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Multidrug resistance reverting activity and antitumor profile of new phenothiazine derivatives

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ABSTRACT

A series of easily affordable phenothiazine derivatives bearing a rigid but-2-ynyl amino side chain were synthesized and tested to evaluate the MDR reverting activity and full antitumor profile. Some compounds endowed with remarkable MDR reverting effect were identified, and the most active one (**6c**) was shown to increase doxorubicin retention in multidrug resistant cells, suggesting a direct interaction with P-glycoprotein. Furthermore, a broad range of cellular activities were observed for different compounds. In particular, the ability of some derivatives to induce antiproliferative effects on resistant cell lines and to interfere with the G_1 phase of the cell cycle, a phase usually not affected by classical antitumor agents, was noted. Moreover, the most cytotoxic compounds of the series were able to induce apoptosis in resistant cell lines, via an atypical pathway of caspase cascade activation, and a synergistic effect in combination with doxorubicin was also found.

1. Introduction

Resistance to multiple chemotherapeutic agents is a common and still unsolved clinical problem in the treatment of cancer. The identification of new experimental strategies involving novel revertant agents or drugs capable of killing resistant cells is, therefore, of utmost interest in cancer research. The phenomenon known as multidrug resistance (MDR) can arise de novo or after exposure of cancer cells even to a single drug and is characterized by resistance to a series of structurally unrelated compounds with different subcellular targets.[1] The best characterized mechanism contributing to MDR is the overexpression of the energy dependent efflux pump, P-glycoprotein (P-gp, also known as ABCB1), a 170 kDa protein belonging to the ATP binding cassette (ABC) superfamily of transporters and capable of preventing drugs, such as anthracyclines or Vinca alkaloids, to reach effective concentrations within the cells.[2] However, other proteins of the same family such as the multidrug resistance associated protein (MRP1, also named ABCC1), the lung resistance protein (LRP), and the breast cancer resistance protein (BCRP, also named ABCG2) have also been implicated.[3–5] A large variety of compounds (anticancer agents, calcium channel blockers, neuroleptics, antiarrhythmics, antimalarial drugs, antifungal agents)[6–9] with the properties of inhibiting P-gp were selected by different approaches and limited common features for the interaction of ligands with this protein were defined: protonable nitrogen, aromatic rings, high lipophilicity, and H-bond interactions.[10,11] Moreover, there are indications that, besides the overall lipophilicity of the molecule, weak interactions, such as those produced by the overlapping of π orbitals of aromatic rings, can play an important role in stabilizing the binding of MDR reverting agents to P-gp.[12,13] Despite extensive studies, the promise of this field of investigations has not been fulfilled yet and there are currently no clinically available reversal agents.

Furthermore, it is increasingly recognized that other tumoral factors can significantly contribute to multidrug resistance such as failure to undergo apoptosis and/or kinetic properties of neoplasias[14,15]: in fact, it is well known that the effects of most classical antiproliferative agents are cell cycle dependent.[16] So, with progresses in understanding the basic molecular mechanisms underlying cell cycle regulation and apoptosis and how these processes are impaired in tumor cells, recent research has been addressed to identify molecules capable of interfering directly with the specific intracellular targets involved such as cyclines, CDK, Bcl-2, IAPs, etc.[17,18]

The chemical structure of phenothiazine (PTZ) provides a valuable molecular template for the development of agents able to interact with a wide variety of biological processes, and synthetic PTZs and/or PTZs-derived agents have proved to be effective in the treatment of a number of medical conditions with widely different etiologies.[19] In particular, these compounds possess some significant characteristics which suggest a potential use as antitumor drugs, because they show an inherent cytotoxicity and

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structural modifications involving the introduction of a nitrogen atom that could lead to more potent cytotoxic agents.[20] The role of PTZs as chemosensitizers in multidrug resistance has been clearly assessed, and several studies have been performed in order to establish the structural features determining their anti-MDR activity.[21,22] Usually, structural modifications that increased lipophilicity of PTZs also increased MDR reverting potency, and it has been found that both the type of substituent in position 2 of the PTZ ring and the length of the alkyl bridge connecting the ring system with the amino group play some role in the reverting activity. In particular, a four carbon atom side chain was superior to alkyl bridges of shorter length, and tertiary cyclic amines, mainly piperazine, were preferred to primary or secondary ones.[23,24]

In this paper, a series of new PTZ derivatives bearing a rigid side chain and different tertiary amines were designed and synthesized in order to assess the MDR reverting effect. The rigid four carbon atom side chain was selected because it had been successfully introduced in EDP 42 (Chart 1), a Verapamil analog with an improved reverting activity[25]; we have also recently reported new MDR modulators bearing the same chain.[26] The amino groups were chosen considering the structure–activity relationship (SAR) studies previously performed on PTZs and Verapamil itself. Moreover, the two-step synthesis of the new compounds proved to be simple and straightforward. In view of the results obtained from the preliminary data on cytotoxicity, the full antitumor profile of PTZ derivatives was also deeply investigated. The structures of the compounds are collected in Table 1.

2. Chemistry

The synthesis of compounds **5a**–**h**, **6a**–**c**, **7a**–**c** is depicted in Scheme 1. The selected PTZ was alkylated with propargyl bromide by means of potassium *tert*-butoxide in DMSO. The final compounds were then obtained via the Mannich reaction, refluxing the propargyl intermediates **2**–**4** with the selected amine and formaldehyde. The amines which were not commercially available were prepared by conventional methods. All the final compounds were characterized by ^{1}H NMR, mass spectra, and elemental analyses.

3. Biology

The PTZ derivatives were tested for cytotoxicity and, afterwards, for reverting activity in combination with doxorubicin (DXR), employing two hematological tumor cell lines, the HL60 and the CCRF/CEM, and their MDR variants HL60R and CEM/VBL300. When a significant cytotoxic effect was found, the influence on the cell cycle and apoptosis induction was evaluated in order to establish the possible mechanism involved.

