# Reversal of ABCB1-related Multidrug Resistance of Colonic Adenocarcinoma Cells by Phenothiazines

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**Abstract.** *Background: The most common mechanism that reduces the efficacy of anticancer agents is overexpression of ATP-binding cassette (ABC) drug transporters. Phenothiazines and structurally-related compounds can sensitize multidrug-resistant (MDR) cells to chemotherapeutics. Materials and Methods: Phenothiazine derivatives were investigated regarding their anticancer and MDR-reversing effect on colonic adenocarcinoma cells. The anti-proliferative and cytotoxic effects of the derivatives were assessed by the thiazolyl blue tetrazolium bromide (MTT) method, the modulation of the ABCB1 activity was measured by rhodamine 123 accumulation assay using flow cytometry. Results: All phenothiazines exhibited potent cytotoxic effect on the sensitive and MDR colon adenocarcinoma cell lines. The inhibition of the ABCB1 transporter was greater in the presence of the phenothiazine derivatives than for the known ABCB1 inhibitor verapamil. Conclusion: It can be concluded that these derivatives show synergism in the presence of doxorubicin and could have potential as ABCB1 inhibitors.*

Colorectal cancer is a common malignancy and despite surgical and chemotherapeutic treatments, it is still the second leading cause of cancer-related deaths in Western Europe, North America, Australia and New Zealand (1, 2). The resistance of tumor cells to cytostatic, structurally unrelated drugs that do not have a common mechanism of action is a phenomenon described as multidrug resistance (MDR) (3). A number of cellular mechanisms are responsible for the MDR of cancer cells. The most common mechanism that reduces the efficacy of anticancer agents is the overexpression of ATP-binding cassette drug transporters (4). Phenothiazines have anticancer properties and are able to reverse the MDR of neoplastic cells by inhibiting the activity of ATP-binding cassette, sub-family B, member 1 protein (ABCB1 or P-glycoprotein) (4).

Previously, 26 new phenothiazine derivatives were synthesized and studied for anticancer activity (6, 7). All 26 phenothiazines had potent anti-proliferative and cytotoxic effects, furthermore, they inhibited the activity of the ABCB1 transporter (P-glycoprotein), and this inhibition was greater than that of the well-known ABCB1 inhibitors thioridazine and verapamil (6).

In the present study, these 26 phenothiazine derivatives were investigated regarding their ABCB1-modulating and apoptosis-inducing properties against MDR colonic adenocarcinoma cells overexpressing the ABCB1 pump. In addition, the most active derivatives were combined with the chemotherapeutic drug doxorubicin and their interaction was studied in MDR colonic adenocarcinoma cells.

## Materials and Methods

*Cell lines.* Human colonic adenocarcinoma cell lines (Colo 205 doxorubicin-sensitive parent and Colo 320/MDR-LRP multidrug-resistant overexpressing ABCB1 (MDR1)-LRP), ATCC-CCL-220.1 (Colo 320) and CCL-222 (Colo 205) were purchased from LGC Promochem, Teddington, UK.

The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM Na-pyruvate and 100 mM Hepes. The cell lines were

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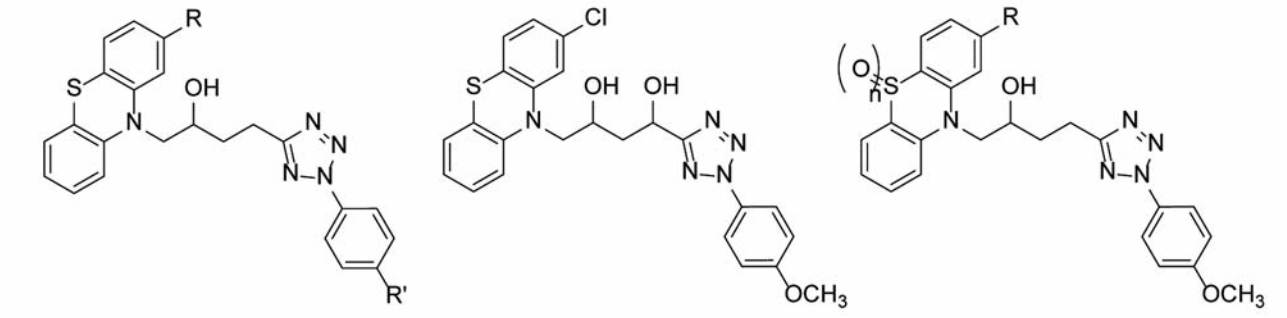
*Key Words*: Phenothiazines, multidrug resistance, MDR, P-glycoprotein (ABCB1), rhodamine 123, colon adenocarcinoma, apoptosis, doxorubicin, verapamil.




