with or without previous incubation with Pgp-170 inhibitors. After fixation with paraformaldehyde the cell imaging was performed with an Axiovert 200 M epifluorescent microscope equipped with the filter set 10 using the Axiovision Software (Carl Zeiss, Jena, Germany). Successive images, were rendered using Adobe Photoshop 5.0 software (Adobe Systems, Mountain View, CA, USA). Bar, 50 μm. Dox, doxorubicin

Table 2 Effect of combination of doxorubicin and nifedipine or nifedipine-like molecules in resistant to doxorubicin A2780/DX3 cells

	5%[a]			0%[b]		
	IC_{50}	% IC_{50} reduction	p value[c]	IC_{50}	% IC_{50} reduction	p value
Nifedipine	0.72±0.22[d]	−52	0.014	1.03±0.13	−32	0.021
1	1.00±0.09	−48	0.021	0.95±0.15	−54	0.021
2	0.06±0.20	−30	0.043	0.76±0.21	−52	0.021

[a] Results obtained by the use of combinations of the concentration of nifedipine, **1** and **2** compounds with doxorubicin giving alone 5% growth inhibition, as calculated by the MTT assay

[b] Results obtained by the use of combinations of doxorubicin with the concentration of nifedipine and nifedipine-like compounds giving alone 0% growth inhibition, as calculated by the MTT assay

[c] P values were calculated according to the Mann-Whitney test for non-parametric data

[d] Data are expressed in μM concentrations

IC_5 of **1** and **2**, the two nifedipine-like compounds showed the ability to significantly reduce the IC_{50} of doxorubicin, with a percent reduction ranging from 30% to 54%, only in resistant cells (Table 2), leaving the IC_{50} of doxorubicin in A2780 unmodified (doxorubicin alone 18±2 nM vs 22± 6 nM and 17±8 nM for **1**; vs 20±8 nM and 19±9 nM for **2**; vs 21±4 nM and 18±7 nM for nifedipine). It must be underlined that the percent reduction for any compound was calculated on the basis of IC_{50} values for doxorubicin alone evaluated for each single experiment.

Determination of apoptosis through morphological analysis of nuclei after DAPI staining and by Annexin-V/PI assay

To determine whether the observed inhibition of cell proliferation caused by the co-treatment of the cells with doxorubicin and the examined compounds was in part due to apoptosis we sought for the presence of typical markers of apoptosis. Therefore we analyzed nuclei fragmentation by DAPI staining, phosphatidylserine externalization by FITC-Annexin-V labeling and membrane permeability by PI staining, respectively.

Staining of segmented nuclei with DAPI showed a significant increase of apoptotic cells, as detected by fluorescence microscopy analysis, after three days of co-treatment with doxorubicin and compounds **1**, **2,** or **3** compared with doxorubicin alone (Fig. 4). Conversely, the incubation of the cells with the examined compounds alone did not modify significantly the observed percentage of segmented nuclei, as compared to the control (1±1% for **1**, 2±1% for **2,** and 3±2% for **3** vs 2±2% for the control).

FACS analysis of A2780/DX3 cells stained with FITC-Annexin-V/PI after co-treatment with doxorubicin and the tested compounds confirmed the increase of apoptosis with respect to the treatment with doxorubicin alone (Figs. 4 and 5). In this case the most effective was compound **2** followed by compounds **3** and **1**. None of them was able to induce significant amount of apoptosis compared to controls when administered individually at the concentration giving 5% inhibition of cell proliferation (control vs compound; for **1**, 12.6±3.1 vs 13.3±3.3; for **2**, 12.2±2.2 vs 14.0±2.3; for **3**, 10.4±4.3 vs 11.6±3.3).

