Multidrug Resistance Reversing Activity of Newly Developed Phenothiazines on P-glycoprotein (ABCB1)-related Resistance of Mouse T-Lymphoma Cells

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Abstract. *Background: Phenothiazines have anticancer properties and are able to reverse the multidrug resistance of neoplastic cells by inhibiting the ATP-binding cassette, sub-family B (MDR/TAP), member 1 protein (ABCB1 or P-glycoprotein) activity. Materials and Methods: A series of new phenothiazine derivatives was investigated regarding their ABCB1-modulating effect on multidrug resistant mouse T-lymphoma cells by rhodamine 123 accumulation assay and real-time ethidium bromide accumulation assay. Results: The phenothiazine derivatives exhibited a potent anticancer effect on the parental cell line and on its multidrug-resistant mouse T-lymphoma subline overexpressing the ABCB1 transporter. The inhibition of the ABCB1 transporter in the presence of the newly-developed phenothiazines was greater than that for the known ABCB1 inhibitors thioridazine and verapamil. Conclusion: Based on the chemical structures and biological activity, compounds with bivalent sulfur atom in the phenothiazine ring demonstrated marked ABCB1-modulating effect, however, other derivatives with halogen or amide substitutions were ineffective.*

Phenothiazines have been in use for the treatment of psychiatric disorders since the 1950s. Other pharmacological and biological activities of phenothiazines have since been described, such as anti-bacterial (1), anti-mycobacterial (2), anti-plasmid (3) and immunomodulatory (4) effects. Phenothiazines and structurally related compounds can sensitize multidrug-resistant (MDR) cells to chemotherapeutics (5). Phenothiazines mostly substituted at position 10 with dialkylaminoalkyl groups, and additionally at position 2 with small groups, exhibit valuable activities such as neuroleptic, anti-emetic, anti-histaminic, anti-puritic, analgesic and anti-helmintic (6). The introduction of different substituents into the phenothiazine skeleton, as well as the modification of the tricyclic ring system, alters their biological activities (7). In addition numerous derivatives of phenothiazine for similar applications have been synthesized and patented (8).

Recently, a series of phenothiazine derivatives was investigated regarding their inhibitory activity against bacterial efflux pump systems. As structures of the studied series of phenothiazines differ from each other by slight modifications, it was relatively easy to observe the structure–activity relationship based on the efflux pump inhibitory properties of the derivatives (9).

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 (ABC) drug transporters (10). Phenothiazines are able to reverse the MDR of neoplastic cells to cytostatic drugs. The lipophilic nature of phenothiazines enables them to easily-penetrate the cell membrane (11).

The presence of the 2-butanol chain is essential in regard to modulation of the ABCB1 protein (P-glycoprotein) by alkylphenothiazines, furthermore the lack of a hydroxyl group significantly reduces the rate of inhibition (12).

The present study provides information regarding this series of new phenothiazine derivatives regarding their ABCB1-modulating effect on MDR mouse T-lymphoma cells.

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Key Words: Phenothiazines, multidrug resistance, P-glycoprotein, ABCB1, ATP-binding cassette protein B1, rhodamine 123, ethidium bromide, thioridazine, verapamil, mouse T-lymphoma cell lines.

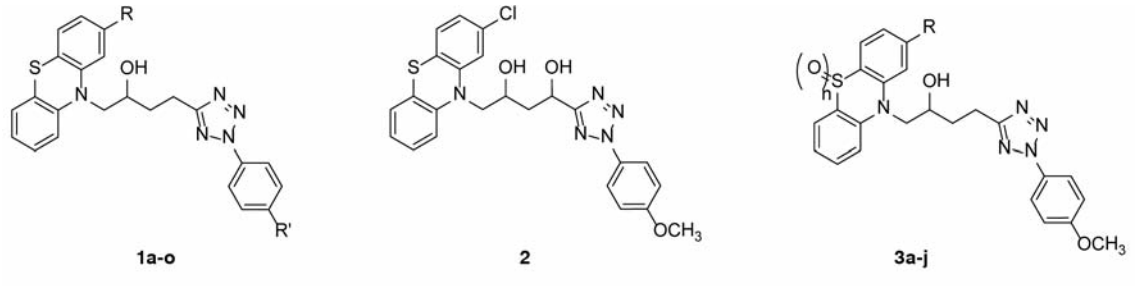


Figure 1. *Structures of N-hydroxyalkyl-2-aminophenothiazines (**1a-o**, **2**, **3a-j**).*