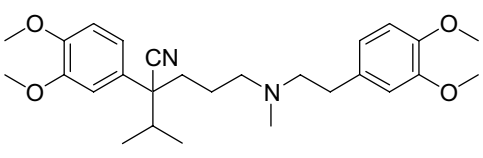
4. Results

4.1. Cytotoxic activity

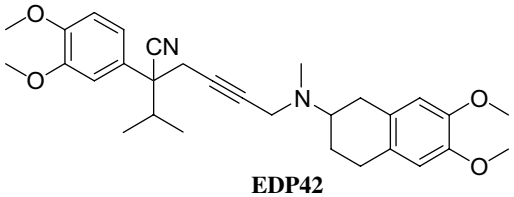
In order to identify the maximal 'non-cytotoxic' concentrations of each PTZ derivative to be used in combination with DXR for MDR modulation, all the synthesized compounds were tested for direct cytotoxicity in a preliminary in vitro assay against two human leukemia cell lines, the HL60 and the CCRF/CEM, and their MDR variants. The results indicated that most compounds were devoid of significant antiproliferative effects on the sensitive cell lines at concentrations ranging from 0.1 to 10 μM, with the only exceptions of **5f** and **5h**, that were able to kill about 50% of the cells exposed when employed at 10 μM (data not shown). A different profile was observed in resistant leukemia cell lines: several compounds showed significant cytotoxic effects, **5f**, **5h**, **5a** and **6a** being the most active ones (Table 2). Furthermore, for these latter molecules the effect appeared to be concentration dependent, with IC_{50} values calculated on the HL60R cell line of 1.1, 5.0, 9.5, and 10 μM, respectively. Thus, these particular compounds showed to induce a higher cytotoxicity in resistant rather than in sensitive tumor cells as reported also for other modulators.[27,28] For a deeper evaluation of this interesting cytotoxic behavior, the effect of these compounds on cell distribution in the different cell cycle phases in the resistant HL60R cells was also measured by flow cytometry. The results, collected in Table 3, indicated, for all the tested compounds, the ability to recruit cells in G_1, a cell cycle phase usually unaffected by the classic anticancer agents.

4.2. MDR modulating activity

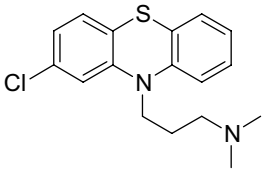
To establish the ability of the compounds to inhibit P-gp activity, non-cytotoxic concentrations were tested in MDR cell lines to assess their ability and to restore DXR sensitivity. The results, collected in Table 4, show a different degree of reverting activity by different compounds. In particular, compounds **5d**, **5e**, and **6c** were the most effective, reducing the IC_{50} of DXR by 4-, 6-, and 8-fold, respectively, in the HL60R cell line. This effect was concentration dependent, being the maximum at the dose of 10 μM (data not shown); overall, the new PTZs were more effective at sensitization than the reference compound chlorpromazine, even if they were less effective than Verapamil (Chart 1 and Table 4). Similar results were obtained in the CEM/VBL300 cell line.



Verapamil



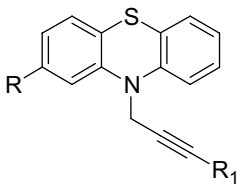
EDP42

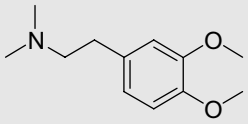
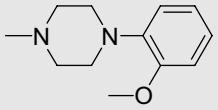
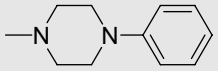
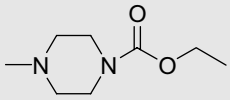
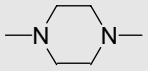
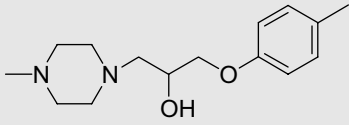
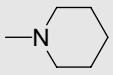
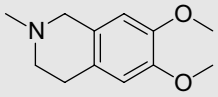
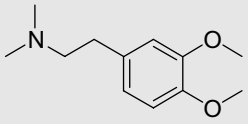
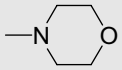
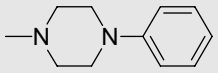
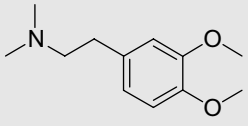
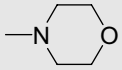
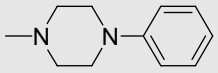


Chlorpromazine

Chart 1.

Table 1
Synthesized compounds



Compound	R	R_1
5a	H	
5b	H	
5c	H	
5d	H	
5e	H	
5f	H	
5g	H	
5h	H	
6a	Cl	
6b	Cl	
6c	Cl	
7a	CF_3	
7b	CF_3	
7c	CF_3	

To assess the real involvement of P-gp in reverting activity, the intracellular accumulation of DXR in sensitive and resistant cell lines was evaluated, with or without the most active compound of the series (**6c**). As shown in Figure 1, this new PTZ derivative was able to significantly increase the accumulation of DXR in resistant cells, even if the number of resistant cells capable of accumulating the drug (about 70%) did not reach the level observed in the sensitive cell line (about 100% accumulating cells).

4.3. Apoptotic effects

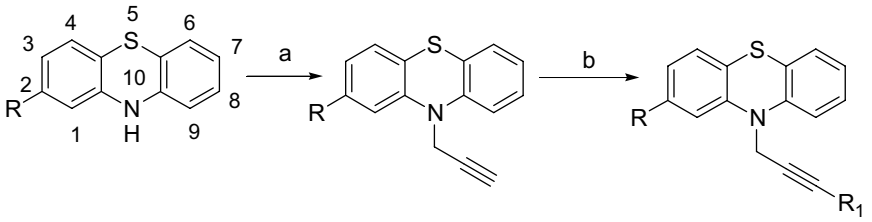
Since some PTZ elicited cytotoxic effects in resistant cells as described above, we wondered whether these effects could be ascribed to apoptosis induction by the compounds in HL60R cells. This finding could be relevant because HL60R cells were shown to be resistant also to apoptosis induction by different stimuli.[15] Interestingly, the morphological analysis showed a concentration-dependent induction of apoptosis by the compounds endowed with a high cytotoxic effect **5a**, **5f**, and **6a** (data not shown). Hence, further studies have been performed, employing different caspase inhibitors, to establish the pathways involved in this phenomenon. As shown in Figure 2, the apoptotic effect was inhibited by the pancaspase inhibitor Z-VAD, but not by other inhibitors such as Z-IETD, Z-LEHD, and Z-DEVD (inhibitors of caspase 8, 9, and 3, respectively). This could mean that PTZ derivatives were able to activate the caspase cascade, but neither the classic extrinsic (caspase 8-mediated) nor the intrinsic (caspase 9-mediated) pathways seem to be implicated.