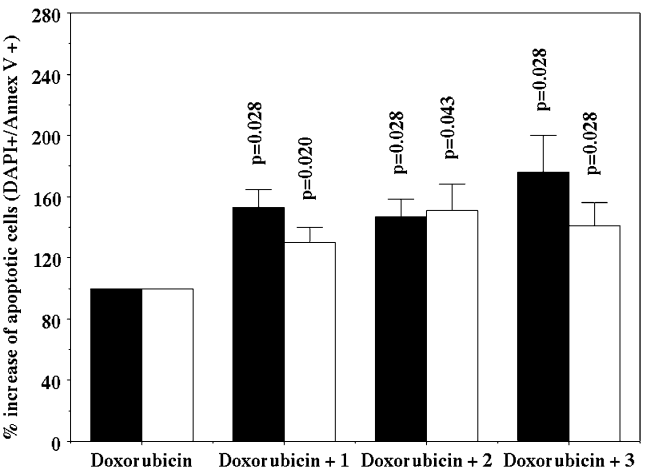

Fig. 4 Histograms represent the percent increase of A2780/DX3 cells showing segmented nuclei (mean ± SE of 6 data, as detected after DAPI staining and microscopic observation, ■) and the percent increase of cells stained with FITC-Annexin-V/PI to make evident the externalized membrane phosphatidylserine (mean ± SE of 5–7 data, □), after co-treatment with doxorubicin and compounds **1**, **2**, or **3** (used at the concentrations giving 5% inhibition of cell proliferation). Percentages were calculated in relation to the percentage of apoptotic cells after doxorubicin alone exposure (100%). P values were calculated according to the Wilcoxon signed rank test for non-parametric data considering the number of apoptotic cells recognized after DAPI staining and the sum of early ($Ann\text{-}V^+/PI^-$) and late ($Ann\text{-}V^+/PI^+$) apoptotic cells. The absolute number (mean ± SE) of DAPI positive apoptotic cells were: for **1** (doxorubicin vs doxorubicin + **1**) 6±1 vs 10±2; for **2** (doxorubicin vs doxorubicin + **2**) 6±1 vs 9±2; for **3** (doxorubicin vs doxorubicin + **3**) 6±1 vs 10±2. The absolute number (mean ± SE) of Annexin-V positive early plus late apoptotic cells were: for **1** (doxorubicin vs doxorubicin + **1**) 26.3±3.0 vs 33.6± 3.6; for **2** (doxorubicin vs doxorubicin + **2**) 18.4±0.4 vs 25.8±3.0; for **3** (doxorubicin vs doxorubicin + **3**) 16.5±1.9 vs 22.4±2

Activation of caspases

Finally, to assess whether the observed potentiation of apoptosis by tested compounds was due to an increased activation of caspases, responsible for triggering this process, we analyzed in a single confirming experiment their activation by flow cytometry using a substrate containing rhodamine linked to two aspartate residues that become fluorescent only after cleavage by caspases.

By this analysis we observed a moderate activation of caspases after co-treatment with doxorubicin and compounds **1**, **2** with respect to doxorubicin alone (Fig. 6). In particular, the most effective compound was **3** followed by **2** and **1** (185%, 136%, and 122%, respectively, expressed as percent increase compared to doxorubicin alone treatment). Moreover, the treatment of the cells with the individual compounds alone did not involve the activation of caspases (data not shown).

Discussion

The main purpose of this study was to investigate the results of interaction of doxorubicin and the now examined nifedipine-like compounds **1** and **2** on Pgp-170 membrane transporter in doxorubicin sensitive A2780 and MDR1 resistant A2780/DX3 cells [29]. These compounds showed a different chemical structure with respect to the series of the diltiazem-like ones [24], being shifted towards different chemical systems, as reported in Introduction. In fact, for example, they show a lower log P and contain pyridine-nitrogen atoms that could cause different chemical interactions as compared to the diltiazem-like compounds previously studied [24].