**1a-o** **2** **3a-j**

| 1 | R | 1 | R |
|---|---|---|---|
| a | H | h | $H_3C$–N N– |
| b | Cl | i | N H |
| c | O N– | j | O N NH |
| d | N– | k | N NH |
| e | H | l | O H N H |
| f | N– | m | O $H_3C$ N H |
| g | N– | n | N H |
| | | o | $CH_3$ N H H |

| 3 | n | R |
|---|---|---|
| a | 1 | H |
| b | 1 | Cl |
| c | 1 | O N– |
| d | 1 | N– |
| e | 1 | $H_3C$–N N– |
| f | 2 | H |
| g | 2 | Cl |
| h | 2 | O N– |
| i | 2 | N– |
| j | 2 | $H_3C$–N N– |

Figure 1. *Structures of N-hydroxyalkyl-2-aminophenothiazines (**1a-o**, **2**, **3**). Commonly, R' is $OCH_3$, except **1e**, where R' is morpholine.*

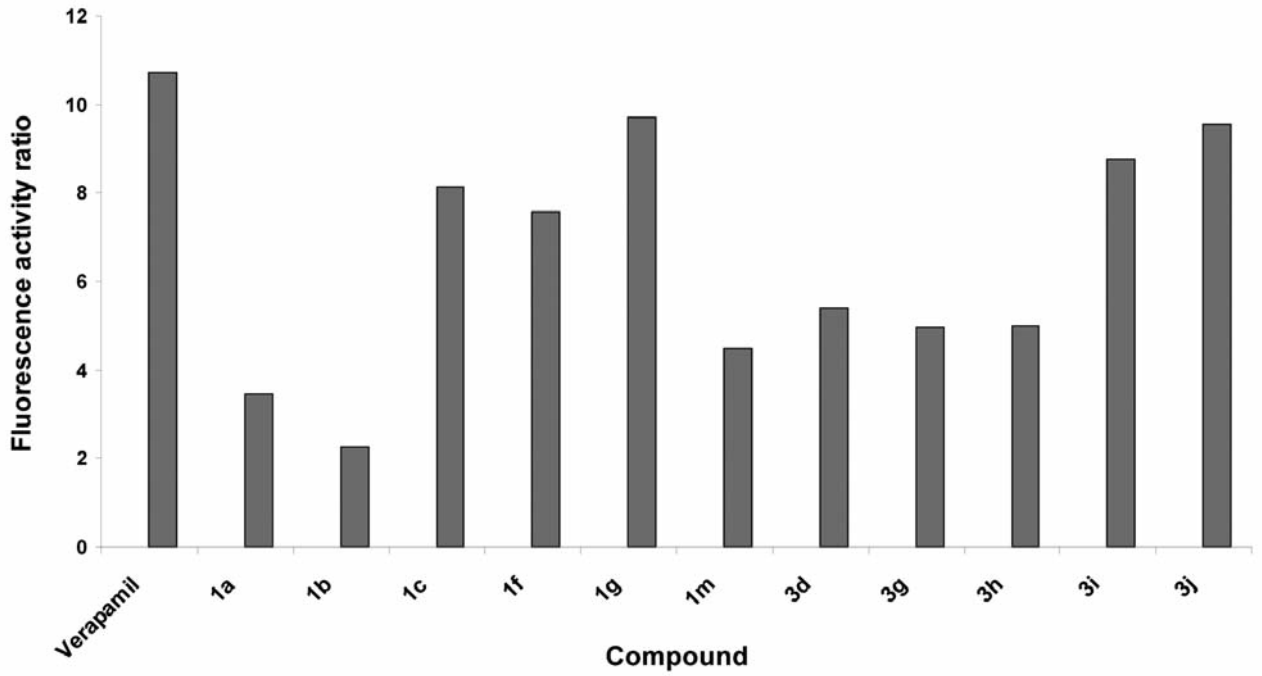



Figure 2. *Rhodamine 123 retention in the presence of the most effective phenothiazine derivatives at 2 μM and verapamil at 20 μM as positive control on multidrug-resistant colonic adenocarcinoma cells (Colo 320).*

incubated in a humidified atmosphere (5% $CO_2$, 95% air) at 37˚C. The semi-adherent human colon cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min at 37˚C.