4.4. Synergistic effects between cytotoxic derivatives and doxorubicin

Furthermore, we tested whether the PTZ compounds that were found to be endowed with a cytotoxic activity could induce synergistic effects when combined with DXR in resistant cell lines. To this purpose, the resistant cells were exposed to increasing concentrations of DXR combined with PTZ compounds at concentrations capable of inducing a moderate cytotoxic effect (less than 50%). As reported in Figures 3 and 4, the cytotoxic effect of all the associations tested appeared significantly higher than the predicted values in both HL60R and CEM/VBL300 cells, demonstrating a synergistic interaction. However, the combinations elicited an additive effect, when DXR was employed at very low concentrations. The synergistic effect did not appear to be related to an increase in apoptotic cell death as shown in Figure 5. On the contrary, most of the treated cells presented necrotic morphologic alterations (data not shown).

5. Discussion

The present study describes the identification and activity of a number of novel compounds derived from phenothiazines designed to selectively circumvent multidrug resistance. From the results obtained, it can be seen that the new derivatives, tested at non-cytotoxic concentrations, showed different efficacies as MDR modulators. The most active compounds appeared to be **5d**, **5e** and **6c**, which were able to significantly, though only partially, restore the sensitivity to DXR in the resistant cell lines. This is in agreement with the accumulation assay: the resistant cells treated with PTZ derivatives showed an increase in DXR retention, without reaching the levels observed in sensitive cell lines. Even if this result could be due to the fact that further increasing in concentrations was limited by PTZs cytotoxicity, it could also be related to the activation of resistance pathways independent from P-gp: indeed, several literature data indicate multiple mechanisms involved in MDR development.[14,15]



Scheme 1. Reagents and conditions: (a) potassium *tert*-butoxide, propargyl bromide, DMSO, rt; (b) selected amine, formaldehyde, $CuSO_4$, EtOH/H_2O, reflux. R_1, see Table 1.

Table 2
Cytotoxicity of PTZ derivatives (10 μM) in resistant HL60R and CEM/VBL300 cells[a]

Compound	Cytotoxicity (%)	
	HL60R	CEM/VBL300
5a	54	41
5b[b]	0	0
5c	25	1
5d	8	15
5e	6	2
5f	97	93
5g	45	28
5h	62	53
6a	52	42
6b	9	7
6c	10	7
7a	36	26
7b	13	0
7c	35	24

[a] Cells viability was evaluated by the MTS test as described in Section 7; hence, % of cytotoxicity was calculated according to the formula $100 - N_t/N_c \times 100$ where N_t is the number of cells in the treated well and N_c is the number of cells in the control.
[b] This compound was tested at 1.0 μM because of low solubility.

Table 3
Effect of PTZ derivatives on cell cycle distribution in HL60R cells[a]

Treatment	G_1 (%)	S (%)	G_2-M (%)
None	37	49	13
5f, 1 μM	54	34	12
5h, 5 μM	57	32	11
5a, 10 μM	67	25	8
6a, 10 μM	64	26	10

[a] The cells were exposed to the compounds at the indicated concentrations for 24 h and examined by flow cytometry as described in Section 7.

From these figures, it is difficult to draw clear structure–activity relationships, because some compounds of this series were tested at different concentrations due to their cytotoxicity, leading to barely comparable data. According to the previously reported SAR studies for PTZs,[23] the most interesting compounds were expected to be **6a**, **6c**, **7a**, and **7c**, bearing a Cl or a CF_3 substituent in position 2 and showing also the highest log*P* value. Moreover, all the derivatives bearing a piperazine amino group were active as MDR modulators, while the morpholine derivatives **6b** and **7b** showed poor activity in this series: this amine led to a decrease in log*P* values and, as expected, a consequent decrease in MDR reverting properties. Unfortunately, compounds **6a**, **7a**, and **7c** were too cytotoxic to be properly evaluated, but compound **6c**, a 2-chlorine PTZ derivative bearing a phenylpiperazine in the side chain, was able to reduce the IC_{50} of DXR 8-fold, and can therefore be considered the most effective of the series as a reverting agent.

Table 4
Influence of PTZ derivatives on DXR cytotoxicity in multidrug resistant cells

Modulator	μM	IC_{50} DXR (μg/ml)[a]		RRI[b]	
		HL60R	CEM/VBL300	HL60R	CEM/VBL300
None	—	6.0	1.0		
Chlorpromazine	10	3.2	0.45	1.9	2.2
Verapamil	10	0.21	0.09	28.0	10.7
5a	1	2.8	0.34	2.1	2.9
5b	1	9.1	0.97	0.66	1.03
5c	10	1.85	0.69	3.2	1.4
5d	10	1.5	0.26	4.0	3.8
5e	10	1.1	0.3	5.9	3.3
5f	0.1	5.64	0.41	1.1	2.4
5g	1	3.9	0.97	1.5	1.03
5h	0.1	6.0	0.44	1.0	2.3
6a	1	2.1	0.63	2.8	1.6
6b	10	2.5	0.52	2.4	1.9
6c	10	0.76	0.13	7.9	7.7
7a	1	2.9	0.6	2.1	1.7
7b	10	3.7	0.71	1.6	1.4
7c	1	2.1	0.78	2.8	1.3

[a] IC_{50}: growth inhibitory concentration 50%.
[b] RRI: resistance reverting index determined as the ratio of the IC_{50} for DXR alone in resistant cells divided by the IC_{50} for DXR in the presence of various PTZs.