Materials and Methods

Compounds. Chemical structures of the tested 26 phenothiazine derivatives are shown in Figure 1, with the aid of Tables I and II. The compounds in Table I were prepared by recently elaborated chemical transformations (12). Thus, derivatives **1a-o** were obtained by protection and Buchwald-Hartwig amination of **1**, where R=Cl, whereas compound **2** was obtained as a by-product of the hydroboration-oxidation transformation of the appropriately-substituted dienylphenothiazine (13). Sulfoxides **3a-e** and sulfones **3f-j** (Table II) were prepared by oxidation of the related phenothiazine **1** with *m*-chloroperoxybenzoic acid (12). The phenothiazine derivatives were dissolved in dimethyl sulfoxide (DMSO; Sigma, Madrid, Spain). Ethidium bromide (EB) was purchased from Sigma.

Cell lines. L5178Y parental mouse T-cell lymphoma cells (ECACC cat. no. 87111908; U.S. FDA, Silver Spring, MD, USA) were transfected with pHa MDR1/A retrovirus (14, 15). The *ABCB1*-expressing cell line was selected by culturing the transfected cells with 60 ng/ml of colchicine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to maintain the MDR phenotype using McCoy's 5A medium (Sigma) supplemented with L-glutamine (Sigma) and antibiotics (penicillin/streptomycin solution, Sigma) at 37˚C and in an atmosphere with 5% CO_2.

Assay for antiproliferative and cytotoxic effect. The effects of increasing concentrations of the compounds on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in 100 μl medium, then 6×10^3 cells for antiproliferative assay, or 2×10^4 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 72 and 24 h, respectively; at the end of the incubation period, 15 μl of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich Chemie GmbH) solution (from a 5 mg/ml stock) were 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 HCl) were added to each well and the plates were further incubated at 37˚C overnight. The cell growth was then determined by measuring the optical density (OD) at 550 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:

$$IC_{50} = 100 - \left[\frac{OD_{\text{sample}} - OD_{\text{medium control}}}{OD_{\text{cell control}} - OD_{\text{medium control}}}\right] \times 100$$

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

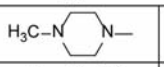
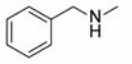
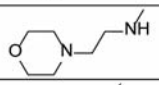
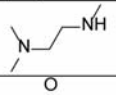
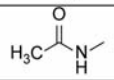
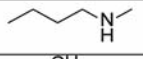
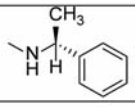
Statistical analyses were conducted using the Student's *t*-test. The accepted level of significance was $p<0.05$. The analyses were performed using GraphPad Prism software (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA).

Flow cytometry assay for evaluation of a compound on the retention of rhodamine 123 by MDR mouse T-cell lymphoma cells. This assay has been fully described elsewhere (16). Briefly, the cells were adjusted to a density of 2×10^6/ml, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 ml aliquots into Eppendorf centrifuge tubes. Test compounds (10 μl) were added at different concentrations (0.2-10 μM), and the samples were incubated for 10 min at room temperature. Next, 10 μl (5.2 mM final concentration) of rhodamine 123 were added to the samples and the cells were incubated for a further 20 min at 37˚C, washed twice and re-suspended in 0.5 ml phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured with a Partec CyFlow flow cytometer (Partec, Münster, Germany). Verapamil was used as a positive control (17) in the rhodamine 123 exclusion experiments. The ratio of the mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared to untreated cells. A fluorescence activity ratio (FAR) was calculated *via* the following equation, on the basis of the measured fluorescence values:

$$FAR = \frac{MDR_{\text{treated}}/MDR_{\text{control}}}{\text{parental}_{\text{treated}}/\text{parental}_{\text{control}}}$$

The results provided are from a representative flow cytometric experiment in which 10,000 individual cells were evaluated. Partec CyFlow flow cytometer research software was used to analyze the recorded data. The data were first presented as histograms and the data were converted to FAR units that define fluorescence intensity, standard deviation, peak channel in the total and in the gated populations.