In particular, the choice of studying these two new molecules was also taken on the basis of our previous results indicating a low activity for these compounds on cardiac functionality (for their cardiovascular activity, see

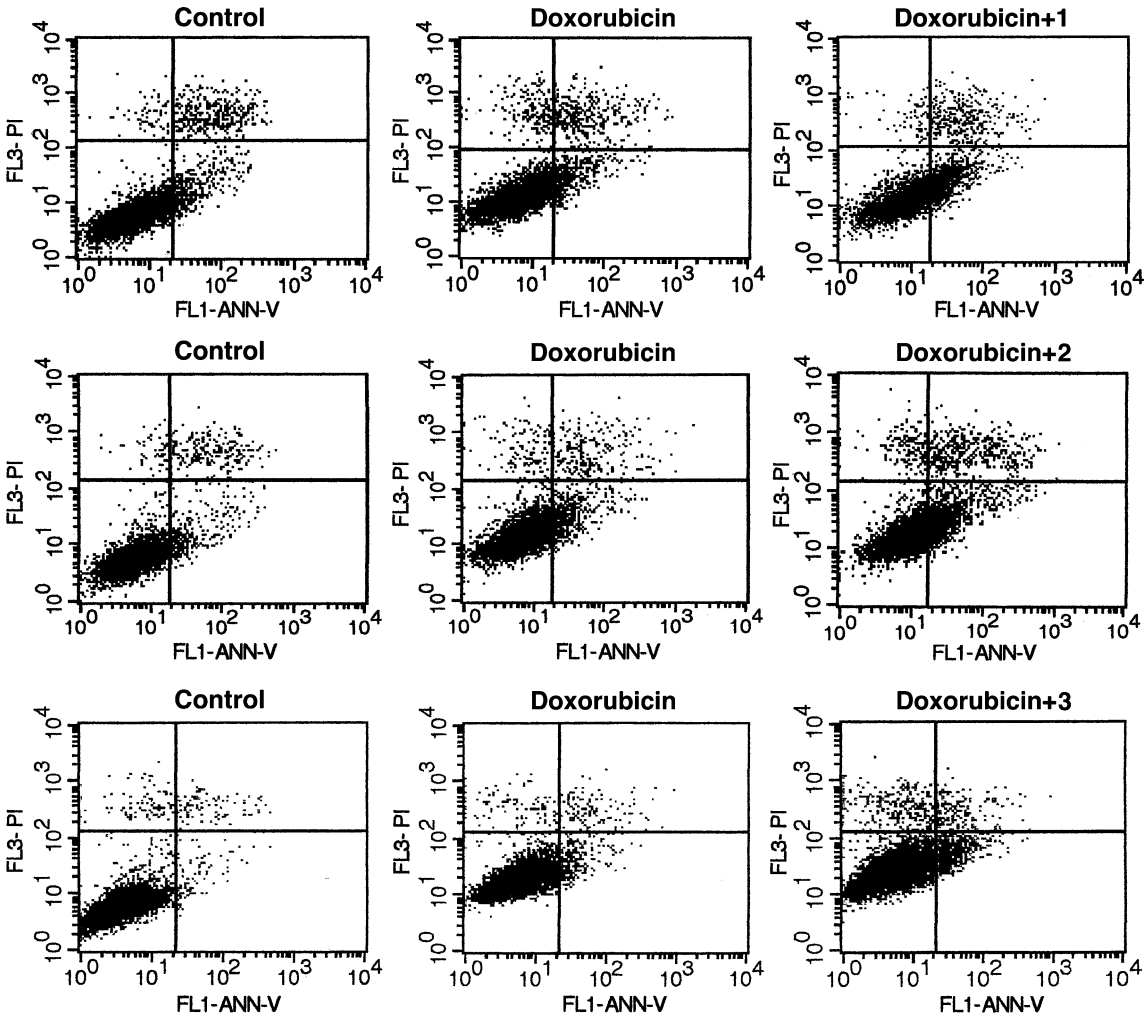

Fig. 5 Representative dotplots of A2780/DX3 treated with doxorubicin plus compounds **1**, **2** and **3** and stained with FITC-Annexin-V/PI. Dotplots of single compounds **1**, **2** and **3** were omitted for simplicity. Mean data pertinent to these dotplots are reported in the paragraph "*Determination of apoptosis through morphological analysis of nuclei after DAPI staining and by Annexin-V/PI assay*" of the "Results" section

data in Table 3). In fact the observation that nifedipine-like compounds **1** and **2** possess a low intrinsic activity on all of the examined parameters of cardiac functionality let us think that they could have also low toxic effect (particularly at cardiac level) once assayed as MDR1 reversing agents in specifically designed *in vivo* experimental models.