*Compounds*. Chemical structures of the previously tested phenothiazine derivatives are shown in Figure 1 (8). The compounds were prepared by recently elaborated chemical transformations (7). Thus, derivatives **1a-o** were obtained by protection and Buchwald-Hartwig amination of **1**, R=Cl whereas **2** was obtained as a by-product of the hydroboration-oxidation transformation of the appropriately substituted dienylphenothiazine. Sulfoxides **3a-e** and sulfones **3f-j** were prepared by oxidation of the related phenothiazine **1** with m-chloroperoxybenzoic acid (m-CPBA). The phenothiazine derivatives were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Madrid, Spain).

*Assay for antiproliferative and cytotoxic effect*. The effects of increasing concentrations of the drugs alone on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 100 μl medium. Then, $5\times10^3$ (for antiproliferative assay) or $2\times10^4$ cells (for cytotoxic assay) in 50 μl of medium, respectively, were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37˚C for 24 and 72 h, respectively; at the end of the incubation period, 15 μl of MTT (thiazolyl blue tetrazolium bromide, Sigma) solution (from a 5 mg/ml stock) was added to each well. After incubation at 37˚C for 4 h, 100 μl of sodium dodecyl sulfate (SDS) (Sigma) solution (10% in 0.01 M HCI) was added to each well and the plates were further incubated at 37˚C overnight. Cell growth was determined by measuring the optical density (OD) at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA).

Inhibition of cell growth was determined according to the formula:

$$IC_{50} = 100 - \left[\frac{OD\,sample \quad OD\,medium\,control}{OD\,cell\,control \quad OD\,medium\,control}\right]\times 100$$

Where $IC_{50}$ is defined as the inhibitory dose that reduces the growth of the cells exposed to the compound by 50%.

*Flow cytometric assay for evaluation of the effects a compound on the retention of rhodamine 123 by ABCB1 (P-glycoprotein) in tumor cells*. This assay has been fully-described previously (8). Briefly, the cells were adjusted to a density of $2\times10^6$/ml, re-suspended in serum-free RPMI-1640 medium and distributed in 0.5 ml aliquots into Eppendorf centrifuge tubes. Test compounds (10 μl) were added at different concentrations, and the samples were incubated for 10 min at room temperature. Next, 10 μl (5.2 μM final concentration) of rhodamine 123 was added to the samples and the cells were incubated for a further 20 minutes at 37˚C, washed twice and re-suspended in 0.5 ml phosphate-buffered saline (PBS) for analysis. The fluorescence uptake of the cell population was measured with a Partec CyFlow flow cytometer (Partec, Münster, Germany). Verapamil was used at 20 μM as a positive control in the rhodamine 123 exclusion experiments. The mean fluorescence intensity (%) was calculated for the treated MDR Colo 320 and sensitive Colo 205 cell lines as compared to untreated cells. A fluorescence activity ratio (FAR)

Table I. *Antiproliferative and cytotoxic activity of phenothiazines on multidrug-resistant Colo 320 colon adenocarcinoma cells.*

| | Antiproliferative effect | | Cytotoxic effect | |
|---|---|---|---|---|
| Derivative | $IC_{50}$ (μM) | SD | $IC_{50}$ (μM) | SD |
| **1a** | 14.83 | 1.15 | 6.97 | 0.50 |
| **1b** | 12.85 | 1.45 | 6.56 | 0.28 |
| **1c** | 7.29 | 0.57 | 6.49 | 0.87 |
| **1d** | 38.85 | 1.42 | 8.46 | 0.06 |
| **1e** | 18.64 | 0.40 | 6.11 | 0.08 |
| **1f** | 21.58 | 1.82 | 6.59 | 0.33 |
| **1g** | 25.16 | 0.53 | 6.60 | 0.52 |
| **1h** | 3.325 | 0.19 | 2.92 | 0.08 |
| **1i** | 41.49 | 1.46 | 5.42 | 0.27 |
| **1j** | 7.50 | 0.95 | 5.03 | 0.39 |
| **1k** | 2.83 | 0.24 | 2.52 | 0.20 |
| **1l** | 11.24 | 2.60 | 6.09 | 0.30 |
| **1m** | 3.60 | 0.16 | 6.56 | 0.47 |
| **1n** | 3.43 | 0.25 | 7.73 | 0.17 |
| **1o** | 2.90 | 0.62 | 4.53 | 0.19 |
| **2** | 3.88 | 0.14 | 5.25 | 0.96 |
| **3a** | 16.91 | 1.79 | 6.56 | 0.19 |
| **3b** | 2.71 | 0.09 | 5.79 | 0.29 |
| **3c** | 3.02 | 0.11 | 7.71 | 0.66 |
| **3d** | 3.09 | 0.23 | 5.99 | 0.08 |
| **3e** | 3.07 | 0.09 | 7.32 | 1.54 |
| **3f** | 4.02 | 0.23 | 5.91 | 0.46 |
| **3g** | 2.83 | 0.04 | 6.14 | 0.86 |
| **3h** | 2.89 | 0.17 | 6.70 | 0.57 |
| **3i** | 4.67 | 0.13 | 8.73 | 0.04 |
| **3j** | 3.59 | 0.33 | 4.98 | 0.31 |
| DMSO (v/v%) | 2.19 | 0.06 | 2.69 | 0.04 |