Table I. *Substituents of N-hydroxyalkyl-2-aminophenothiazines (**1a-o**).*

Compound 1	R	R'	Compound 1	R	R'
a	H	OCH_3	h		OCH_3
b	Cl	OCH_3	i		OCH_3
c		OCH_3	j		OCH_3
d		OCH_3	k		OCH_3
e	H		l		OCH_3
f		OCH_3	m		OCH_3
g		OCH_3	n		OCH_3
			o		OCH_3

EB accumulation assay. The cells were adjusted to a density of 2×10^6 cells/ml, centrifuged at 2000 ×*g* for 2 min and re-suspended in PBS at pH 7.4. The cell suspension was distributed in 90 μl aliquots into 0.2 ml tubes. The tested phenothiazines were individually added at different concentrations in 5 μl of their stock solutions and the samples were then incubated for 10 minutes at 25˚C. Verapamil was used as a positive control (17). After this incubation, 5 μl (1 μg/ml final concentration) of EB (20 μg/ml stock solution) were added to the samples and the tubes were placed in a Rotor-Gene 3000™ thermocycler with real-time analysis software (Corbett Research, Sydney, Australia) and the fluorescence monitored on a real-time basis. Prior to the assay, the instrument was programmed for temperature (37˚C), appropriate excitation and emission wavelengths of EB (530 nm bandpass and 585 nm highpass, respectively), and the time and number of cycles for the recording of the fluorescence (18). The results were evaluated by Rotor-Gene Analysis Software 6.1 (Build 93) provided by Corbett Research.

Results

The derivatives inhibited cell proliferation ($IC_{50}\approx1.5$ μM), but the difference between the parental and MDR cells overexpressing ABCB1 was not significant (Student's *t*-test, $p>0.05$). In addition, the IC_{50} values of cytotoxicity were similar for the parental and MDR cells ($IC_{50}\approx5$ μM) (Student's *t*-test, $p>0.05$).

ABCB1-modulating properties were evaluated by comparing the accumulation of the specific fluorescent substrate of ABCB1, rhodamine-123, in MDR cells, where P-glycoprotein expression is high with that in parental cells. The resulting FAR values are summarized in Table III. Compounds with FAR values greater than 1 were considered to be active P-glycoprotein inhibitors, and those with FAR values greater than 10 as strong MDR modulators.

Table II. *Substituted sulfoxide and sulfone derivatives of N-hydroxyalkyl-2 aminophenothiazines (**3a-j**).*

Compound 3	n	R
a	1	H
b	1	Cl
c	1	
d	1	
e	1	
f	2	H
g	2	Cl
h	2	
i	2	
j	2	

All the phenothiazines investigated in the present study inhibited the activity of P-glycoprotein, and based on the

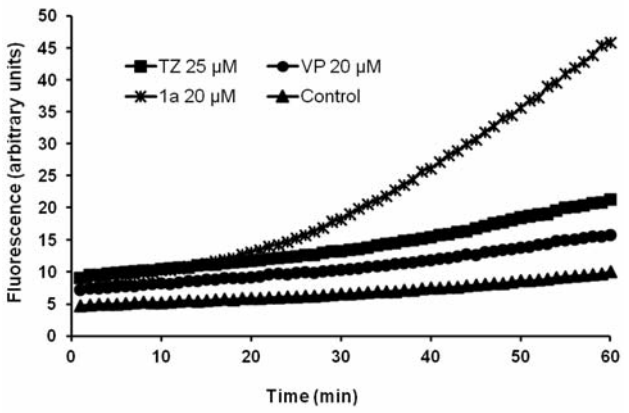


Figure 2. *Comparison of ethidium bromide accumulation by multidrug-resistant mouse T-lymphoma cells overexpressing the ATP-binding cassette transporter protein B1 in the presence of thioridazine (TZ), verapamil (VP), and the phenothiazine derivative **1a**. The graphs were obtained from a single monitoring by the fluorescence assay.*

