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Effect of Thioridazine Stereoisomers on the Drug Accumulation of Mouse Lymphoma and Human Prostate Cancer Cell Lines *In Vitro*

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Abstract. *Background: Cancer cells become refractory to chemotherapy as a consequence of their overexpression of multidrug transporters. Materials and Methods: The anticancer and multidrug resistance (MDR) reversal effects of the racemic form and the two enantiomers of thoridazine were investigated on a mouse T-lymphoma cell line over-expressing the ATP-binding cassette, subfamily-B (MDR/TAP), member 1 (ABCB1) transporter (also known as P-glycoprotein) and on human PC3 prostate cancer cell line by 3-(4.5-dimethylthiazolyl-2)-2.5-diphenyl tetrazolium bromide (MTT) assay. The modulation of ABCB1 transporter activity was studied by rhodamine123 accumulation, the apoptosis-inducing effect was investigated using fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide. Results: The thioridazine racemic and (+) and (–) enantiomers were similarly effective. Drug accumulation by MDR mouse T-lymphoma cells was moderately modified in the presence of thioridazine derivatives. Thioridazine induced apoptosis of the MDR cancer cell line, but there was no significant apoptotic effect on the PC3 cell line. Conclusion: Apparently, the chirality of thioridazine has no importance in the inhibition of MDR phenotype of cancer cells.*

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The multidrug resistance (MDR) of tumor cells is often associated with the over-expression of membrane-localized transporter (ABCB1; P-glycoprotein). This protein reduces the intracellular concentration of anticancer drugs by expelling the drug from the cell. Therefore, it was supposed that inhibition of the efflux mechanism may improve cancer chemotherapy by combination with anticancer drugs and resistance modifiers. Previously, six different stereoisomers of phenothiazines such as levo-, and dextro-mepromazine enantiomers, (+) and (-) butaclamols, *cis*- and *trans*-clopenthixol were studied and their ABCB1 inhibitory effects were compared in the hope that central nervous system (CNS)-inactive or less active stereoisomers may be applied as resistance modifiers for use in clinical practice (1).

In our previous studies, the effects of different stereoisomers of phenothiazines were studied on the reversal of MDR of cancer cells. It was found that for tricyclic compounds there is no stereoselectivity on the MDR reversal effects mediated by the ABCB1 membrane transporter (1). Other data show the importance of stereoselective binding on *cis* and *trans* isomers of clopenthixols (2). Similar results were obtained by comparison of the inhibitory effects of levo- and dextro-mepromazine on *Eschericia coli* ATPase and mouse serum cholinesterase (2). It was suggested that the investigated tumor cells possess binding sites for phenothiazines, recognizing the tricyclic structure, but cannot distinguish the position of the rotatable side chain of the two

enantiomers (3). On the contrary, the extremely hydrophobic butaclamol isomers without polar side chain exhibited some stereoselective effects on the inhibition of MDR of mouse lymphoma cells (1). Inhibitory effects in different biological systems *e.g.* plasmid replication in some bacterial systems by *cis* (Z)- and *trans* (E)-clopenthixols were also different. The effect of levo- and dextro-enantiomers of mepromazines was practically the same. On the contrary, the inhibition of mouse serum acetyl cholinesterase or butyryl-cholinesterase was apparently more stereospecific than the inhibition of ATPase or total serum cholinesterase (2). Some differences were found in antibacterial effects of different *cis*(Z)- and *trans*(E)-thioxanthenes, +/– *cis* and +/– *trans* phenylpiperidines (4, 5). The trans compounds were, with few exceptions, slightly more potent than the cis compounds. Similar contradictions were found between the fine chemical structures of stereoisomers of verapamil and carotenoids and their inhibitory effects on the MDR of tumor cells *in vitro* (8).

In the present work, we attempted to clarify the stereo-specificity of the ABCB1 efflux protein by comparing two stereoisomers of thioridazine to the parental, racemic compound in mouse lymphoma cells transfected by the human *ABCB1* gene for their ability to inhibit the activity of human ABCB1 expressed by an MDR mouse lymphoma cell line.