Both **1** and **2**, when tested for the inhibition of cell proliferation, showed an extremely low effect, with IC_{50}s much higher than 100 μM on both sensitive A2780 and resistant A2780/DX3 cells and well different from the respective IC_{50} values for doxorubicin. When co-administered with doxorubicin at the inactive or slightly active concentrations giving 0% and 5% inhibition of cell proliferation both **1** and **2** were able to potentiate by a significant 30–54% doxorubicin activity, as evaluated considering the IC_{50} parameter. The inhibition of Pgp-170 activity was also demonstrated by cytofluorimetric and microscopic analyses that showed, in the presence of nifedipine-like compounds **1** and **2,** a +150% and +128% accumulation of co-administered doxorubicin, respectively, similar to that observed using a comparable nifedipine concentration (+160%). In this context it is also worth of note that no higher accumulation was observed when we used A2780 sensitive cells as target, that express membrane Pgp-170 at extremely low levels.

On the base of these results our nifedipine-like compounds could appear less active in terms of doxorubicin accumulation (about 40% of doxorubicin accumulation in sensitive A2780 cells) than some of our previously described diltiazem-like compounds [24], other than in general. Nevertheless it must be underlined that both molecules **1** and **2** are active 5 to 7 more times than the considered diltiazem-like molecules and 10–15 times more active than nifedipine itself on a molar basis. These data, together with the observation that compounds **1** and **2** at equitoxic concentrations affect doxorubicin antiproliferative activity similarly to the parent nifedipine or to the active diltiazem-like compounds [24], may imply the existence of other cytotoxic mechanisms of action different from the inhibition of Pgp-170, still to be determined, and possibly acting sinergistically with the mechanism here studied. On the other hand the fact that both **1** and **2** do not increase the doxorubicin activity reaching that observed in A2780 cells, in terms of MTT activity and drug accumulation, is in part counteracted by the observation that also nifedipine, an active compound that has reached the clinical phase studies [18, 19], has a similar activity in our experimental conditions.

The membrane expression of Pgp-170, studied by the anti-MDR1 MM4.17 monoclonal antibody (kindly provided by Dr. M. Cianfriglia), was not down-regulated in the presence of active concentration of both nifedipine-like compounds (data not shown), thus confirming that the intracellular accumulation of doxorubicin was not a consequence of the Pgp-170 down-regulation.

Finally, concerning the evaluation of hydrophobicity of **1** and **2,** we calculated their log P (ChemBioDraw 11.0 software), as 1.75 and 1.72, respectively. It is of note that an appropriate value of log P (in the 3–5 range) together with the presence of one or more coplanar rings have been proposed as important characteristics of chemosensitizers acting as competitive inhibitor of Pgp-170 [6, 30, 31]. Our results seem to indicate that notwithstanding their more hydrophilic characters also compounds **1** and **2** with a

