Inhibition of MDR1 Activity in Vitro by a Novel Class of Diltiazem Analogues: Toward New Candidates

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Received June 30, 2008

The reversal of multidrug resistance by 22 molecules [8-aryl-8-hydroxy-5-*R*′-8*H*-[1,4]thiazino[3,4-*c*]-[1,2,4]oxadiazol-3-ones (**1a**−**i)** and 8-aryl-8-alkoxy-5-methyl-8*H*-[1,4]thiazino[3,4-*c*][1,2,4]oxadiazol-3-ones (**2a**−**m**)] related to myocardial-calcium-channel-modulator diltiazem was studied in multidrug resistant A2780/DX3 and their sensitive counterpart A2780 cells. MTT, cytofluorimetry assays, and fluorescence microscopy analyses were used to define activity and accumulation of doxorubicin with or without the diltiazem-like modulators. Of the 22 molecules, **1a**, **2f**, **2g**, and **2m** were able to overcome the established criteria for the selection in A2780/DX3 cells (IC_{50} reduction ≥25%), but only **2f**, **2g**, and **2m** caused a significant increase of intracellular accumulation of doxorubicin. In conclusion, experiments lead to the identification of three diltiazem-like molecules able to increase the intracellular accumulation of doxorubicin by inhibiting the MDR1 function, thus potentiating its antiproliferative activity in multidrug resistant A2780/DX3 cells.

Introduction

Localized malignancies are usually effectively treated by surgery and radiotherapy. However, chemotherapy is the preferred treatment for patients with metastatic tumors with the addition of immunotherapy or biological response modifiers that may improve the response rate in selected cases.

Despite recent improvements, effectiveness of chemotherapy is still low for most cancer types such as ovary, colon, lung, kidney, pancreas, and liver cancers. One of the major clinical and pharmacological causes of this failure is the development of drug resistance by the tumor cells. In particular, acquired cellular resistance is the cause of disease relapse and is characterized by cellular mechanisms that provide the tumor cells with the ability to resist chemotherapy.[1−5] Acquired cellular resistance has been extensively studied by using cell lines in most cases selected by exposure to increasing concentrations of cytotoxic agents.

Tumor cells may develop resistance to a single drug or to a family of drugs sharing similar mechanisms of action. Moreover, cells may also acquire cross-resistance to functionally and structurally unrelated drugs commonly used in cancer chemotherapy. This phenomenon is known as "classic" multidrug resistance (MDR).[4,6−8]

Several members of the superfamily of ATP-binding cassette (ABC) transporters are involved in MDR in vitro. ABC Transporters are membrane proteins, which contain two transmembrane-spanning domains (TMDs) and two nucleotide-binding domains (NBDs) that couple transport with hydrolysis of ATP. Three main subfamilies of ABC transporters are involved in multidrug resistance: ABCB (ABCB1/MDR1/Pgp-170), ABCC (ABCC1/MRP1, ABCC2/MRP2), and probably also ABCC3−6, ABCC10−11, and ABCG (ABCG2/MXR/BCRP).[6,7]

Nevertheless, overexpression of Pgp-170 is the most common cause of multidrug resistance in many types of tumors, including those of the ovary and of the breast. Pgp-170 is the product of the ABCB1 (or MDR1) gene, located on chromosome 7q21. It is a 170 kDa phosphoglycoprotein that has 12 transmembrane domains (TMDs) and 2 ATP-binding sites.[9,10] Pgp-170 is an ATP-driven efflux transporter that promotes the efflux of neutral to slightly cationic hydrophobic xenobiotics from the cells. It is expressed in several normal tissues, where it carries out protective functions such as the regulation of the excretion of metabolites into bile and urine and limitations of the entry of drugs into central nervous system, testis, blood cells, and through the placenta.[8,11]

However, Pgp-170 overexpression is frequently found in human solid and hematologic cancers and it could be considered a marker of chemoresistance or reduced survival of patients affected by several tumors like leukemias, lymphomas, osteosarcomas, and small-cell lung, breast, and ovarian cancers.[12−15]

In tumor tissues, Pgp-170 acts like an efflux pump that extrudes chemotherapeutic agents from tumor cells, resulting in decreased intracellular drug concentrations and loss of efficacy. Several studies have shown that the expression of Pgp-170 is characterized by cross-resistance to natural-product-based chemotherapeutics, including taxanes, anthracyclines, *Vinca* alkaloids, podophyllotoxins, and camptothecins.[16,17]

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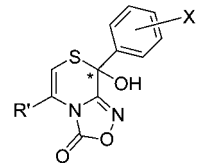
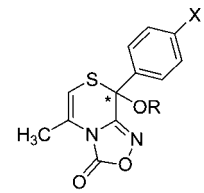
10.1021/jm801195k CCC: $40.75
Published on Web 12/18/2008

Chart 1



8-Aryl-8-hydroxy-5-R'-8*H*-[1,4]thiazino[3,4-*c*][1,2,4]oxadiazol-3-ones

1a: R' = X= H
1b: R' = H, X = p-Cl
1c: R' = CH_3, X = H
1d: R' = CH_3, X = *p*-CH_3
1e: R' = CH_3, X = *p*-OCH_3
1f: R' = CH_3, X = *p*-NO_2
1g: R' = CH_3, X = *m*-Cl
1h: R' = CH_3, X = *m*-NO_2
1i: R' = CH_3, X = *m*-CF_3