FARs, the most potent derivatives were **1c**, **1g**, **1j**, **1k**, **1f**, **1h**, **1d**, **3f**, and **3i** (Table III). These compounds were able to reverse the P-glycoprotein-related resistance of MDR cells and exhibited remarkable effects at low concentration (0.2 μM) by increasing the intracellular accumulation of the ABCB1 substrate rhodamine 123 (FAR=3.12-6.8 at 0.2 μM concentration). The derivatives **1k**, **1j**, **1g**, **1m**, **1c** (FAR=77.29-50.01), and **1o**, **1f**, **1l**, **1d**, **1a**, **3i**, **1b**, **2**, **3f** and **1n**, were found to be active ABCB1 inhibitors at the concentration of 2 μM (FAR=43.94-30.36).

To demonstrate the efficacy of these new derivatives, an example is depicted in Figure 2, namely the ABCB1-modulating effect of compound **1a** compared to the activity of well-known ABCB1-inhibiting agents verapamil and thioridazine. The real-time accumulation of the ABCB1 substrate EB in the presence of resistance modifiers was monitored. As shown by Figure 2, compound **1a** had greater effect on MDR mouse T-lymphoma cells than verapamil and thioridazine.

Discussion

Compounds that inhibit the function of MDR efflux proteins might improve the cytotoxic activity of anticancer chemotherapy, for this reason, systematic research for pharmacophor structures is a promising strategy aiming to increase the efficacy of drugs against ABCB1-related MDR in cancer. In the present study, a series of new phenothiazine derivatives was developed and systematically evaluated.

It is well-known that phenothiazines have ABCB1-modulatory effects (5, 19, 20), as well as anti-bacterial, anti-fungal, anti-cancer, anti-viral, anti-inflammatory, anti-malarial, anti-filarial, trypanocidal, anti-convulsant, analgesic, immunosuppressive and

Table III. *Rhodamine123 accumulation in multidrug-resistant mouse T-lymphoma cells overexpressing the ATP-binding cassette transporter protein B1.*

Compound	Concentration (μM)	Fluorescence activity ratio
Verapamil	20.4	8.63
1 a	0.2	0.93
	2	35.32
1 b	0.2	1.61
	2	32.28
1 c	0.2	6.81
	2	50.01
1 d	0.2	3.58
	2	36.83
1 e	0.2	1.73
	2	29.13
1 f	0.2	5.90
	2	40.47
1 g	0.2	3.93
	2	54.34
1 h	0.2	3.37
	2	5.88
1 i	0.2	1.91
	2	23.25
1 j	0.2	4.24
	2	71.87
1 k	0.2	3.88
	2	77.29
1 l	0.2	1.15
	2	39.89
1 m	0.2	1.25
	2	50.78
1 n	0.2	1.35
	2	30.36
1 o	0.2	1.70
	2	43.94
2	0.2	1.13
	2	32.00
3 a	0.2	0.80
	2	1.70
3 b	0.2	0.67
	2	16.34
3 c	0.2	0.79
	2	10.19
3 d	0.2	1.22
	2	26.79
3 e	0.2	1.03
	2	6.58
3 f	0.2	0.97
	2	14.68
3 g	0.2	3.13
	2	30.79
3 h	0.2	1.49
	2	21.3
3 i	0.2	3.12
	2	32.9
3 j	0.2	0.93
	2	21.10

MDR-reversal properties. The reasons for the biological effects of phenothiazines are the interactions of the pharmacophoric substituent and interaction of the multicyclic ring system (π–π interaction, DNA-intercalating properties); in addition their lipophilic character facilitates their penetration through biological membranes (7).

In the present work, 26 new phenothiazine derivatives were investigated and all of them had potent antiproliferative and cytotoxic effect. 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.

Comparison of structures with their biological efficacy in the case of compounds with bivalent sulfur atom in the phenothiazine ring reveals that all derivatives exhibited marked ABCB1-modulatory effects due to the presence of a secondary amine moiety, whereas other derivatives, *e.g.* halogen- or amide-substituted compounds seem to be ineffective. With sulfoxides and sulfones, however, such a relationship was not apparent.

Acknowledgements

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