Materials and Methods

The thioridazine hydrochloride is a racemic mixture of enantiomers with an asymmetric carbon at position 2 in the piperidyl ring (Figure 1). The racemic thioridazine was purchased from Sigma-Aldrich, Vallensbak Strand, Denmark. Thioridazine in its ordinarily clinically prescribed form is a racemic mixture of equal amounts of the (+) and (–) enantiomers (Figure 1). The enantiomers were separated by resolution of the commercially available thioridazine racemate according to the procedure described (6). Racemic thioridazine hydrochloride (0.05 mol), 125 ml water, 100 ml 33% NaOH and 250 ml diethyl ether were stirred at room temperature until a clear two-phase system was obtained. The organic phase was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give the free base, yielding 17.8 g (98%). The free base was then dissolved in 25 ml HPLC-grade acetone and mixed with a solution of 0.05 mol di-p-toluyl-L-tartaric acid with stirring. The solution was stirred at room temperature overnight, forming a white suspension of the (–) salt, which was isolated by centrifugation, washed thoroughly with HPLC-grade acetone and dried at 40˚C. The crude (–) salt was recrystallized from 70% ethanol to yield 16.5 g of the di-*p*-toluyl-L-tartrate of the (–)-enantiomer after drying. This salt was suspended in a mixture of 150 ml water, 50 ml 33% NaOH and 125 ml diethylether and stirred at room temperature until a clear two-phase system was obtained. The organic phase was separated, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give (–)-thioridazine as the free base, yielding 7.3 g (82%). with specific rotation: –29˚ in. ethanol. The (–) base was dissolved in a small volume, and converted into the hydrochloride by the addition of 6 M HCl in diethyl ether. The hydrochloride crystallized upon further addition of diethylether and was filtered and dried *in vacuo*, yielding 6.8 g (67%). The acetone

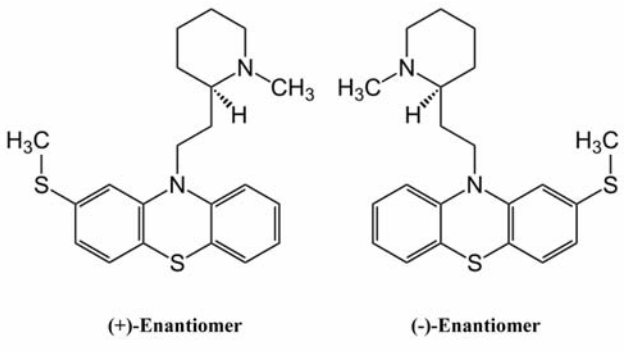

Figure 1. *Enantiomers of thioridazine.*

filtrates and the mother liquor from the recrystallization of the (–)-tartrate were concentrated *in vacuo* to dryness and converted to the crude (+)-thioridazine base, which was resolved by salt formation with di-*p*-toluyl-D-tartaric acid similar to the procedure used for the (–)-isomer. The free (+) base had specific rotation of +25˚ and was converted to the hydrochloride as described for the (–)-enantiomer, yielding 3.4 g (33 %).

Cell cultures. The L5178 mouse T-cell lymphoma cells (ECACC cat. no. 87111908, obtained from FDA, Silver Spring, MD, USA) were transfected with pHa ABCB1 retrovirus, as previously described by Cornwell *et al.* (7, 9). ABCB1-expressing cell line L5178Y was selected by culturing the infected cells with colchicine. L5178 (parent) mouse T-cell lymphoma cells and the L5178Y human ABCB1-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat inactivated horse serum, 200 mM L-glutamine, and penicillin-streptomycin mixture in 100 U/l and 10 mg/l concentration, respectively. The detection of MDR Pgp was performed by monoclonal antibody (9). The cell lines were incubated in a humidified atmosphere (5% CO_2, 95% air) at 37˚C. The prostate cancer cell line PC-3 (ATCC® CRL-1435) was grown in RPMI-1640 medium with 2 mM L-glutamine and 10% fetal bovine serum supplemented with antibiotics. Adherent human cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min.

Assay for antiproliferative effects. The effects of increasing concentrations of compounds on cell growth were determined in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 100 μl in McCoy's 5A or RPMI-1640 medium, respectively. For the antiproliferative assay, 6×10^3 cells of mouse T-cell lymphoma cell line or 1×10^4 PC3 of prostate cancer cells in 100 μl of medium were added to each well. For the cytotoxic assay, 2×10^4 cells of the mouse T-cell lymphoma cell line or PC3 cells in 100 μl of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37˚C, for 72 h for the antiproliferative effect assay and for 24 h for the cytotoxic effect assay. At the end of the incubation period, 20 μl of 3-[4.5-dimethylthiazol-2-yl]-2.5 diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) solution (from a 5 mg/ml stock) was added to each well, then after 4 h, 100 μl of 10% sodium dodecyl sulfate (SDS) (Sigma) in 0.01 M HCl was measured into each well. The culture plates were further incubated at 37˚C overnight. The cell growth was determined by measuring the optical

density (OD) at 550 nm (ref. 630nm) with a Multiscan EX ELISA reader (Thermo Labsystem, Cheshire, WA, USA). Inhibition of cell growth was determined according to the formula:

$$\%inhibition = 100 - \left[\frac{OD\,sample - OD\,medium\,control}{OD\,cell\,control - OD\,medium\,control}\right] \times 100$$