8-Aryl-8-alkoxy-5-methyl-8*H*-[1,4]thiazino[3,4-*c*][1,2,4]oxadiazol-3-ones

2a: R = CH_3, X = Cl
2b: R = CH_3, X = Br
2c: R = CH_2CH_3, X = Br
2d: R = $(CH_2)_3CH_3$, X = Cl
2e: R = $(CH_2)_3CH_3$, X = Br
2f: R = $(CH_2)_4CH_3$, X = Cl
2g: R = $(CH_2)_4CH_3$, X = Br
2h: R = $(CH_2)_{14}CH_3$, X = Cl
2i: R = $(CH_2)_{17}CH_3$, X = Cl
2j: R = $(CH_2)_{17}CH_3$, X = Br
2k: R = $CH_2CH{=}CHCH_2CH_3$ (*E*-isomer), X = Cl
2l: R = $CH_2CH{=}CHCH_2CH_3$ (*E*-isomer), X = Br
2m: R = $CH_2CH{=}CHCH_2CH_3$ (*Z*-isomer), X = Cl

Several Pgp-170 inhibitors used in combination with drugs pumped out by Pgp-170 can determine the preservation of a constant intracellular drug concentration, restoring the effectiveness of these therapeutics. The most efficient Pgp-170 inhibitors can be classified into several structurally different species: calcium blocking agents such as verapamil, diltiazem, and nifedipine; cyclosporines such as cyclosporine A; hormones or antihormones such as progesterone and tamoxifen; isoquinoline alkaloids such as tetrandrine; and other agents such as vinblastine.[5,7] The first generation inhibitors verapamil, diltiazem, and cyclosporine A have successfully antagonized Pgp-170 both in vitro and in vivo.[4] However, clinical trials have been disappointing due to the considerable toxicity of the concentration necessary to inhibit the Pgp-170-machinery. Despite extensive research and clinical development, today none of the known Pgp-170 inhibitors[18] provides satisfying results due to their negative effects in vivo and their high dose-dependent toxicities or pharmacokinetic side effects.[19−21]

Recently, one of the main lines of this pharmacological research has investigated the rational design of new chemical compounds using the first-generation drugs as a template structure with the aim of generating effective molecules with low toxicity. Thus, in the present study, we analyzed the role of 22 synthesized compounds related to diltiazem (**1a**−**i**, **2a**−**m,** Chart 1) in order to evaluate their capacity to strengthen the anticancer activity of doxorubicin on sensitive and MDR-resistant ovarian A2780 and A2780/DX3 cancer cells, modulating the Pgp-170 mechanism.

As a matter of fact some of us have recently evidenced that both 8-aryl-8-hydroxy-5-*R′*-8*H*-[1,4]thiazino[3.4-*c*][1,2,4]oxadiazol-3-ones[22] or 8-aryl-8-alkoxy-5-methyl-8*H*-[1,4]thiazino-[3.4-*c*][1,2,4]oxadiazol-3-ones **2**[22,23] show LTCC[a] blockers activity. They are in some way structurally related to diltiazem and in some instances are even more active than diltiazem itself [e.g., compare EC_{50} of **2c** (0.04 μM) to that of diltiazem (0.79 μM)]. Furthermore, **2c** elicited a potent negative inotropic effect on isolated tissues without affecting chronotropy and vasorelaxant activity, being on the whole more selective of diltiazem.[23]

The results obtained in the present study suggest that some of these new molecules obtained by chemical synthesis may improve the efficacy of doxorubicin; therefore we propose that they should be considered as possible candidates for in vivo studies on the treatment of multidrug resistant cancers expressing Pgp-170.

Results

Inhibition of Cell Proliferation of Diltiazem-like Derivatives. The analysis of the inhibition of cell proliferation obtained by the treatment with diltiazem-like compounds **1** and **2** and their parent compound (diltiazem) showed that none of them possesses a significant inhibiting activity. Because the maximal concentration used for evaluating the inhibiting activity of our molecules was 100 μM, it must be underlined that the calculated IC_{50} values, ranging from 108 ± 18 μM (**2g**, A2780) to 166 ± 16 μM (**2a**, A2780) were broadly approximated. As showed in Table 1, the most active compounds on A2780 sensitive cells were **2l**, **2m**, and **2k**, with IC_{50}s ranging from 59 ± 14 to 55 ± 9 μM, while on resistant A2780/DX3 the most active compounds were **1i** and **2d**, with IC_{50}s of 71 ± 2 and 49 ± 7 μM, respectively. Anyway, also for these more active molecules, the IC_{50} values were quite high, especially if compared to the mean IC_{50}s calculated for doxorubicin, which were on average 0.02 ± 0.01 and 2.21 ± 0.22 μM for A2780 and A2780/DX3, respectively.

On the basis of the same experiments, we also calculated the IC_0 and IC_5 of our molecules. They are listed in Table 2 and were used to perform the experiments of cotreatment with doxorubicin.