$IC_{50}$: Half-maximal inhibitory concentration; SD: standard deviation; v/v%: volume solute per volume of total solution after mixing.

was calculated *via* the following equation, on the basis of the measured fluorescence values:

$$FAR = \frac{Colo\ 320\ treated\ /\ Colo\ 320\ control}{Colo\ 205\ treated\ /\ Colo\ 205\ control}$$

*Apoptosis assay.* The assay was carried out using Annexin V-FITC Apoptosis Detection Kit (Calbiochem, Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The density of the MDR colonic adenocarcinoma cell suspension was adjusted to approximately $1\times10^6$ cells/ml. The cell suspension was distributed into 0.5 ml aliquots (5×105 cells) to a 24-well microplate and incubated overnight at 37˚C with 5% $CO_2$.

On the following day, the medium was removed and fresh medium was added to the cells. The cells were incubated in the absence and presence of the compounds for 3 h at 37˚C, compound 12*H*-benzo[α]phenothiazine (M627) was used as positive control at a final concentration of 20 μM (9). The culture medium was removed, the cells were washed with PBS and fresh medium was added to the cells. The 24-well plates were incubated overnight at 37˚C with 5% $CO_2$.

Table II. *Type of interaction between phenothiazine derivatives and doxorubicin against multidrug resistant (MDR) colonic adenocarcinoma (Colo 320) cells*

| Derivative | Ratio[a] | CI[b] | Interaction |
|---|---|---|---|
| **1c** | 12:1 | 0.31 | Synergism |
| **3d** | 12:1 | 0.14 | Strong synergism |
| **3g** | 12:1 | 0.17 | Strong synergism |
| **3h** | 12:1 | 0.11 | Strong synergism |
| **3i** | 12:1 | 0.10 | Strong synergism |
| **3j** | 12:1 | 0.29 | Strong synergism |

[a]Data are shown as the best combination ratio between the tested compounds and doxorubicin. [b]Combination index (CI) values at the 50% growth inhibition dose ($ED_{50}$) were determined by the CompuSyn software to plot four to five data points at each ratio. CI values were calculated by means of the median-effect equation, where CI<1, CI=1 and CI>1 represent synergism, additive effect (*i.e.*, no interaction), and antagonism, respectively.

After the incubation period, the cells were removed gently from each well using a cell scraper, then centrifuged at 2000 ×*g* for 2 min at room temperature, the supernatant was removed and the cells were re-suspended in fresh serum-free medium.

After this procedure, the apoptosis assay was carried out according to the rapid protocol of the kit. The fluorescence was analyzed immediately using a Partec CyFlow flow cytometer (Partec).

*Checkerboard combination assay.* A checkerboard microplate method was applied to study the effect of drug interactions between phenothiazine derivatives and the chemotherapeutic drug doxorubicin on MDR colonic adenocarcinoma cells overexpressing the ABCB1 transporter.

The dilutions of doxorubicin were made in a horizontal direction in 100 μl, and the dilutions of the phenothiazines vertically in the microtiter plate in 50 μl volume. The cells were re-suspended in culture medium and distributed into each well in 50 μl containing $1\times10^4$ cells. The plates were incubated for 72 h at 37˚C in a $CO_2$ incubator. The cell growth rate was determined after MTT staining, as described above. Combination index (CI) values at 50% of the growth inhibition dose ($ED_{50}$), were determined using CompuSyn software (www.combosyn.com, ComboSyn, Inc., Paramus, NJ. 07652 USA) to plot four to five data points to each ratio. CI values were calculated by means of the median-effect equation (10), where CI<1, CI=1, and CI>1 represent synergism, additive effect (or no interaction), and antagonism, respectively (10).