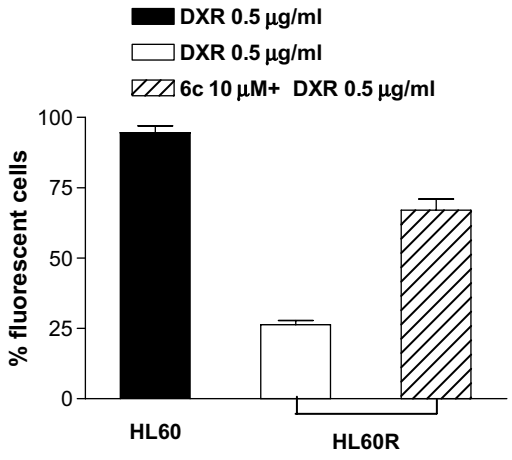


Figure 1. Influence of **6c** on DXR accumulation in HL60R cells. HL60R cells were exposed for 1 h to DXR alone (white bar) or to **6c** for 1 h followed by a 1-h exposure to DXR (hatched bar). The black bar shows DXR accumulation in the sensitive HL60 cells exposed to DXR alone. After treatment the cells were washed with cold PBS and examined by flow cytometry. Fluorescence of cells exposed to **6c** alone was similar to the control.

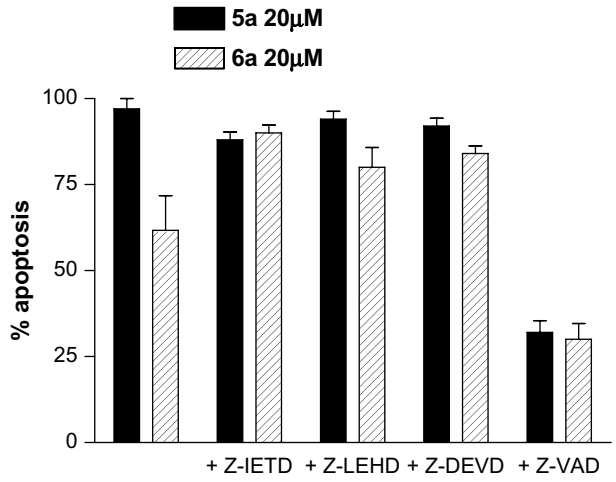


Figure 2. Effect of several caspase inhibitors on PTZ induced apoptosis in HL60R cells. Cells were pretreated with caspase inhibitors for 2 h and then exposed to the PTZ derivatives at the concentrations indicated for 48 h. Apoptosis was evaluated morphologically as described in the text.

Particularly interesting results were obtained with the introduction of a *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-methyl-amino group, the same of Verapamil, on the butynic side chain as in compounds **5a**, **6a**, and **7a**. While it was difficult to evaluate the reverting properties of these three compounds, they showed remarkable direct cytotoxicity in MDR cell lines, the ability to trigger apoptosis and a synergistic effect with DXR. Similarly, various authors reported that a number of modulators were selectively toxic to MDR cells.[27] This effect was independent from the ability of the compounds to reverse resistance, even though P-gp overexpression was considered a relevant factor. Furthermore, some modulators were reported to induce cytotoxicity by causing ATP depletion in MDR cells.[28] In our work, the ability of the compounds to induce apoptosis in an apoptosis resistant cell line was a very relevant finding that still needs to be fully explained at the molecular level. Finally, it is worth noting that our results suggest that these latter compounds, although not suitable as MDR modulators due to a high cytotoxic activity, could be successfully combined with DXR inducing synergistic effects. In our view, this interesting synergism could be partially ascribed to the ability of PTZs and DXR to act on different phases of the cell cycle as well as to an increase in intracellular DXR concentration induced by PTZs with a potentiation of DXR cytotoxicity.

6. Conclusions

A series of readily affordable PTZ derivatives bearing a rigid side chain and different amines were synthesized tested to evaluate the MDR reverting activity and antitumor profile. The results showed that some compounds were endowed with a remarkable MDR reverting effect, compound **6c** being the most interesting. Furthermore, several molecules, such as **5a**, **5f**, **5h** and **6a**, proved to be capable of inducing cytotoxic effects in resistant cell lines by interfering with the cell cycle in the G_1 phase, usually not affected by classical antitumor agents. Finally, it was interesting to note that the cytotoxic compounds demonstrated to trigger apoptosis via an atypical pathway of caspase activation and to induce synergistic effects when combined with DXR in resistant cell lines.