Growth Inhibition of A2780 and A2780/DX3 Cells by Doxorubicin Associated with Tested Compounds. As cited in the previous paragraph, in the absence of cotreatment with compounds **1** and **2**, A2780 and A2780/DX3 treated with doxorubicin, showed IC_{50}s that were on average 0.02 ± 0.15 and 2.21 ± 0.22 μM, respectively (range for A2780/DX3: 1.41−2.59 μM), with a mean resistance index of 93 ± 22. When cells were cotreated with doxorubicin and the IC_0 and IC_5 of our derivatives, only four molecules overcome the strict rules for selection. In particular, **1a** caused a maximal reduction of the IC_{50} of doxorubicin of 36% (IC_0), **2m** of 47% (IC_5), **2f** of 39% (IC_0), and **2g** of 43% (IC_5) (Table 3). It must be underlined that the percent reduction for any compound was calculated on

[a] Abbreviations: LTCC, L-type calcium channel; EC_{50}, 50% effective concentration (concentration required for 50% of efficiency); IC_{50}, 50% inhibitory concentration (concentration required for 50% of inhibition); IC_0, 0% inhibitory concentration (maximal applied concentration without effect on cell growth); IC_5, 5% inhibitory concentration (concentration required for 5% of inhibition); SE standard error.

Table 1. Concentrations of Diltiazem and Diltiazem-like Compounds Inhibiting 50% Proliferation (IC_{50}) in Sensitive A2780 and Multidrug Resistant A2780/DX3 Cells

	IC_{50} μM)	
compd	A2780	A2780/DX3
Diltiazem	163 ± 23	≫100
1a	≫100	≫100
1b	≫100	≫100
1c	≫100	≫100
1d	≫100	≫100
1e	≫100	≫100
1f	≫100	≫100
1g	≫100	≫100
1h	159 ± 53	129 ± 52
1i	60 ± 2	71 ± 2
2a	166 ± 16	113 ± 11
2b	148 ± 44	≫100
2c	≫100	≫100
2d	89 ± 25	49 ± 7
2e	82 ± 12	78 ± 15
2f	≫100	≫100
2g	108 ± 18	≫100
2h	≫100	≫100
2i	≫100	≫100
2j	≫100	≫100
2k	55 ± 12	88 ± 14
2l	59 ± 14	111 ± 7
2m	55 ± 9	103 ± 22

Table 2. Micromolar Concentrations of Diltiazem and Diltiazem-like Compounds Inhibiting 5% and 0% Proliferation in Sensitive A2780 and Multidrug Resistant A2780/DX3 Cells

	A2780		A2780/DX3	
compd	5%	0%[a]	5%	0%
Diltiazem	2.8	1.3	20.6	2.2
1a	4.4	0.6	32.1	1.2
1b	0.48	0.14	3.02	0.81
1c	100	10	49.4	6.2
1d	28.8	6.1	31.1	0.5
1e	40.1	15.4	26.7	2.25
1f	7.7	2.1	2.9	1.5
1g	24.3	1.97	3.89	0.84
1h	3.95	3.1	15.9	3.7
1i	18.3	5.3	12.6	1.9
2a	28.4	13.1	11.7	3.0
2b	21.6	9.4	8.9	4.2
2c	3.3	1.5	7.8	1.0
2d	19.7	12.2	2.2	1.0
2e	7.7	1.2	3.2	1.0
2f	7.8	2.9	19.0	4.0
2g	2.60	0.20	41.8	15.6
2h	2.1	0.72	14.6	4.5
2i	4.74	2.22	6.74	2.40
2j	0.9	0.23	4.9	1.88
2k	1.26	0.15	2.5	0.3
2l	1.9	1.3	2.45	1.23
2m	3.50	2.20	20.7	2.4

[a] The concentration 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 the cell growth.

the basis of IC_{50} values for doxorubicin alone evaluated for each specific experiment.

Determination of Doxorubicin Accumulation by Flow Cytometry. Once selected, the **1a**, **2f**, **2g**, and **2m** derivatives were analyzed for their ability to counteract the efflux of doxorubicin from A2780 and A2780/DX3 cells. The results of the flow cytometric analysis of their reversing activity in A2780/DX3 cells are summarized in Table 4. In our experimental conditions, diltiazem caused a mean percent increase (±SE) accumulation of doxorubicin of 699 ± 75%, compared to

Table 3. Effect of Combination of Diltiazem and Diltiazem-like Compounds with Doxorubicin in Multidrug Resistant A2780/DX3 Cells

	A2780/DX3					
	5% [1]		p value [2]	0% [3]		p value
Compound	IC_{50} (μM)	% IC_{50} variation		IC_{50} (μM)	% IC_{50} variation	
Diltiazem	0.53±0.28	-84		0.92±0.36	-72	
1a	2.13±0.64	-18	NS	1.66±0.50	-36	0.025
1i	1.35±0.35	-4		1.11±0.07	-22	
1h	2.81±0.33	10		2.03±0.45	-21	
1c	1.38±1.25	-2		1.18±0.24	-16	
2a	2.34±0.67	2		2.10±0.79	-8	
1b	2.62±0.18	10		2.46±0.39	3	
1d	2.27±0.14	-1		2.47±0.28	4	
1g	2.42±0.36	7		2.41±0.44	7	
1e	2.55±0.71	12		2.49±0.64	10	
1f	1.96±0.24	-10		2.75±0.28	26	
2m	0.92±0.33	-47	0.029	1.51±0.29	-9	NS
2g	0.98±0.17	-43	0.007	1.18±0.31	-31	0.100
2f	1.36±0.22	-38	0.029	1.34±0.12	-39	0.023
2l	1.76±0.16	-23		2.10±0.42	-8	
2j	1.71±0.17	-22		1.74±0.17	-21	
2i	1.75±0.31	-20		1.66±0.06	-24	
2d	1.76±0.26	-16		1.66±0.30	-21	
2h	1.97±0.52	-13		1.95±0.51	-14	
2b	2.13±0.17	-9		2.13±0.43	-9	
2c	1.98±0.48	-8		2.28±0.53	6	
2k	1.77±0.10	-1		1.84±0.20	2	
2e	1.59±0.29	3		1.41±0.31	3	

[a] Results obtained by the use of combinations with doxorubicin of the concentration of diltiazem-like compounds giving alone 5% growth inhibition, as calculated by the MTT assay. [b] Only reduction of IC50s >25% and statistically significant were considered. Kruskal−Wallis and Mann−Whitney tests for nonparametric data were used. [c] Results obtained by the use of combinations with doxorubicin of the concentration of diltiazem-like compounds giving alone 0% growth inhibition, as calculated by the MTT assay.