IC_{50} was calculated using non-linear regression curve fitting of log(inhibitor) *vs*. response and variable slop with a least squares (ordinary) fit, using the GraphPad Prism software. Differences between the enantiomers were evaluated by one-way analysis of variances (ANOVA) with Bonferroni post-test for comparing all pairs of results, by the GraphPad Prism software.

Assay for determination of activity of the thioridazine compounds against the human ABCB1-expressing mouse T-cell lymphoma cell lines. The L5178 (parental) and L5178Y (MDR) cells were grown in McCoy's 5A medium containing 10% heat-inactivated horse serum, L-glutamine and antibiotics. 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. The tested compounds were added at the final concentrations of 2.5 and 5 μg/ml and the samples were incubated for 10 min at room temperature. Verapamil (Sigma-Aldrich, St. Louis, MO, USA.) was applied as positive control at 10 μg/ml (7). Next, 10 μl (5.2 μM final concentration) of the indicator rhodamine 123 (Sigma, St Louis, MO, USA) was 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 (PBS) for analysis. The fluorescence of the cell population was measured with a Partec CyFlow flow cytometer (Partec, Germany). The mean fluorescence intensity as a percentage was calculated for the treated MDR and parental cell lines as compared with the untreated cells. The fluorescence activity ratio (FAR) was calculated using the following equation on the basis of the measured fluorescence values:

$$FAR = \frac{MDR_{\,treated}/MDR_{\,control}}{parental_{\,treated}/\,parental_{\,control}}$$

Apoptosis assay. Apoptosis induction in the presence of the studied compounds in human ABCB1 carrying mouse lymphoma and PC3 prostate cancer cell lines was determined. The cells were adjusted to a density of 1×10^6/ml and were distributed in 0.5 mL aliquots to microcentrifuge tubes. The apoptosis inducer 12*H*-benzo[α]phenothiazine (M627) was applied at a final concentration of 25 μg/ml as positive control.

Thioridazine (racemic and enantiomers) was applied to human ABCB1-expressing mouse lymphoma cells concentrations of 0.25, 2.5, 5 and 10 μg/ml. To assess the effect of thioridazine on apoptosis induction in PC3 prostate cancer cells, two concentrations of thioridazine (20 and 40 μg/ml) were selected. Wells containing no M627 or thioridazine compounds served as untreated controls. The cells were incubated at 37˚C for 1, 2 and 3 h. The samples were then centrifuged and washed with serum-free medium and re-suspended in 0.5 ml culture medium and distributed into the 24 wells of the culture plates and further incubated for 24 h at 37˚C, in 5% CO_2. The apoptosis assay was carried out using Annexin V-FITC Apoptosis Detection Kit (Cat. No. PF 032 from Calbiochem) according to the manufacturer's instructions. The fluorescence of cell population was analysed immediately using a Partec CyFlow flow cytometer.

Results

The antiproliferative effects (inhibitory concentration - IC_{50}) of the racemate and (+) and (–) enantiomers of thioridazine on MDR mouse lymphoma cells are summarized in Figure 2. No significant differences were found among the three compounds for antiproliferative activity against the MDR mouse lymphoma cell line. The effect of the racemic, and the (+) and (–) enantiomers of thioridazine was studied on the retention of rhodamine 123 in the MDR mouse lymphoma cell line at 0.25 and 2.5 μg/ml (Table I). At the concentration of 0.25 μg/ml of thioridazine and of its two enantiomers, moderate inhibition of ABCB1 expressed by the MDR mouse T-cell lymphoma cell line takes place, while at 2.5 μg/ml stronger inhibition was observed, which seems to be promising, without apparent stereospecificity. Similarly, low concentrations were applied in the apoptosis induction assay and the potency of apoptosis induction in L5178Y and PC3 cells was dependent on the duration of induction (Tables II and III).

Discussion

The isolation and stability of enantiomers is dependent on the local milieu (10).