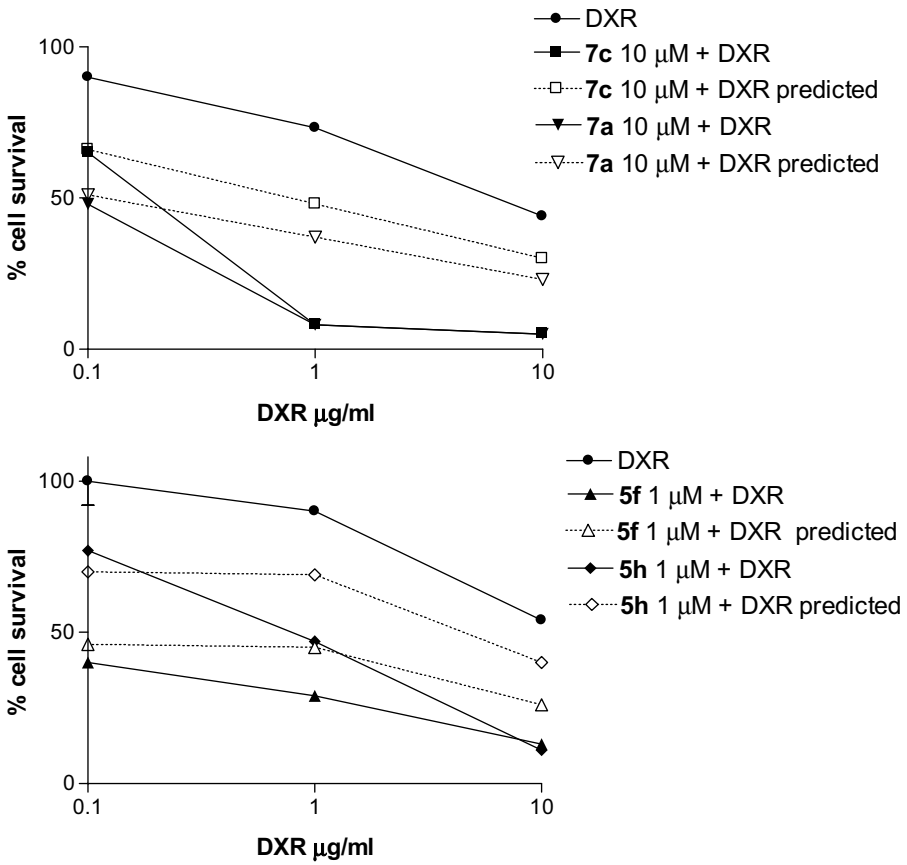


Figure 3. Effects of PTZ derivatives + DXR in HL60R cells. Cells were treated with the PTZ derivatives and after 1 h increasing concentrations of DXR were added. Cytotoxicity was evaluated after 72 h by MTS. Dashed lines indicate additive effect calculated according to the fractional product method of Webb. Survival values of combinations laying below the additivity lines represent a synergistic effect.

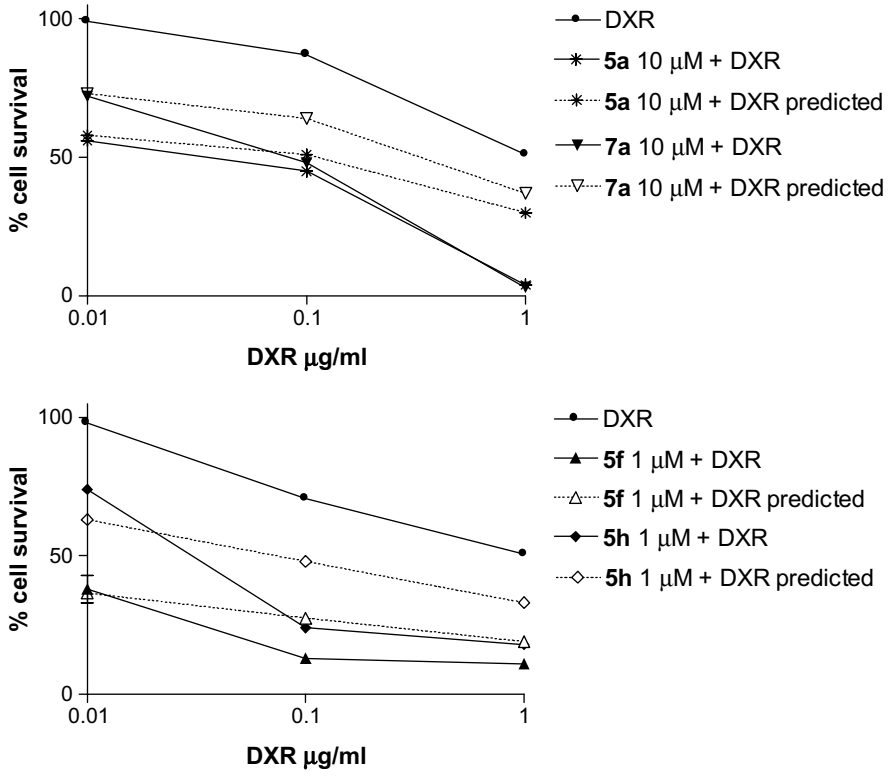


Figure 4. Effects of PTZ derivatives + DXR in CEM/VBL300 cells. Cells were exposed to the PTZ derivatives for 1 h and then increasing concentrations of DXR were added. Cytotoxicity was evaluated by MTS after 72 h. Dashed lines indicate additive effect calculated according to the fractional product method of Webb. Survival values of combinations laying below the additivity lines represent a synergistic effect.

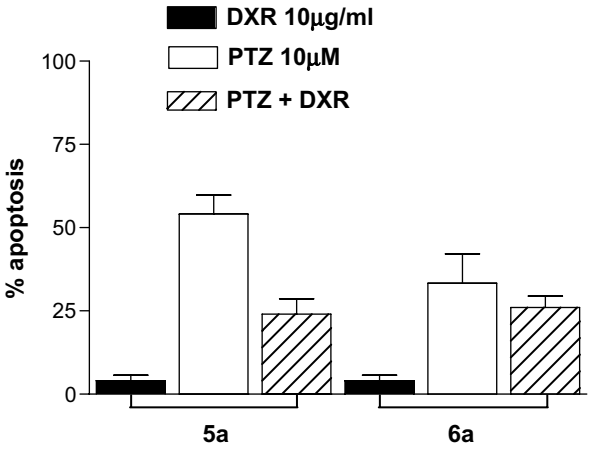


Figure 5. Influence of PTZ derivatives on DXR induced apoptosis in HL60R cells. Cells were exposed to the PTZ derivatives for 1 h followed by 48 h treatment with DXR. Apoptosis was evaluated by fluorescence microscopy.

7. Experimental

7.1. Chemistry: general methods

All melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Gemini 300 spectrometer in $CDCl_3$ solutions, with Me_4Si as the internal standard. Mass spectra were recorded on a V.G. 7070 E spectrometer or on a Waters ZQ 4000 apparatus operating in electrospray (ES) mode. Elemental analyses were within 0.4% of theoretical value unless otherwise indicated. Compounds were named following IUPAC rules as applied by AUTONOM, a PC software for systematic names in organic chemistry, Beilstein-Institut and Springer.

7.1.1. 10-Prop-2-ynyl-10*H*-phenothiazine (2)

Four grams (0.036 mol) of potassium *tert*-butoxide were added to a suspension of phenothiazine (5 g, 0.025 mol) in 15 mL of DMSO and the mixture was stirred at room temperature for 30 min. The solution obtained was then added dropwise to a solution of propargyl bromide (4 mL, 0.034 mol) in DMSO, stirred at room temperature for 3 h and poured into ice. The aqueous solution was extracted with methylene chloride, which was then washed with water, dried with Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (cyclohexane/methylene chloride 9:1), to yield 3.8 g (65%) of **2**, mp 80–82 °C (ligroin; lit[29] 92 °C). ^{1}H NMR: δ 2.45 (t, J = 3.0 Hz, 1H), 4.50 (d, J = 3.2 Hz, 2H), 6.90–7.30 (m, 8H arom).