Table 4. Evaluation of Doxorubicin Intracellular Accumulation after Co-treatment with Diltiazem and Diltiazem-like Compounds by Flow Cytometric Analyses

	% intracellular doxorubicin accumulation
Diltiazem	699 ± 75[a]
1a	NA
2f	248 ± 11
2g	52 ± 6
2m	429 ± 29

[a] Data express the mean ± SE (no = 2) of the percent increase of doxorubicin accumulation (MFI) relative to doxorubicin alone treatment. NA, no increased accumulation.

doxorubicin alone and calculated by the ratio of MFI values, while the diltiazem-like related compounds **2f**, **2g**, and **2m** caused a percent increase of intracellular doxorubicin content of 248 ± 11%, 52 ± 6%, and 429 ± 29%, respectively. It is worth noting that the addition of compound **1a** to the anticancer drug did not cause any significant increase of the intracellular doxorubicin concentration, as shown in Figure 1, reporting the representative histograms of flow cytometric analysis of **2f**, **2g**, **2m**, **1a**, and diltiazem. Furthermore, it should also be noted that none of the tested substances caused a significant accumulation of doxorubicin in sensitive A2780 cells (range of variation of doxorubicin content: −8% to 16%, mean 5%). Similarly, none of unselected tested compounds significantly increased the accumulation of doxorubicin in resistant A2780/DX3 cells (% increase accumulation: ≤48%). Roughly, the accumulation in these cases reflected the activity of single substances evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-