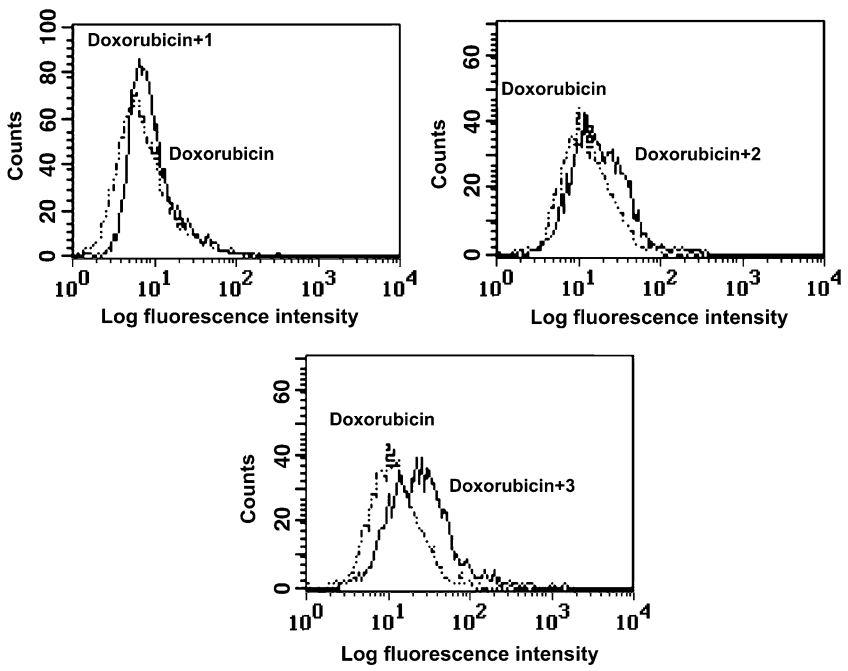

Fig. 6 Histograms of flow cytometric analysis of activation of caspase complex obtained using the CaspScreen Flow Cytometric Apoptosis Detection kit in A2780/DX3 cells. Only histograms obtained after treatment with doxorubicin alone and after co-treatment with doxorubicin and **1**, **2**, or **3** were reported. Nifedipine—and diltiazem-like compounds **1**, **2**, and **3** showed histograms superimposed to those obtained for untreated controls while doxorubicin showed about 93% increase of MFI compared to controls (data not shown)

Table 3 Cardiovacular activity of compounds **1–3**, of nifedipine, and of diltiazem[a,b]

	% decrease (M ± SEM)		EC_{50} of inotropic neg. activity		EC_{30} of chronotropic neg. activity		Vasorelaxant activity		
	Negative inotropic activity[c]	Negative chronotropic activity[d]	EC_{50}[e] (μM)	95% conf. lim. ($\times 10^{-6}$)	EC_{30}[f] (μM)	95% conf. lim. ($\times 10^{-6}$)	Activity[f] (M ± SEM)	IC_{50}[e] (μM)	95% conf. lim. ($\times 10^{-6}$)
Nifedipine[a]	97.0±2.0[g]	85.0±4.2[h]	0.26	0.19–0.36	0.025	0.019–0.031	82.0±1.3[k]	0.009	0.003–0.02
1[a]	42.0±0.2	25.0±1.5[j]					44.0±3.7		
2[a]	46.0±1.3	36.0±1.7[g]					28.0±1.5[i]		
Diltiazem[b]	78.0±3.5[h]	94.0±5.6[k]	0.79	0.70–0.85	0.07	0.064–0.075	88.0±2.3	2.6	2.2–3.1
3[b]	67.0±2.4[i]	5.0±0.2	1.31	0.97–1.43			10.0±0.8[i]		

[a] From reference [25]

[b] From reference [23]

[c] Activity: decrease in developed tension in isolated guinea-pig left atrium at 10^6 M, expressed as percent changes from the control (n=4–6). The left atria were driven at 1 Hz. The 10^{-6} M concentration gave the maximum effect for most compounds

[d] Activity: decrease in atrial rate in guinea-pig spontaneously beating isolated right atria at 5×10^{-5} M, expressed as percent changes from the control (n=6–8). Pretreatment heart rate ranged from 170 to 195 beats/min. The 5×10^{-5} M concentration gave the maximum effect for most compounds

[e] Calculated from log concentration-response curves [Probit analysis according to Litchfield and Wilcoxon (Tallarida, R. J.; Murray, R. B. Manual of Pharmacologic Calculation with Computer Programs, 2nd ed.; Springer-Verlag: New York; 1987)] with n=6–8. When the maximum effect was <50%, the EC_{50} inotropic, EC_{30} chronotropic and IC_{50} values were not calculated

[f] Activity: percent inhibition of calcium-induced contraction on K^+-depolarized guinea-pig aortic strip at 10^{-4} M. The 10^{-4} M concentration gave the maximum effect for most compounds

[g] At 10^{-5} M

[h] At 5×10^{-7} M

[i] At 10^{-5} M

[j] At 5×10^{-6} M

[k] At 10^{-6} M

dihydropyridine scaffold decorated by 4-substituted imidazo[2,1-*b*]thiazole may act as inhibitors of Pgp-170.