7.1.2. 2-Chloro-10-prop-2-ynyl-10*H*-phenothiazine (3)

Using the previous procedure, 4.0 g (60%) of **3** were obtained, mp 79–81 °C (ligroin; lit[30] 112–115 °C). ^{1}H NMR: δ 2.55 (t, J = 3.1 Hz, 1H), 4.50 (d, J = 3.2 Hz, 2H), 6.95–7.40 (m, 7H arom).

7.1.3. 10-Prop-2-ynyl-2-trifluoromethyl-10*H*-phenothiazine (4)

Using the previous procedure, 5.3 g (70%) of **4** were obtained, mp 84–86 °C (ligroin). ^{1}H NMR: δ 2.55 (t, J = 3.0 Hz, 1H), 4.50 (d, J = 3.2 Hz, 2H), 6.95–7.20 (m, 7H arom).

7.2. General procedure for the synthesis of compounds 5a–h, 6a–c, and 7a–c

A suspension of formaldehyde (0.21 mL, 0.02 mol), the selected amine (0.02 mol), and 0.05 g of $CuSO_4$ was added to a solution of

the propynyl derivative (**2**–**4**) (0.7 g, 0.02 mol) in 10 mL of water/ethanol 1:1, and the mixture was refluxed for 24 h. After cooling, 15 mL of ammonia was added and the mixture was extracted with diethylether, dried with Na_2SO_4, and evaporated to dryness, to give an oily compound which was purified by flash chromatography. Where indicated, the oxalate salt was prepared dissolving the compound in ethanol and adding the equivalent amount of oxalic acid. After stirring for 30 min at rt, the mixture was filtered and diethyl ether was added until crystallization occurred. The compounds were obtained with yields ranging from 55% to 70%.

7.2.1. [2-(3,4-Dimethoxyphenyl)ethyl]methyl-(4-phenothiazin-10-yl-but-2-ynyl)amine (5a)

The compound was purified by flash chromatography (toluene/acetone 4:1) and converted to the oxalate salt, mp 185–187 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.35 (s, 3H), 2.65–2.75 (m, 4H), 3.45 (s, 2H), 3.80 (s, 3H), 3.85 (s, 3H), 4.60 (t, J = 4.2 Hz, 2H), 6.65–7.30 (m, 11H, arom). MS: *m/z* (rel. abundance) 444 (M^+, 16.4%), 32 (100%), 266 (70.7%). Anal. ($C_{27}H_{28}N_2O_2S$) Calcd: C, 72,94; H, 6,35; N, 6,30; found: C, 72,92; H, 6,36; N, 6,29.

7.2.2. 10-{4-[4-(2-Methoxyphenyl)piperazin-1-yl]but-2-ynyl}-10*H*-phenothiazine (5b)

The compound was purified by flash chromatography (toluene/acetone 9:1) and converted to the oxalate salt, mp 137–139 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.75–2.85 (m, 4H), 3.15–3.25 (m, 4H), 3.45 (s, 2H), 3.90 (s, 3H), 4.60 (s, 2H), 6.90–7.30 (m, 12H arom). MS: *m/z* (rel. abundance) 441 (M^+, 7.7%), 44 (100%), 136 (67.7%). Anal. ($C_{27}H_{27}N_3OS$) Calcd: C, 73.44; H, 6.16; N, 9.52; found: C, 73.42; H, 6.15; N, 9.53.

7.2.3. 10-[4-(4-Phenylpiperazin-1-yl)but-2-ynyl]-10*H*-phenotiazine (5c)

The compound was purified by flash chromatography (toluene/acetone 4:1) and converted to the oxalate salt, mp 118–120 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.65–2.75 (m, 4H), 3.15–3.25 (m, 4H), 3.40 (t, J = 4.1 Hz, 2H), 4.55 (t, J = 4.1 Hz, 2H), 6.80–7.30 (m, 13H arom). MS: *m/z* (rel. abundance) 411 (M^+, 33.9%), 198 (100%), 56 (20.1%). Anal. ($C_{26}H_{25}N_3S$) Calcd: C, 75.88; H, 6.12; N, 10.21; found: C, 75.90; H, 6.10; N, 10.22.

7.2.4. 4-(4-Phenothiazin-10-yl-but-2-ynyl)piperazine-1-carboxylic acid ethyl ester (5d)

The compound was purified by flash chromatography (toluene/acetone 4:1) and converted to the oxalate salt, mp 157–159 °C (methanol/diethylether). ^{1}H NMR (free base): δ 1.25–1.35 (m, 3H), 2.45–2.55 (m, 4H), 3.40 (t, J = 4.2 Hz, 2H), 3.50–3.65 (m, 4H), 4.10–4.20 (m, 2H), 4.60 (t, J = 4.2 Hz, 2H), 6.90–7.20 (m, 8H arom). MS: *m/z* (rel. abundance) 407 (M^+, 23.0%), 198 (100%), 45 (58.9%). Anal. ($C_{23}H_{25}N_3O_2S$) Calcd: C, 67.79; H, 6.18; N, 10.31; found: C, 67.81; H, 6.20; N, 10.33.

7.2.5. 10-[4-(4-Methylpiperazin-1-yl)but-2-ynyl]-10*H*-phenothiazine (5e)

The compound was purified by flash chromatography (toluene/ethyl acetate 3:2) and converted to the oxalate salt, mp 216–220 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.40 (s, 3H), 2.55–2.80 (m, 8H), 3.40 (t, J = 5.4 Hz, 2H), 4.60 (t, J = 5.4 Hz, 2H), 7.00–7.35 (m, 8H arom). MS: *m/z* (rel. abundance) 349 (M^+, 16.7%), 198 (100%), 150 (24.9%). Anal. ($C_{21}H_{23}N_3S$) Calcd: C, 72.17; H, 6.63; N, 12.02; found: C, 72.15; H, 6.60; N, 12.00.