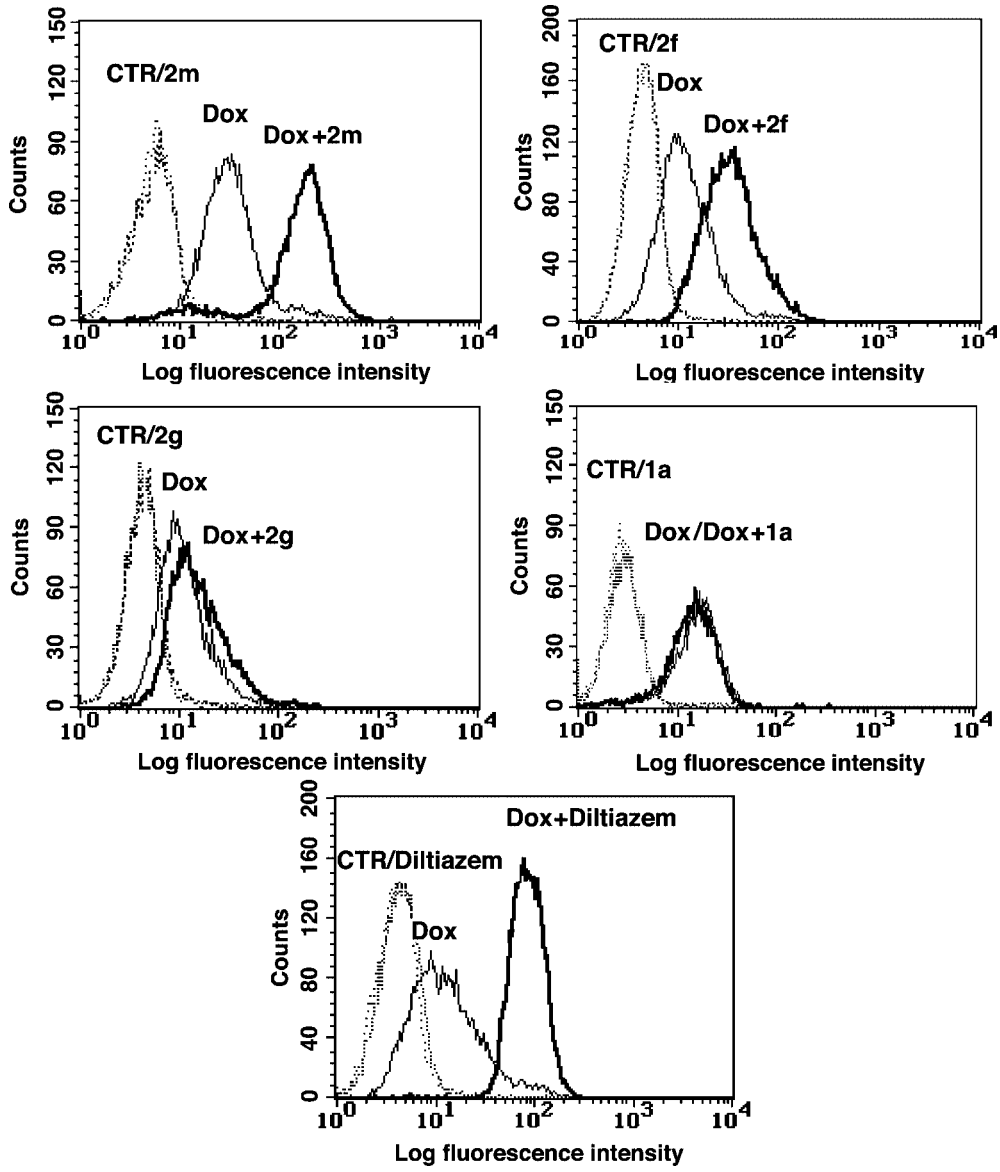


Figure 1. Representative histograms of flow cytometric analysis of diltiazem and diltiazem-like compounds. All histograms are superimposed in order to make evident the shift of doxorubicin content after combination with diltiazem or diltiazem-like compounds. CTR, control; dox, doxorubicin. In these experiments, cells were treated with the selected molecules or diltiazem using concentrations five 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 diluted in PBS containing 2% sucrose, the fluorescence intensity of cells was determined by flow cytometry using 488 nm excitation and 575 nm bandpass filter for doxorubicin detection.

lium bromide] assay. It is also important to remark that when the A2780/DX3 cells were treated with 10 μM doxorubicin, drug accumulation was about 6-fold lower than with the sensitive A2780 cells. In Figure 1, some representative histograms of flow cytometric analysis of **2f**, **2g**, **2m**, **1a**, and diltiazem are shown.

Determination of Doxorubicin Accumulation by Microscopic Analysis. Intracellular accumulation of doxorubicin was confirmed by microscopy analysis of A2780/DX3 cells cotreated with the selected substances. Also in this case, in the presence of compounds **2f**, **2g**, and **2m**, the cells accumulated more doxorubicin than when they were treated with the anticancer drug alone (Figure 2). Microscopy confirmed that compound **1a** did not cause an increase of intracellular accumulation of doxorubicin (data not shown). According to the flow cytometry data, this analysis also showed no apparent drug accumulation when sensitive A2780 cells were cotreated with different substances. Similarly, when A2780/DX3 cells were cotreated with some compounds not selected by the MTT assay (data not shown), they showed limited accumulation of doxorubicin in a mode reflecting their specific activity in the inhibition of cell proliferation as revealed by the MTT test.

Immunofluorescence Analysis of Pgp Expression. None of the selected compounds was able to down-regulate membrane Pgp-170 expression as detected by flow cytometry when they were used at the concentrations giving the maximal inhibition of cell proliferation (data not shown).

Discussion

Multidrug resistance, the biological phenomenon by which tumor cells exposed to a single anticancer drug may become resistant with time to a number of structurally and functionally unrelated compounds, is one of the main culprits of the failure of anticancer chemotherapy. This form of resistance is linked to the overexpression of the cell-membrane ATP-dependent

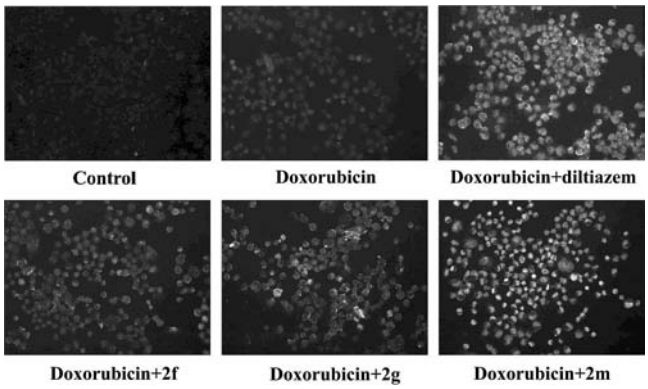


Figure 2. Microscopy evaluation of doxorubicin accumulation in A2780/DX3 cells after cotreatment with diltiazem-like compounds. Cells were treated as described for the cytofluorimetric studies in the presence of doxorubicin at 10 μM with or without previous incubation with diltiazem or diltiazem-like compounds. After fixation with paraformaldehyde in PBS containing 2% sucrose, 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). Successive images were rendered using Adobe Photoshop 5.0 software (Adobe Systems, Mountain View, CA). Magnification, 20X.

transporter Pgp-170. Pgp-170 belongs to the ATP-binding cassette family of transporters, and its overexpression was demonstrated to be frequently involved in human solid and hematologic cancer chemoresistance. Moreover, Pgp-170 may be considered as a bad prognostic parameter in several tumors such as leukemias, lymphomas, osteosarcomas, small-cell lung, breast, and ovarian cancers.[12−15] As already described, Pgp-170 acts as an efflux pump, which may avoid the accumulation of toxic concentrations of anticancer drugs into the malignant cells.[8,9] This molecule is present in many normal epithelial cells, such as those of colon, liver, kidney, and pancreas, and is highly expressed in structures like the placenta and the blood−brain barrier where it is involved in the excretion of toxic exogenous compounds, thus protecting the brain and the fetus from toxic damages.[8,24]

In several tumors, such as those derived from epithelial tissues, the expression of Pgp-170 is generally high and may be responsible for some forms of intrinsic resistance. On the other hand, disregulation of the Pgp-170 expression may also be the cause of frequent forms of acquired resistance. In this case, those cells displaying/having on the cell membrane huge amounts of this drug transporter are selected by a single anticancer drug of the class of anthracyclines, taxanes, *Vinca* alkaloids, podophyllotoxins, or camptothecins.[16,17] This selection allows the tumor to grow and become resistant toward a series of other chemotherapeutics belonging to other classes of anticancer drugs.