Finally, in spite of the relatively recent controversial opinions on the real role of apoptosis in clinical setting [32] the concept that this process may play a crucial role for the response to anticancer chemotherapy [33–35] has become a milestone for the anticancer drug research and for new chemotherapy-based therapeutic procedures. It is well known that apoptosis represents an important physiological pathway of cell death other than one of the possible main modalities of cell death after chemotherapy, the other one being necrosis. As many other anticancer drugs, doxorubicin is able to trigger apoptosis in tumor and normal tissues both *in vitro* and *in vivo* [36–38]. This is the reason why we decided to study this important mechanism of cell death *in vitro* when the topoisomerase inhibitor was co-administered together with the tested nifedipine or diltiazem-like compounds. This was also important to demonstrate that both **1** and **2** and also the diltiazem-like compound **3** do not represent possible inhibitors of this important mechanism.

On the whole, our results indicate that apoptotic triggering of doxorubicin, as detected by FITC-Annexin-V/PI assay and DAPI staining, is significantly potentiated by the addition of slightly active concentrations of the diltiazem-like **3** and of the nifedipine-like **1** and **2**. At these concentrations no one of our compounds was able to induce apoptosis in a significant extent compared to untreated controls. These results were confirmed, although in a small extent, by the cytofluorimetric analysis of caspases' activity.

In conclusion our data show that nifedipine-like **1** and **2** increase the intracellular concentration of doxorubicin, likely by competing for the efflux mediated by the Pgp-170 transporter, and together with the diltiazem-like compound **3** potentiate its antiproliferative and pro-apoptotic activity. Although the activity of these compounds may not seem to be particularly high it must be underlined that in term of doxorubicin accumulation and increase of doxorubicin antiproliferative activity their efficiency was similar to that of nifedipine while it is highly predictable a very lower cardiac activity. All these properties, in particular their low activity on cardiac functionality, may have potential therapeutic relevance prompting us for future modifications of chemical structures in order to obtain more active substances and *in vivo* studies.

Acknowledgements This research was supported by funds from "Ministero dell'Istruzione, dell'Università e della Ricerca" (FIRB 2001, PRIN-2005034305_001, PRIN-20078J9L2A_005) and from the University of Bologna. Dr. M. Croce is recipient of a fellowship by "Fondazione italiana per la lotta al neuroblastoma".