## Results

The phenothiazine derivatives exhibited more potent cytotoxic effects on the resistant colon adenocarcinoma cells than on the sensitive Colo 205 cell line. The $IC_{50}$ values of the derivatives on the sensitive Colo 205 cell line can be divided into four groups: with $IC_{50}$ of more than 100 μM: **1i, 3f, 3g, 3h, 3i**; $IC_{50}$ of between 20 and 55 μM: **1a, 1b, 1d,**

| Samples | Concentration | **Early apoptosis (%)** | **Late apoptosis and necrosis (%)** | **Cell death (%)** |
|---|---|---|---|---|
| **Control** (annexin V-FITC and propidium iodide staining) | - | 10.60 | 5.96 | 3.10 |
| **M627** | 20 μM | 44.01 | 15.60 | 3.02 |
| **3j** | 20 μM | 22.80 | 16.60 | 11.50 |

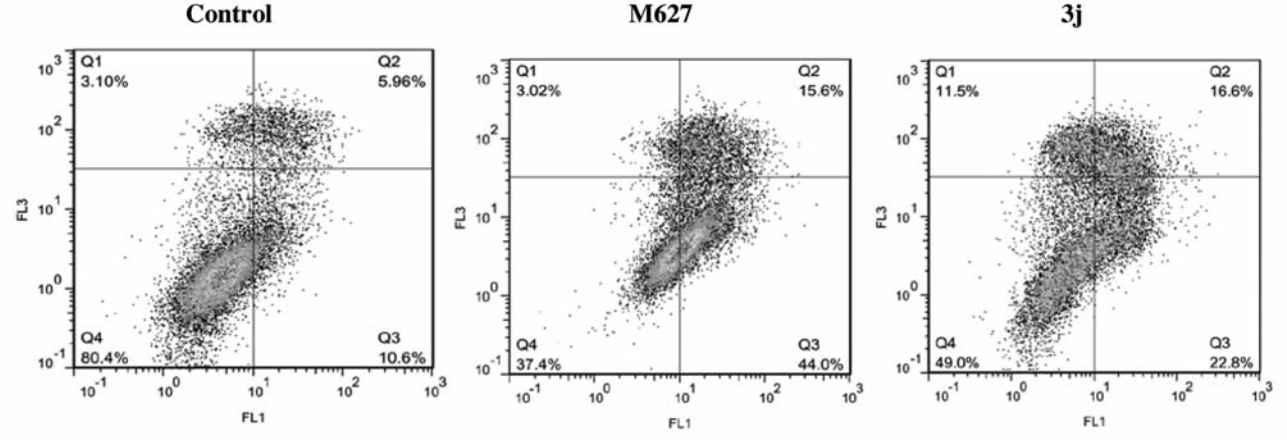



Figure 3. *Apoptosis induction in Colo 320 cells by the most effective phenothiazine derivative 3j. M627: 12H-benzo[α]phenothiazine (positive control)*

**1f, 1g, 1l, 1n, 3a, 3b, 3c, 3e**; $IC_{50}$ of between 10 and 19 μM: **1c, 1j, 1m, 2, 3d, 3j**; and $IC_{50}$ of between 5 and 9 μM: **1e, 1h, 1k**. The compounds were more selective for the MDR Colo 320 cells, as shown by their lower $IC_{50}$ values. Based on the $IC_{50}$, the compounds can be divided into three categories: with $IC_{50}$ of between 10 and 40 μM: **1a, 1b, 1d, 1e, 1f, 1g, 1i, 1l, 3a**; $IC_{50}$ of between 3 and 10 μM: **1c, 1h, 1j, 1m, 1n, 2, 3c, 3d, 3e, 3f, 3i, 3j**; and $IC_{50}$ of less than 3 μM: **1k, 1o, 3b, 3g, 3h** (Table I).