Many ways to overcome MDR resistance have been explored in the two last decades, in particular the inhibition of Pgp-170 activity has been considered as a central field of research and tens of new substances of different chemical origin have been studied in vitro and in vivo for their ability to block reversibly or irreversibly the pump function of Pgp-170.[18] In spite of these efforts, only a few of them reached the clinical-test phase.

Of these compounds, those derived from the modification of L-type calcium channel blockers like verapamil, nifedipine, or diltiazem, which otherwise represent a class of molecules synthesized to obtain different characteristics in terms of cardiovascular profiles and antihypertensive properties, are a group of compounds endowed with significant features in terms of inhibition of the Pgp-170 activity. Nevertheless, compounds such as dexverapamil, emopamil, and even nifedipine or diltiazem[20,25] displayed some characteristics that limited their clinical activity. In particular, because of their low binding affinity, at doses necessary to enable the inhibition of Pgp-170, they also inhibit the metabolism and excretion of anticancer drugs, thus causing the onset of toxic phenomena leading to the necessity of a drug−dose reduction.[26] Moreover, it has been reported that their affinity for other enzyme systems and transporters may cause unexpected pharmacokinetic interactions with the coadministered anticancer drugs.[27]

It is in this context that we started a series of experiments in order to analyze the ability of different diltiazem-like derivatives, some of which already demonstrated their good characteristics in terms of cardiodepressant activity,[22,23] to compete with doxorubicin, a known target of Pgp-170 activity, determining its accumulation into A2780/DX3 cells made multidrug-resistant by the continuous exposure to doxorubicin.

Thus, we analyzed the behavior of two series of 8-aryl-8-hydroxy-5-*R′*-8*H*-[1,4]thiazino[3.4-*c*][1,2,4]oxadiazol-3-ones (**1a**−**i**) and 8-aryl-8-alkoxy-5-methyl-8*H*-[1,4]thiazino-[3.4-*c*][1,2,4]oxadiazol-3-ones (**2a**−**m**). They represent two sets of derivatives showing interesting LTCCs blocker activity, whose EC_{50}s, for the active compounds, range between 0.04 and 6.58 μM.

In the first series (**1a**−**i**), the 8*H*-[1,4]thiazino[3.4-*c*][1,2,4]-oxadiazol-3-one scaffold is decorated by different 8-aryl groups, containing as X substituents *meta* and *para* groups with very different electronic properties and effects on the hydrophilic−hydrophobic balance: from the strongly electron-donating *para*-methoxy group (σ_p −0.28 and σ_p^+ −0.78, respectively) to the strongly electron-withdrawing *para*-nitro group (σ_p +0.81 and σ_p^- +1.25, respectively). Decoration can concern also the 5-*R′* substituent, which usually is a methyl group (**1c**−**i**) or a hydrogen atom (**1a,b**). Interestingly enough, all of these compounds are practically inactive with the only exception of **1a**, whose interaction target site is supposedly different from that involved in all of the other active compounds (see below), as also suggested by its significant hydrophilicity (log *P* 0.13). In this series, the calculated log *P* values change only from 0.13 to 1.26.

In the second series (**2a**−**m**), the base structure seems more constant also if the "added" decoration is able to strongly affect the molecular size and the hydrophilic−lipophilic balance as well. In every case, the 8-aryl-5-methyl-8*H*-[1,4]thiazino[3.4-*c*]-[1,2,4]oxadiazol-3-one scaffold is characterized by the fact that the aryl group always contains a *para*-halogen atom (Cl in **2a**, **2d**, **2f**, **2h**, **2i**, **2k**, and **2m**; Br in **2b**, **2c**, **2e**, **2g**, **2j**, and **2l**), while the system is decorated by very different 8-alkoxy groups. They range from short to long linear saturated chains (from C_1 to C_{18}), but also unsaturated chains of medium length (C_5, *E* as well as *Z*) have been considered. The structural variations in the decoration cause very large alterations in the hydrophilic−lipophilic balance, the log *P* values ranging between 1.6 and 10.

All of the substances analyzed were initially tested for their ability to inhibit cell proliferation. Our data clearly show that none of these molecules had IC_{50} values lower than 55 μM, thus indicating their quite low inhibiting activity, also considering that the IC_{50} values for doxorubicin were on average 0.02 and 2.21 μM for A2780 and A2780/DX3, respectively.

Moreover, to select the substances most active in terms of potentiation of doxorubicin activity in A2780/DX3 resistant cells, we arbitrarily decided to consider as active only those compounds that decreased the IC_{50} for doxorubicin by a value

higher than 25% at the inactive or slightly active concentrations, giving 0% or 5% inhibition of cell proliferation. It is important to underline that the choice to select these concentrations to study the effect of our substances on doxorubicin treatment was due to our effort to isolate their effect on Pgp-170. To this regard, it must be also noted that 9 out of 22 tested diltiazem-like molecules showed IC_5 values higher for sensitive A2780 than for resistant A2780/DX3 cells. This apparently unexpected result could be in part explained by considering the possible molecular differences between sensitive and resistant cells probably involving molecular targets other than Pgp-170, the fact that we do not know the possible interaction of our diltiazem-like compounds with other vital molecular constituents of these cells, and finally, that probably not all our substances are in any case efficient substrate for Pgp-170, as our MTT and cytofluorimetric assays suggest.