7.2.6. 1-[4-(4-Phenothiazin-10-yl-but-2-ynyl)piperazin-1-yl]-3-*p*-tolyloxy-propan-2-ol (5f)

The compound was purified by flash chromatography (toluene/ethylacetate 1:1) and converted to the hydrochloride salt, mp 232–235 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.30 (s, 3H), 2.50–2.75 (m, 8H), 2.85–2.95 (m, 2H), 3.40 (s, 2H), 4.00 (t, J = 3.8 Hz, 2H), 4.20–4.30 (m, 1H), 4.55 (s, 2H), 6.60–7.25 (m, 12H arom). ES-MS: *m/z* 500 (M+1). Anal. ($C_{30}H_{33}N_3O_2S$) Calcd: C, 72,11; H, 6,66; N, 8,41; found: C, 72.09; H, 6.70; N, 8.40.

7.2.7. 10-(4-Piperidin-1-yl-but-2-ynyl)-10*H*-phenotiazine (5g)

The compound was purified by flash chromatography (toluene/acetone 4:1) and converted to the oxalate salt, mp 191–193 °C (methanol/diethylether). ^{1}H NMR(free base): δ 1.35–1.45 (m, 2H), 1.60 (t, J = 4.2 Hz, 4H), 2.50 (t, J = 4.2 Hz, 4H), 3.35 (t, J = 3.7 Hz, 2H), 4.55 (t, J = 3.7 Hz, 2H), 6.90–7.30 (m, 8H arom). ES-MS: *m/z* 335 (M+1). Anal. ($C_{21}H_{22}N_2S$) Calcd: C, 75.41; H, 6.63; N, 8.38; found: C, 75.40; H, 6.65; N, 8.40.

7.2.8. 10-[4-(6,7-Dimethoxy-3,4-dihydro-1*H*-isoquinolin-2-yl) but-2-ynyl]-10*H*-phenothiazine (5h)

The compound was purified by flash chromatography (petroleum ether/ethylacetate 9:1) and crystallized from ethanol, mp 136–138 °C. ^{1}H NMR: δ 2.90 (d, J = 3.2 Hz, 4H), 3.55 (t, J = 3.4 Hz, 2H), 3.70 (s, 2H), 3.80 (s, 3H), 3.85 (s, 3H), 4.50 (t, J = 3.4 Hz, 2H), 6.55–7.35 (m, 10H arom). ES-MS: *m/z* 443 (M+1). Anal. ($C_{27}H_{26}N_2O_2S$) Calcd: C, 73.27; H, 5.92; N, 6.33; found: C, 73.25; H, 5.94; N, 6.30.

7.2.9. [4-(2-Chlorophenothiazin-10-yl)but-2-ynyl]-[2-(3,4-dimethoxyphenyl)ethyl]methylamine (6a)

The compound was purified by flash chromatography (toluene/acetone 4:1) and converted to the oxalate salt, mp 165–167 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.45 (s, 3H), 2.65–2.75 (m, 4 H), 3.50 (t, J = 3.2 Hz, 2H), 3.80 (s, 3H), 3.85 (s, 3H), 4.50 (t, J = 3.3 Hz, 2H), 6.65–7.20 (m, 10H arom). MS: *m/z* (rel. abundance) 478 (M^+, 14.3%), 95 (100%), 327 (68.4%). Anal. ($C_{27}H_{27}ClN_2O_2S$) Calcd: C, 67.70; H, 5.68; N, 5.85; found: C, 67.72; H, 5.67; N, 5.86.

7.2.10. 2-Chloro-10-(4-morpholin-4yl-but-2-ynyl)-10*H*-phenothiazine (6b)

The compound was purified by flash chromatography (toluene/acetone 4:1) and converted to the oxalate salt, mp 182–184 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.55–2.65 (m, 4H), 3.40 (d, J = 4.2 Hz, 2H), 3,70–3.80 (m, 4H), 4.55 (d, J = 4.3 Hz, 2H), 6.90–7.25 (m, 7H arom). MS: *m/z* (rel. abundance) 370 (M^+, 31.1%), 45 (100%), 232 (92.0%). Anal. ($C_{20}H_{19}ClN_2OS$) Calcd: C, 64.77; H, 5.16; N, 7.55; found: C, 64.78; H, 5.14; N, 7.56.

7.2.11. 2-Chloro-10-[4-(4-phenylpiperazin-1-yl)but-2-ynyl]-10*H*-phenothiazine (6c)

The compound was purified by flash chromatography (toluene/acetone 9:1) and converted to the oxalate salt, mp 101–103 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.65–2.75 (m, 4H), 3.20–3.35 (m, 4H), 3.40 (t, J = 3.5 Hz, 2H), 4.50 (t, J = 3.5 Hz, 2H), 6.90–7.30 (m, 12H arom). MS: *m/z* (rel. abundance) 445 (M^+, 8.6%), 32 (100%), 233 (65.6%). Anal. ($C_{26}H_{24}ClN_3S$) Calcd: C, 70.02; H, 5.42; N, 9.42; found: C, 70.00; H, 5.44; N, 9.43.

7.2.12. [2-(3,4-Dimethoxyphenyl)ethyl]methyl-[4-(2-trifluoromethyl-phenothiazin-10-yl)but-2-ynyl]amine (7a)

The compound was purified by flash chromatography (toluene/acetone 4:1) and converted to the oxalate salt, mp 133–135 °C (methanol/diethylether). ^{1}H NMR: δ 2.35 (s, 3H), 2.65–2.75 (m, 4H), 3.50 (t, J = 4.2 Hz, 2H), 3.80 (s, 3H), 3.85 (s, 3H), 4.55 (t, J = 4.2 Hz, 2H), 6.65–7.40 (m, 10H arom). MS: *m/z* (rel. abundance) 512 (M^+, 16.8%), 361 (100%), 32 (97.4%). Anal. ($C_{28}H_{27}F_3N_2O_2S$) Calcd: C, 65.61; H, 5.31; N, 5.47; found: C, 65.60; H, 5.32; N, 5.45.