The presented equilibrium between (+) and (–) isomers influences the binding affinities of the enantiomers to different ligands or receptors. The presented study asked the question "Does the racemate thioridazine and its two enantiomers express different activities against cancer cell lines?" This question was based upon observations published by others, which suggested that "the (–)-TZ enantiomer had slightly more catalepsy (neuroleptic)" (11). Nevertheless, because a variety of pharmacological studies reported by Svendsen *et al*. (11) indicated significantly greater binding of the (–)-TZ enantiomer to the D1 receptor of the rodent brain, we, as well as others (12, 13) studied the activity of the racemate TZ which consists of equal concentrations of the (+) and (–) enantiomers and the separate activity of each enantiomer against cancer cell lines.

Our results clearly demonstrate that the racemate TZ and its two enantiomers have essentially the same activity against cancer cell lines with respect to the inhibition of replication, induction of apoptosis and inhibition of ABCB1.

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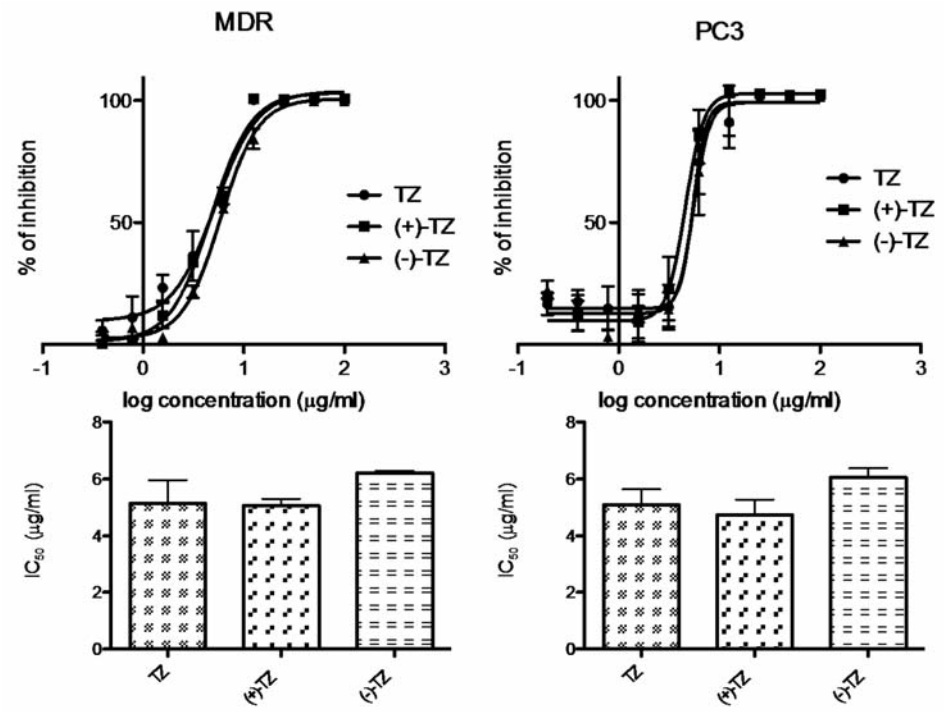

Compounds	Mean IC_{50} (µg/ml)			
	L5178Y	SE	PC3	SE
TZ Racemate	5.14	0.83	5.09	0.56
(+)-Enantiomer	5.06	0.24	4.74	0.54
(-)-Enantiomer	6.23	0.07	6.05	0.34

Figure 2. *Antiproliferative effect of thioridazine enantiomers on L5178Y mouse lymphoma and PC3 prostate cancer cells. IC_{50} was calculated using non-linear regression curve fitting of log (inhibitor) vs. response and variable slope with a least squares (ordinary) fit, using GraphPad Prism software. The mean IC_{50} was calculated based on three independent experiments. SE: Standard error of the mean. Differences between the enantiomers were evaluated by one-way analysis of variances (ANOVA) with Bonferroni post-test for comparing all pairs of results, by using the GraphPad Prism software.*

Table I. *Reversal of the multidrug resistance of L5178Y MDR mouse lymphoma cells in the presence of low doses of thioridazine.*

	Concentration µg/ml	FSC	SSC	Mean FL1	FAR
L5178Y (Parental)	-	1573	674	52.4	-
L5178Y (ABCB1 expressing)	-	1601	629	0.545	-
Verapamil	10	1587	628	14.9	27.34
Thioridazine racemate	0.25	1601	625	2.65	4.86
	2.50	1609	618	34.7	63.66
(+)-Enantiomer	0.25	1590	626	1.73	3.17
	2.50	1614	626	33.5	61.46
(-)-Enantiomer	0.25	1602	636	1.35	2.47
	2.50	1614	631	29.8	54.67

FSC: Forward scatter count of cells in the samples (cell size ratio); SSC: Side scatter count of cells in the samples; FL-1: Mean fluorescence intensity of the cells. FAR: fluorescence activity ratio values were calculated by using the equation given in the Material and Methods.