Our strict conditions allowed the selection of only four compounds that were then "verified" for their ability to increase the intracellular concentration of doxorubicin. The cotreatment of cells with these molecules, in particular **2f**, **2g**, **2m**, and doxorubicin, allowed a significant accumulation of the anticancer drug into the cells, which was particularly high when **2m** was used. It is also worth noting that no accumulation was observed when we used A2780 sensitive cells as targets while when we treated the resistant cells with some of the unselected compounds, in general, we observed lower (also if significant) intracellular accumulation of doxorubicin.

The expression of Pgp-170, studied by flow cytometry using an anti-MDR1 monoclonal antibody, was not down-regulated by the culture of the cells in the presence of the active compounds. This confirmed that the mechanism of action of diltiazem-like compounds was not linked to loss of Pgp-170 from the cellular membrane but was a consequence of the increase of drug accumulation.

Many authors agreed on the fact that hydrophobicity, along with the presence of two or more coplanar aromatic rings,[8] is an important characteristic of chemosensitizers acting as competitive inhibitor of Pgp-170.[28,29] To this regard, it should be noticed that while **2f**, **2g**, and **2m**, characterized by an alkyl chain of medium length (all of them possessing a C_5 skeleton and showing calculated log *P* values in the 3.1−3.7 range), seem to satisfy this assumption, the substantially more hydrophilic **1a** (log *P* 0.13), notwithstanding the presence of the aromatic rings, does not seem to follow the same rule. On the basis of this information and of the fact that, in contrast with **1a**, compounds **2f**, **2g**, and **2m** caused a significant increase of intracellular doxorubicin concentration after cotreatment and, furthermore, that **1a** seems to be more active at lower concentrations in terms of potentiation of doxorubicin inhibition of cell proliferation, we may advance the hypothesis that compound **1a** might influence doxorubicin activity by a mechanism of action different from the competitive interaction with Pgp-170.

Interestingly enough, our data demonstrate that some of our compounds **1** and **2**, previously synthesized with the aim of obtaining hits with LTCCs blocker activity, have also a significant reversing effect on MDR resistance and that this is probably due to a competitive effect with the binding and efflux of doxorubicin at Pgp-170 level. Future studies will be devoted to the analysis in vivo of the activity of the molecules here reported and to the modification of their structures in order to obtain new and more active compounds.

Conclusion

Considering the whole of the obtained results, an attempt of correlation between cardiovascular activity, chemical structure of the tested compounds, and their inhibition of MDR1 activity can be carried out.

As a matter of fact, all of the tested compounds **1** and **2** show significant or good negative inotropic (34−95% decrease) and low chronotropic (2−34% decrease) and vasorelaxant activity (2−19, 37%).

Considering the four most promising compounds, three points must be mentioned. (1) They show quite different hydrophobic character, as their log *P* range from 0.13 for **1a** to 3.1−3.7 for **2f**, **2g**, and **2m** (different target sites or different molecular mechanisms in the two class of compounds could be hypothesized) while compounds with very high hydrophobic character (**2j**, log *P* 10) seem essentially nonefficient. (2) Concerning their negative inotropic effects, one can observe that all of the four compounds are very different in term of potency. As a matter of fact, while the most active **2m** compound shows a significant potency (EC_{50}, 1.31 μM), **1a** seems to be less potent (EC_{50} 6.58 μM) and **2f** and **2g** are still less potent (EC_{50} not calculated). In terms of inhibition of MDR1 activity and consequent intracellular doxorubicin increase, compound **1a** proved to be inactive. In any case, the fact that it was able to potentiate the activity of inhibition of cell proliferation of doxorubicin in resistant cells suggests a different, still unknown, mechanism of action. Part of our future work will be devoted to clarifying this mechanism. (3) All of the three active compounds **2** contain a C_5 linear saturated or unsaturated acetal chain and, moreover, the presence of a chlorine or of a bromine atom in the *para* position of the 8-aryl ring seems to moderately affect the MDR1 activity. Interestingly enough, the *E*-isomers **2k** and **2l** are inactive while the *Z*-isomer **2m** is the most active compound, thus furnishing a first indication on the stereoselectivity of the target site.

The results of this study appear promising, and we think that our next target should be the identification of a structure−activity relationship able to guide us in the search for more active compounds. A structural and functional model of Pgp-170 transporter is far from being completed, as currently only low to medium resolution three-dimensional structures are available, together with a description of a number of binding sites with different characteristics mapped for Pgp-170[30] by means of radioactively tagged photoaffinity drug analogues. Nevertheless, further studies will be devoted to the analysis of the possibly different mechanism of interaction of compounds **2** with Pgp-170 in the perspective of designing new molecules with an even better activity.

Keeping in mind the Lipinski's rule-of-five[31] all of the three hits we found (**2f**, **2g**, and **2m**) obey its four conditions (log *P* values, number of HB-donor and HB-acceptor centers, MW) and appear as probable leads, i.e., good candidates for reaching the state of effective drugs in the "war" against MDR.

We are confident that computational methods (virtual screening, docking, and so on) will be useful to reach the goals proposed above and to address us toward the synthesis of more and more active compounds.