7.2.13. 10-(4-Morpholin-4-yl-but-2-ynyl)-2-trifluoromethyl-10*H*-phenothiazine (7b)

The compound was purified by flash chromatography (toluene/acetone 4:1) and converted to the oxalate salt, mp 103–105 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.55–2.65 (m, 4H), 3.35 (d, J = 3.5 Hz, 2H), 3.70–3.85 (m, 4H), 4.56 (d, J = 3.5 Hz, 2H), 7.00–7.30 (m, 7H arom). MS: *m/z* (rel. abundance) 404 (M^+, 10.6%), 32 (100%), 74 (94.9%). Anal. ($C_{21}H_{19}F_3N_2OS$) Calcd: C, 62.36; H, 4.74; N, 6.93; found: C, 62.38; H, 4.75; N, 6.92.

7.2.14. 10-[4-(4-Pheny-piperazin-1-yl)but-2-ynyl]-2-(trifluoromethyl)-10*H*-phenothiazine (7c)

The compound was purified by flash chromatography (toluene/acetone 9:1) and converted to the oxalate salt, mp 114–116 °C (methanol/diethylether). ^{1}H NMR (free base): δ 2.65–2.75 (m, 4H), 3.15–3.25 (m, 4H), 3.40 (t, J = 3.4 Hz, 2H), 4.55 (t, J = 3.4 Hz, 2H), 6.90–7.40 (m, 12H arom). MS: *m/z* (rel. abundance) 479 (M^+, 5.7%), 45 (100%), 120 (17.1%). Anal. ($C_{27}H_{24}F_3N_3S$) Calcd: C, 67.62; H, 5.04; N, 8.76; found: C, 67.62; H, 5.03; N, 8.75.

7.3. Cell culture and treatment

The human acute promyelocytic cell line HL60, the human lymphoblastic leukemia cell line CCRF/CEM, and their MDR variants HL60R and CEM/VBL300 were employed in the study. HL60R cells express P-glycoprotein and are about 300-fold resistant to the cytotoxic effects of the anticancer drug DXR; in addition, this cell line shows a high degree of resistance to apoptosis induction by different chemically unrelated agents. CEM/VBL300 cells also express P-glycoprotein and are about 300-fold resistant to vinblastine. Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated FCS (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 2 mM of L-glutamine (Gibco) at 37 °C in a humidified 5% CO_2 atmosphere. DXR was purchased from Sigma–Aldrich S.r.l. (Milano, Italy). The caspase inhibitors Z-IETD-FMK (Z-Ile-Glu-Thr-Asp-fluoromethylketone), Z-LEHD-FMK (Z-Leu-Glu (OMe)-His-Asp (OMe)-fluoromethylketone), Z-DEVD-FMK (Z-Asp (OMe)-Glu (OMe)-Val-Asp (OMe)-fluoromethylketone), and Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) were purchased from Alexis Biochemicals (Laufelfingen, Switzerland). All the PTZ derivatives were dissolved in DMSO. However, because of poor water solubility and compound precipitation, PTZ concentrations in samples could not exceed 10 μM.

7.4. Cell viability and apoptosis

Cytotoxicity was assessed by the trypan blue dye exclusion test or the MTS-assay. In brief, cells were seeded at a density of 0.2×10^6 cells/ml in 24-well plates and exposed to the modulators and after 1 h to doxorubicin. After 72 h of exposure, aliquots of cells were harvested, stained with trypan blue and counted by light microscopy. Alternatively, cells were seeded at a density of 1×10^5 per milliliter in 96-well microtiter plates, treated as described above and cell viability was evaluated measuring the reduction of the tetrazolium compound (MTS) with an assay kit (Promega, Madison, WI) according to the supplier manual. The absorbance was recorded using a spectrophotometer at λ 595 nm. All samples were measured in triplicate in at least three independent experiments. Resistance index was calculated as the ratio between the DXR IC_{50} (inhibitory concentration 50%) in the resistant cells and that in the parental sensitive cell line. The resistance reverting index (RRI) was determined as the ratio of the IC_{50} for DXR alone divided by the IC_{50} for DXR in the presence of the maximal "non-cytotoxic" concentrations of various modulators, that is those that produced $\leqslant$10% inhibition of growth in resistant cells when employed alone.

To study the combined effects of DXR plus concentrations of the cytotoxic PTZs that alone produced $\leqslant$50% growth inhibition, the fractional product method of Webb was applied.[31] Experimental dose–response curves were compared with the theoretical lines of additivity. These predicted curves were created by calculating a predicted value (c) for each drug combination according to the equation $c = a \times b/100$, where a and b indicate cell survival values with single agents. When the cytotoxic effects of combinations and the predicted values overlapped, the interaction was considered additive, while it was considered synergistic when the cytotoxic effect was higher than the predicted value.

Apoptosis was evaluated by fluorescence microscopy according to the method of Duke and Cohen.[32] After treatment with the drugs for 48 h, cells were centrifuged and the pellet was resuspended in 25 μl of a dye mixture containing acridine orange and ethidium bromide. Live and apoptotic cells were identified by fluorescence microscopy. The caspase inhibitors were added 2 h before drug treatment. Alternatively, apoptosis was evaluated by flow cytometry as the percentage of hypodiploid nuclei accumulated in the sub-G_0–G_1 peak after labeling with propidium iodide.[33]

7.5. Cell cycle analysis

The effects of the compounds on the cell cycle were studied by flow cytometry. Briefly, the cells were washed once in ice-cold PBS and resuspended at 1×10^6/ml in a hypotonic fluorochrome solution containing propidium iodide (Sigma) 50 μg/ml in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40 (Sigma). After 30 min of incubation in this solution, the samples were filtered through nylon cloth, 40 μm mesh, and their fluorescence was analyzed as single-parameter frequency histograms by using a FACSort (Becton Dickinson, Mountain View, CA, USA). A minimum of 10,000 events for each sample was collected in list mode. The distribution of cells in the cell cycle was analyzed with the ModFit LT program (Verity Software House, Inc.).

7.6. Determination of DXR accumulation

Intracellular DXR concentrations were evaluated by flow cytometry. In brief, cells at a concentration of 5×10^5 cells/ml were exposed to the PTZ for 1 h and then to DXR 0.5 μg/ml for 1 h. Cells were then washed twice in cold PBS, resuspended in fresh medium at a concentration of 1×10^6 cells/ml and anthracycline fluorescence was immediately examined using the FACScan with the appropriate filter.

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