References

1. Abolhoda A, Wilson AE, Ross H, Danenberg PV, Burt M, Scotto KW (1999) Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin. Clin Cancer Res 5:3352–3356
2. Kourti M, Vavatsi N, Gombakis N, Sidi V, Tzimagiorgis G, Papageorgiou T, Koliouskas D, Athanassiadou F (2007) Expression of multidrug resistance 1 (MDR1), multidrug resistance-related protein 1 (MRP1), lung resistance protein (LRP), and breast cancer resistance protein (BCRP) genes and clinical outcome in childhood acute lymphoblastic leukemia. Int J Hematol 86:166–173
3. Hille S, Rein DT, Riffelmann M, Neumann R, Sartorius J, Pfützner A, Kurbacher CM, Schöndorf T, Breidenbach M (2006) Anticancer drugs induce mdr1 gene expression in recurrent ovarian cancer. Anticancer Drug 17:1041–1044
4. Sharom FJ (2008) ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics 9:105–127
5. Sarkadi B, Homolya L, Szakács G, Váradi A (2006) Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. Physiol Rev 86:1179–1236
6. Li X, Li JP, Yuan HY, Gao X, Qu XJ, Xu WF, Tang W (2007) Recent advances in P-glycoprotein-mediated multidrug resistance reversal mechanisms. Meth Find Exp Clin Pharmacol 29:607–617
7. Sharom FJ (2006) Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABCB1). Biochem Cell Biol 84:979–92
8. Fromm MF (2004) Importance of P-glycoprotein at blood-tissue barriers. Trends Pharmacol Sci 25:423–429
9. Mechetner E, Kyshtoobayeva A, Zonis S, Kim H, Stroup R, Garcia R, Parker RJ, Fruehauf JP (1998) Levels of multidrug resistance (MDR1) P-glycoprotein expression by human breast cancer correlate with in vitro resistance to taxol and doxorubicin. Clin Cancer Res 4:389–398
10. Lum BL, Gosland MP, Kaubisch S, Sikic BI (1993) Molecular targets in oncology: implications of the multidrug resistance gene. Pharmacotherapy 13:88–109
11. Avedaño C, Menéndez JC (2002) Inhibitors of multidrug resistance to antitumor agents (MDR). Curr Med Chem 9:159–193
12. Krishna R, Mayer LD (2000) Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR, and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci 11:265–283
13. Ferry DR, Traunecker H, Kerr DJ (1996) Clinical trials of P-glycoprotein reversal in solid tumors. Eur J Cancer 32A:1070–1081
14. Fischer V, Rodríguez-Gascón A, Heitz F, Tynes R, Hauck C, Cohen D, Vickers AE (1998) The multidrug resistance modulator Valspodar (PSC 833) is metabolized by human cytochrome P450 3A. Implications for drug-drug interactions and pharmacological activity of the main metabolite. Drug Metab Dispos 26:802–811
15. Wandel C, Kim RB, Kajiji S, Guengerich P, Wilkinson GR, Wood AJP (1999) Glycoprotein and cytochrome P-450 3A inhibition: dissociation of inhibitory potencies. Cancer Res 59:3944–3948
16. Vergouwen MD, Vermeulen M, de Haan RJ, Levi M, Roos YB (2007) Dihydropyridine calcium antagonists increase fibrinolytic activity: a systematic review. J Cereb Blood Flow Metab 27:1293–1308
17. Sahney S (2006) A review of calcium channel antagonists in the treatment of pediatric hypertension. Paediatr Drugs 8:357–373
18. Holmes FA, Lopez A, Mavligit G, Fraschini G, Frye D, Hortobagyi GN (1997) Secondary drug resistance in breast cancer: failure to reverse with oral nifedipine. Int J Cancer 73:184–186
19. Philip PA, Joel S, Monkman SC, Dolega-Ossowski E, Tonkin K, Carmichael J, Idle JR, Harris AL (1992) A phase I study on the reversal of multidrug resistance (MDR) in vivo: nifedipine plus etoposide. Br J Cancer 65:267–270
20. Li X, Lu WL, Liang GW, Ruan GR, Hong HY, Long C, Zhang YT, Liu Y, Wang JC, Zhang X, Zhang Q (2006) Effect of stealthy liposomal topotecan plus amlodipine on the multidrug-resistant leukaemia cells in vitro and xenograft in mice. Eur J Clin Invest 36:409–418
21. Li X, Ruan GR, Lu WL, Hong HY, Liang GW, Zhang YT, Liu Y, Long C, Ma X, Yuan L, Wang JC, Zhang X, Zhang Q (2006) A novel stealth liposomal topotecan with amlodipine: apoptotic effect is associated with deletion of intracellular Ca2+ by amlodipine thus leading to an enhanced antitumor activity in leukemia. J Control Release 112:186–198
22. Budriesi R, Cosimelli B, Ioan P, Lanza CZ, Spinelli D, Chiarini A (2002) Cardiovascular characterization of [1, 4]thiazino[3, 4-c][1, 2, 4]oxadiazol-1-one derivatives: Selective myocardial calcium channel modulators. J Med Chem 45:3475–3481
23. Budriesi R, Carosati E, Chiarini A, Cosimelli B, Cruciani G, Ioan P, Spinelli D, Spisani R (2005) A new class of selective myocardial calcium channel modulators. 2. Role of the acetal chain in oxadiazol-3-one derivatives. J Med Chem 48:2445–2456
24. Viale M, Cordazzo C, Cosimelli B, de Todero D, Castagnola P, Aiello C, Severi E, Petrillo G, Cianfriglia M, Spinelli D (2009) Inhibition of MDR1 activity in vitro by a novel class of diltiazem analogues: towards new candidates. J Med Chem 52:259–66 see also SciBX, doi:10.1038/scibx.2009.44
25. Budriesi R, Ioan P, Locatelli A, Cosconati S, Leoni A, Ugenti MP, Andreani A, Di Toro R, Bedini A, Spampinato S, Marinelli L, Novellino E, Chiarini A (2008) Imidazo[2, 1-b]thiazole system: A scaffold endowing dihydropyridines with selective cardiodepressant activity. J Med Chem 51:1592–1600
26. Phillips AP (1949) Hantzsch's pyridine synthesis. J Am Chem Soc 71:4003–4007
27. Andreani A, Granaiola M, Leoni A, Locatelli A, Morigi R, Rambaldi M, Garaliene V, Welsh W, Arora S, Farruggia G, Masotti L (2005) Antitumor activity of new substituted 3-(5-imidazo[2, 1-b]thiazolylmethylene)-2-indolinones and study of their effect on the cell cycle. J Med Chem 48:5604–5607
28. Hussain RF, Nouri AME, Oliver RTD (1993) A new approach for measurement of cytotoxicity using colorimetric assay. J Immunol Methods 60:89–96
29. Novi M, Ottone M, Dell'Erba C, Barbieri F, Chiavarina B, Maccagno M, Viale M (2004) 1, 4-Bis(1-naphthyl)-2, 3-dinitro-1, 3-butadiene a novel anticancer compound effective against tumor cell lines characterized by different mechanisms of resistance. Oncol Rep 12:91–96
30. Qi J, Yang CZ, Wang CY, Wang SB, Yang M, Wang JH (2002) Function and mechanism of pyronaridine: a new inhibitor of P-glycoprotein-mediated multidrug resistance. Acta Pharmacol Sin 23:544–550
31. Beck WT, Qian XD (1992) Photoaffinity substrates for P-glycoprotein. Biochem Pharmacol 43:89–93
32. Gonzales VM, Fuertes MA, Alonso C, Perez JM (2001) Is cisplatin-induced cell death always produced by apoptosis? Mol Pharmacol 59:657–663