Experimental Section

Chemistry. The compounds tested were stable and fully characterized 8-aryl-8-hydroxy-5-*R*′-8*H*-[1,4]thiazino[3.4-*c*][1,2,4]-

oxadiazol-3-ones **1**[22] or 8-aryl-8-alkoxy-5-methyl-8*H*-[1,4]thiazino[3.4-*c*][1,2,4]oxadiazol-3-ones **2**.[22,23]

The synthesis of **1** was achieved by treating the relevant 6-aryl-5-nitrosoimidazo[2,1-*b*][1,3]thiazoles with hydrochloric acid at reflux in ethanol: the obtained hemiacetals **1** contained a chiral carbon, but their separation in the relevant enantiomers was prevented by their easy enantiomerization. Synthesis of this quite interesting new class of condensed heterocycles was set up more than 10 years ago by some of us.[32−34]

Their transformation into the acetals was realized by reaction with the proper primary alcohol in the presence of catalytic amounts of *para*-toluensulfonic acid in refluxing toluene.[22,23] In the instance of methyl acetals **2a** and **2b**, a mixture of methanol and trimethyl orthoformate was used as alkylating agent. The obtained acetals **2** contain a stable chiral carbon,[23] and the enantiomeric separation has been realized in the instance of **2c**.[35,36] The acetals **2k** and **2m** are a couple of diastereomeric racemates, namely the *E*- and *Z*-isomers, respectively. The enantiomeric separation of **2m** is actually in progress.

Both classes of compounds **1** and **2** have been recently tested for their LTCCs blocker activity,[22,23] and some of them show "in vitro" activity comparable or significantly (20 times) higher than that of diltiazem.

Chemicals. Doxorubicin was obtained in clinical form from Ebewe Italia (Rome, Italy) and diluted in normal saline to the opportune concentrations. Because of the low water solubility of some **1** and **2**, all of the compounds were first dissolved in 100% dimethylsulfoxide (DMSO) and then diluted in fetal calf serum (final concentration DMSO 1−6%). Diltiazem was diluted in water.

Cells. 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. Y. M. Rustum and obtained by exposure to increasing concentrations of doxorubicin) were maintained in complete medium containing 0.1 μM doxorubicin. Two to three days before experiments, doxorubicin was removed from the medium.

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 for 6−8 h. To evaluate the concentrations of **1** and **2** compounds 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). Reference compound diltiazem was also tested. Three days later, 50 μL of MTT (Sigma, St. Louis, MO) solution (2 mg/mL in PBS) was added to each well and incubated for 4 h at 37 °C. Microplates were centrifuged at 275 g for 5 min, culture medium carefully aspirated, and 100 μL of 100% DMSO added. Complete and homogeneous solubilization of formazan crystals was achieved after 20 min of incubation and shaking of well contents. The absorbance was measured on a microculture plate reader 400 ATC (SLT Labinstruments, Austria) at 540 nm.[37] 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 to the opportune concentrations in a medium containing the various molecules (diltiazem and compounds **1** and **2**) at the specific concentrations calculated by the previous MTT analysis for both A2780 and A2780/DX3 cells. 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 also contained concentrations of diltiazem or of diltiazem-like molecules **1** and **2** equal to that already contained in the wells. By this kind of treatment, we obtained a pretreatment 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 4−6 times. Only **1** and **2** compounds giving a significant decrease of doxorubicin IC_{50} higher than 25% were selected for the following experiments.

Cytofluorimetric Study of Intracellular Accumulation of Doxorubicin. A2780 and A2780/DX3 cells were plated in 25 cm^2 flasks at 1×10^6 and 1.5×10^6 cells/flask, respectively, in 10 mL. Then 24 hours later, when cells reached about 75−85% confluence, they were treated with **1** and **2** or with diltiazem drug using concentrations five times higher than that giving 5% inhibition of cell proliferation, as calculated in the MTT assay. After 2 h, doxorubicin was added 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, and the incubation performed for additional 2 h. Cells were then detached by trypsin at 37 °C for 5 min, 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 diltiazem or **1** and **2** were assayed as well. 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).

Microscopy Study of Intracellular Accumulation of Doxorubicin. 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 (usually after 2 day culture), they were treated as described for the cytofluorimetric studies except for the doxorubicin concentration that was 10 μM for both cell lines. Once 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 **1** and **2** or with diltiazem) 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) using the Axiovision Software (Carl Zeiss, Jena, Germany).

Immunofluorescence Study of Pgp-170 Expression. A2780 and A2780/DX3 cells were treated for 72 h with the concentrations giving 5% or 0% inhibition of cell proliferation of selected compounds in order to study their down-regulating activity of Pgp-170 expression. Cells were then harvested and washed 3 times with PBS plus 2% fetal calf serum. Pelletted 2.5×10^5 cells were then incubated at 4 °C with 25 μL of anti-Pgp MM4.17[38] monoclonal antibody, washed 3 times with PBS plus 2% fetal calf serum, and incubated again with a FITC-labeled goat antimouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). After three more washes in washing buffer, cells were ready to be analyzed by flow cytometry (FACScan, BD Biosciences, Milano, Italy).

Statistics. The Kruskal−Wallis and the Mann−Whitney tests for nonparametric data were used for statistical analysis (StatView 4.5 software, Abacus Concepts Inc., Burlington, MA). The resistance indexes were calculated as the ratio between the mean IC_{50} of resistant and that of sensitive wild-type cells. The partition coefficient log *P* was calculated by the software Chemdraw Pro (version 10.1).

Acknowledgment. This research was supported by funds from MIUR (FIRB 2001, PRIN-2005034305_001, PRIN-20078J9L2A_005) and from the Universities of Bologna, Genova, and Napoli.

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JM801195K