Table II. *Apoptosis induction of ABCB1 expressing MDR cells by thioridazine and its enantiomers after 1 h incubation.*

Treatment		Gated events %		
	Concentration µg/ml	Early apoptosis	Late apoptosis and necrosis	Cell death
Untreated control		5.74	2.8	0.164
M627	50	61.6	34.3	0.107
Thioridazine racemate	2.5	19.9	1.15	0.18
	5.0	48.1	2.52	0.2
(+)-Enantiomer	2.5	19.4	1.8	0.193
	5.0	40	3.58	0.378
(–)-Enantiomer	2.5	9.6	1.84	0.164
	5.0	54.3	3.81	0.458
DMSO	5 µl	6.2	2.17	0.128

Table III. *Apoptosis induction of PC3 cells by thioridazine and its enantiomers after 1 h incubation.*

Treatment	Concentration µg/ml	Gated events %		
		Early apoptosis	Late apoptosis and necrosis	Cell death
Untreated control		0.776	2.03	5.49
DMSO	5%	1.1	1.92	3.8
M627	25	42	4.75	2.92
Thioridazine racemate	20	1.72	3.01	1.41
	40	7.98	3.31	3.6
(+)-Enantiomer	20	1.75	4.44	2.35
	40	3.11	3.48	5.35
(–)-Enantiomer	20	1.76	2.29	1.44
	40	12.3	1.83	3.15

described by Svendsen *et al*. in 1988 (11). Optical isomers such as those of thioridazine's exhibit significant differences in their affinities for receptor sites, biotransformation and binding to serum and tissue proteins. The decomposition of the racemate thioridazine in the human body into its enantiomers has been measured (12). In these investigations, the (–)-enantiomer was found at higher concentrations than the (+)-enantiomer in the different tissues. The general possibility for using resolution of commercially available racemates in therapeutics for anxiolytic effect has been described by Baumann *et al*. (13) and underline the very important investigations and statements made by Ariëns *et al*. in 1986 (14).

The role of chirality of other compounds known to affect ABCB1 such as the enantiomers of verapamil has been examined. These latter studies indicated that both L- and D-forms of verapamil were equal in their ability to reverse the *in vitro* resistance of MDR leukemic cells to drugs such as to vincristine (15). The mechanism of action of thioridazine was analyzed by Spengler *et al*. in detail and they showed that the racemate thioridazine was able to induce apoptosis of MDR mouse lymphoma cells (16).

These latter studies did not examine the differential effects of the two TZ enantiomers. Nevertheless, the observations reported are consistent with those obtained by others with cervical and endometrial cancer cell lines (17).

In MDR cancer cells, the efflux pump mechanism is responsible for treatment failures; therefore, the inhibition of such mechanisms given simultaneously with anticancer drugs can result in an effective anticancer chemotherapy. However, we have to consider that normal cells also contain the ABC transporters, *e.g*. for detoxification. Hence in order to avoid the toxic side-effect of resistance-reversal compounds, drugs with selective inhibition of the MDR in cancer cells are needed. To achieve this effect, three classes of known neuroleptic drugs with active and inactive stereoisomers were tested for MDR efflux pump inhibition on mouse T-lymphoma cell lines (1). Their anti-proliferative effects on sensitive and MDR cancer cell cultures were compared to the classic resistance modifier verapamil. Consistent with our present results, enantiomers of phenylalkylamines are equally potent inhibitors of drug transport by P-glycoprotein (1). However, CNS-active and -inactive butaclamol enantiomers present with slightly different effects on the reversal of the MDR phenotype which means that drug binding might have weak enantio-selectivity for P-glycoprotein (1) suggesting that some enantiomers of compounds that inhibit ABCB1 can be exploited as adjuvants to cytostatic chemotherapy of cancer in order to increase the efficacy of the cancer agent. However, in the current study, the separate use of one enantiomer of TZ versus the other does not appear to provide any advantage over racemic mixture of TZ for the adjuvant therapy of MDR cancer.

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