33. Kerr JF, Winterford CM, Harmon BV (1994) Apoptosis. Its significance in cancer and cancer therapy. Cancer 73:2013–2026
34. Fuster JJ, Sanz-González SM, Moll UM, Andrés V (2007) Classic and novel roles of p53: prospects for anticancer therapy. Trends Mol Med 13:192–199
35. Gadducci A, Cosio S, Muraca S, Genazzani AR (2002) Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications. Eur J Gynaecol Oncol 23:390–396
36. Lerma-Díaz JM, Hernández-Flores G, Domínguez-Rodríguez JR, Ortíz-Lazareno PC, Gómez-Contreras P, Cervantes-Munguía R, Scott-Algara D, Aguilar-Lemarroy A, Jave-Suárez LF, Bravo-Cuellar A (2006) In vivo and in vitro sensitization of leukemic cells to adriamycin-induced apoptosis by pentoxifylline. Involvement of caspase cascades and IkappaBalpha phosphorylation. Immunol Lett 103:149–158
37. Yoshimoto Y, Kawada M, Ikeda D, Ishizuka M (2005) Involvement of doxorubicin-induced Fas expression in the antitumor effect of doxorubicin on Lewis lung carcinoma in vivo. Int Immunopharmacol 5:281–288
38. Wu S, Ko YS, Teng MS, Ko YL, Hsu LA, Hsueh C, Chou Y, Liew CC, Lee YS (2002) Adriamycin-induced cardiomyocyte and endothelial cell apoptosis: in vitro and in vivo studies. J Mol Cell Cardiol 34:1595–1607