The phenothiazine derivatives showed more potent cytotoxic effects on the resistant colonic adenocarcinoma cells than on the sensitive Colo 205 cell line. The phenothiazine derivatives **1n, 3b, 3c, 3g, 3h**, and **3i** had no cytotoxic effect on the sensitive Colo 205 cells because their $IC_{50}$ values were above 100 μM. The derivatives **1f, 1l, 3d**, and **3f** inhibited the growth of Colo 205 cells to a certain extent ($IC_{50}$ between 40 and 50 μM). Furthermore, derivatives **1a, 1c, 1e, 1g, 1m, 1o, 2, 3a, 3f**, and **3j** had potent cytotoxic activity ($IC_{50}$ between 15 and 30 μM). The most active derivatives had $IC_{50}$ values between 4 and 15 μM: **1b, 1d, 1h, 1i, 1j, 1k** (data not shown). Interestingly, the phenothiazine derivatives exerted more potent cytotoxic activity against the ABCB1-expressing MDR colonic adenocarcinoma cells, suggesting that the compounds may act on the ABCB1 transporter. The cytotoxic activity of the compounds expressed as the $IC_{50}$ ranged between 2.5 and 9 μM, the most active derivative being 1k ($IC_{50}$=2.5 μM) (Table I).

Out of the 26 phenothiazine derivatives, 13 **(1a-c, 1f, 1g, 1m, 2, 3d, 3f-j)** demonstrated potent ABCB1-modulating effect because these compounds increased the intracellular rhodamine 123 concentration of the MDR colonic adenocarcinoma cells at 2 and 20 μM. As shown in Figure 2, the most active derivatives induced rhodamine 123 accumulation at 2 μM compared to ABCB1 modulator verapamil at 20 μM (FAR=10.7). It is noteworthy that out of the 13 ABCB1-modulating phenothiazine derivatives, only compound **3j** was able to induce apoptosis of MDR colonic

adenocarcinoma cells at 20 μM, however, its early apoptosis-inducing activity was lower compared to that of the positive control 12*H*-benzo[α]phenothiazine (Figure 3) (9).

Based on the ABCB1-modulating activity of the lead compounds, six derivatives were selected for combination studies with doxorubicin. Compound **3j** had the highest FAR value at 2 μM of 9.55, in addition, the other selected derivatives had FAR values of between 4.97 and 8.77 at 2 μM. The lead compounds 1**c, 3d, 3g, 3h, 3i**, and **3j** were studied regarding their effect in combination with doxorubicin. As shown in Table II, all compounds exhibited synergistic activity with doxorubicin at the ratio of 12:1 (phenothiazine derivative:doxorubicin). It can be concluded that the lead compound of the phenothiazine series was compound **3j** because this derivative was a very potent ABCB1 modulator and apoptosis inducer, furthermore, it showed synergism with the anticancer drug doxorubicin against MDR colonic adenocarcinoma cells.

## Discussion

Phenothiazines have proven to be a special group of compounds with extraordinary anticancer (10, 12, 13, 14) and antibacterial activities (4, 15). Besides their various biochemical effects (16), phenothiazines may be promising candidates for combined anticancer chemotherapy (17).

The investigated *N*-hydroxyalkyl-2-aminophenothiazines show promising antiproliferative and cytotoxic effects on MDR colonic adenocarcinoma cells. The type of substituents used in their synthesis can strongly influence the anticancer activity of the derivatives. From our understanding of the relationship between structural diversity and activity, we have concluded that the compounds substituted with secondary amines (morpholine, diethylamine or *N*-methylpiperazine) in the second position of the phenothiazine ring **(3c, 3d, 3f)** are the most selective candidates. Furthermore, the oxidation state of the sulfur atom (sulfoxide or sulfone) leads to remarkable selectivity **(3h, 3i, 3j)** according to their $IC_{50}$ data. The compounds containing primary amines or acid amide in the second position of the ring, such as **1c, 1h, 1j, 1m,** 1n resulted in lower activity compared to the aforementioned molecules. It can be assumed that the modulation of similar structural details (secondary amines in the second position, 2-hydroxyl group in the alkyl chain, sulfoxide or sulfone) of the chosen lead compounds **(1c, 3d, 3g, 3h, 3i, 3j)** is responsible for the efficacy of their ABCB1 modulatory role and synergism with doxorubicin. According to the cytotoxic and antiproliferative activity, furthermore the ABCB1 modulating and apoptosis inducing activity of the compounds, it can be observed that the oxidation of sulfur atom of 1h resulted in the lead molecule **3j**. It can be concluded that this structural modification increased the biological activity. To understand this effect further binding assays could reveal more biophysical and biochemical information on the anticancer activity of these compounds.

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